CORONARY VASCULAR DYSFUNCTION IN OBESE TYPE 2 DIABETIC MICE

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This dissertation entitled

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Obesity and type 2 diabetes have reached epidemic proportions worldwide. It is known that diabetics have a significantly increased risk for developing cardiovascular disease, especially coronary artery disease. The coronary circulation is regulated cooperatively by numerous systems including the vasodilator nitric oxide (NO) system and the vasoconstrictor endothelin-1 (ET-1) and $\alpha_1$-adrenoceptor ($\alpha_1$-AR) systems. Diabetes-related dysfunction in each of these systems is suggested from previous research.

The present study uses a common diet-induced model of obesity and type 2 diabetes in C57BL/6J mice to examine these alterations in the coronary circulation. Specifically, a Langendorff isolated mouse heart system is utilized to measure changes in coronary function related to type 2 diabetes. The NO, ET-1 and $\alpha_1$-AR systems are analyzed individually. In addition, examination of the interactions between these systems is performed mainly through pharmacologic methods utilizing inhibitors along with receptor agonists and antagonists. Studies are also performed to characterize the metabolic abnormalities associated with obesity and type 2 diabetes in this mouse model.

Type 2 diabetic mouse hearts exhibit reduced NO bioavailability resulting from enhanced scavenging by superoxide anion. Normal cGMP-mediated vasodilator mechanisms are maintained in this model. The role of endogenous ET-1 in
vasoconstriction due to NO inhibition is absent in diabetes. The contribution of ET-1 in basal coronary tone is enhanced in diabetes possible due to enhanced ET$_B$ receptor-mediated responses. Vasoconstriction to exogenous ET-1 appears to be ET$_B$ receptor dependent in control but not diabetic mice. The overall vasoconstriction to ET-1 is enhanced in diabetic mice. The $\alpha_1$-AR system appears to be functionally unaltered under baseline conditions. However, ET$_A$ receptors may reduce $\alpha_1$-AR-mediated vasoconstriction in diabetes and suggests a possible upregulation of this system.

The results suggest significant vascular dysfunction influenced by numerous regulatory systems and their interactions. Overall, a shift in the balance of vasodilator and vasoconstrictor responsiveness toward vasoconstriction in the coronary circulation is demonstrated. This may result from reduced NO bioavailability and/or upregulation of vasoconstrictor pathways. In summary, diabetes has a multifactorial deleterious effect on coronary vascular function in obese diabetic mice that partly parallels the human condition.

Approved:

Richard E. Klabunde

Associate Professor of Biomedical Sciences
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<tr>
<td>8-br-cGMP</td>
<td>8-bromo-cGMP</td>
</tr>
<tr>
<td>α₁-AR</td>
<td>Alpha(one)-adrenoceptor</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ADMA</td>
<td>Asymmetric dimethylarginine</td>
</tr>
<tr>
<td>ADO</td>
<td>Adenosine</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6J Mice</td>
</tr>
<tr>
<td>BH₄</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
</tr>
<tr>
<td>BQ-123</td>
<td>Selective ETₐ antagonist</td>
</tr>
<tr>
<td>BQ-788</td>
<td>Selective ETᵦ antagonist</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine-3’5’-monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine-3’5’-monophosphate</td>
</tr>
<tr>
<td>CPP</td>
<td>Coronary perfusion pressure</td>
</tr>
<tr>
<td>CVR</td>
<td>Coronary vascular resistance</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DDAH</td>
<td>Dimethylarginine dimethylaminohydrolase</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>Effective drug concentration to achieve 50% of the maximal response</td>
</tr>
<tr>
<td>ECE</td>
<td>Endothelin converting enzyme</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EPI</td>
<td>Epinephrine</td>
</tr>
<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>ETₐ</td>
<td>Endothelin type A receptor</td>
</tr>
<tr>
<td>ETᵦ</td>
<td>Endothelin type B receptor</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>GC</td>
<td>Guanylyl cyclase</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter-4</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HFHSC</td>
<td>High-fat, high-simple carbohydrate</td>
</tr>
<tr>
<td>IFG</td>
<td>Impaired fasting glucose</td>
</tr>
<tr>
<td>IGT</td>
<td>Impaired glucose tolerance</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>IRL-1620</td>
<td>Selective ETᵦ agonist</td>
</tr>
<tr>
<td>KHB</td>
<td>Krebs-Henseleit buffer</td>
</tr>
<tr>
<td>L-NAME</td>
<td>Nω-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
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<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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NE: Norepinephrine
NO: Nitric oxide
NOS: Nitric oxide synthase
O$_2^-$: Superoxide anion
ONOO$^-$: Peroxynitrite
PE: Phenylephrine
PGH$_2$: Prostaglandin H$_2$
PIVD: Post-ischemic vasodilation
PKA: Protein kinase A
PKC: Protein kinase C
PLC: Phospholipase C
ROS: Reactive oxygen species
SNAP: (±)-S-nitroso-N-acetylpenicillamine
SOD: Superoxide dismutase
STZ: Streptozotocin
T1DM: Type 1 diabetes mellitus
T2DM: Type 2 diabetes mellitus
TXA$_2$: Thromboxane A$_2$
VSMC: Vascular smooth muscle cell
I. BACKGROUND AND SIGNIFICANCE

A. Diabetes Mellitus

Overview and Classification. Diabetes mellitus is a chronic disease of glucose metabolism afflicting more than 20 million Americans and is currently the sixth leading cause of death in the United States (48). However, it is likely that diabetes is underreported as a cause of death due to the many pathologies precipitated by this disease that become the immediate cause of death. A recent report estimates that 1 in 3 males and 2 in 5 females born in 2000 will be diagnosed with diabetes during their lifetime (198). Worldwide, the total number of people with diabetes is projected to increase from 171 million in 2000 to 366 million in 2030 (325).

Conventionally, diabetes is classified as either type 1 or type 2, with the latter accounting for approximately 95% of all cases. Type 1, or insulin-dependent, diabetes mellitus (T1DM) occurs upon cessation of insulin production by the pancreas and is most prevalent in juveniles. Type 2, or non-insulin-dependent, diabetes mellitus (T2DM) is due to insulin resistance in target cells and is associated with elevated blood insulin levels. This form is most common in adults; however childhood diagnoses are increasing, especially in minority populations (48, 232). Ultimately, both types of diabetes result in decreased cellular utilization of glucose leading to hyperglycemia.

Fasting blood glucose levels in a healthy human are typically less than 100 mg/dl. Clinically, diabetes is diagnosed in a patient who presents with fasting blood glucose levels greater than or equal to 126 mg/dl or who has a blood glucose level greater than 200 mg/dl following a two hour oral glucose tolerance test (1). However, an intermediate
group of patients with blood glucose levels higher than normal but not high enough to meet the criteria for diabetes has recently been recognized as “pre-diabetic”. This group maintains fasting blood glucose levels greater than 100 mg/dl but less than 126 mg/dl (1). Patients in this group are deemed as having impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) if the diagnosis was done using an oral glucose tolerance test. IFG and IGT are not clinically significant entities by themselves but are risk factors indicating an increased likelihood for future diabetes development and cardiovascular disease (1).

Pathogenesis of Type 2 Diabetes. The pathogenesis of T2DM is a complex multifactorial process. Due to its complexity, the exact sequence of events leading to the onset of overt T2DM is not fully understood. It is widely accepted, however, that genetic and environmental factors play a cooperative role in this process. Studies using a wide range of techniques including twin studies and spontaneously diabetic animals have noted definitive genetic predispositions to diabetes (22, 51). However, these predispositions may not be autonomous in the development of diabetes but rather dependent on an environmental stimulus. Current research has shown that while a strong genetic component for diseases such as obesity and diabetes exists, the dramatically increased prevalence of these diseases is most likely due to an energy balance shifted toward energy intake and away from energy expenditure (352). The link between obesity and T2DM is well established and elegant investigations are beginning to show the biological basis for this association. In fact, about 60-90% of T2DM patients are overweight or obese thereby providing ample support for the term “diabesity” used to describe obesity-
related T2DM (13, 84). Zimmet has suggested that obesity-related T2DM derives from the ‘collision of our ‘hunter-gatherer’ genes with our new twentieth-century way of life’ (353). It should be mentioned, however, that while most T2DM patients are obese, most obese patients do not develop overt T2DM thereby providing strong support for the presence of an underlying genetic predisposition in those who progress to T2DM.

The classic dogma concerning the onset of T2DM holds that early in insulin resistance relatively normal blood glucose levels are maintained, due to compensatory insulin secretion from pancreatic beta-cells. This is not sustained, however, as insulin resistance worsens and the elevated insulin secretion becomes inadequate to overcome the impaired insulin action. Eventually this leads to beta-cell “exhaustion” and impaired insulin release. The fall in insulin secretion would thereby decompensate blood glucose regulation and produce hyperglycemia in the absence of hyperinsulinemia.

Early studies reported a hyperinsulinemic response two hours following a meal in diabetic subjects (219). Hence, it was thought that insulin resistance was a primary defect and led to beta-cell dysfunction. However, it has been revealed that this hyperinsulinemic response two hours after a glucose challenge is compensatory for what we now know as a reduced first phase insulin release that occurs thirty minutes post-challenge (302). Current evidence places more emphasis on both insulin resistance and beta-cell dysfunction acting in concert and that beta-cell dysfunction must be present for even minimal increases in blood glucose (161). The nature of the primary beta-cell defect remains unknown. Research has not yet elucidated which of these two defects precedes the other. This issue is complicated by results showing that each defect has the
ability to cause the other. For example, chronic hyperglycemia, which mimics impaired beta-cell insulin release, causes a reduction in beta-cell glucose sensitivity through a process known as glucotoxicity (229, 345). Also, sustained hyperinsulinemia, indicative of insulin resistance, can lead to reduced insulin release and action (70, 71).

_Obesity and Type 2 Diabetes._ Pertinent to this discussion is the relationship between obesity and the onset of T2DM or “diabesity”. As noted above, obesity is a significant risk factor for the development of insulin resistance and T2DM. In fact, insulin resistance is seen early in the obese phenotype as evidenced by increased blood glucose levels coupled with a compensatory increase in blood insulin levels (83). Obesity is also accompanied by elevated plasma free fatty acids (FFA) which have been shown to cause defects in glucose-stimulated insulin release as well as inhibit insulin gene expression in the presence of elevated glucose levels (128). Therefore, with worsening insulin resistance and the subsequent hyperglycemia, elevated FFAs contribute to defects in beta-cell insulin production and release. This phenomenon has been termed lipotoxicity and is thought to arise not only from elevated plasma FFA but from the accumulation of FFA esterification metabolites (229). The obesity-mediated rise in plasma FFA is partly due to increased glucose uptake by adipocytes as tissue insulin sensitivity falls thereby stimulating FFA production and release and increasing adiposity. Elevated plasma FFA also promotes hyperglycemia through stimulation of hepatic gluconeogenesis while reducing tissue glucose uptake and storage (270). Therefore, the increased blood glucose and FFA levels indicative of obesity are integral factors for the development of T2DM as described above. It should be noted that an elevation of plasma
FFA is seen in the insulin resistant state regardless of obesity due to the reduced inhibition of insulin on FFA production (244). Overall, obesity significantly increases the risk of developing T2DM due to the combined effects of glucotoxicity and lipotoxicity, or “glucolipotoxicity” on insulin production, release and activity. Many of these defects also play an integral role in the deleterious effects of T2DM on multiple organs systems resulting in pathologies including various vascular diseases, metabolic disorders, neuropathies, and end-organ failure, particularly renal failure (103, 106).

B. Vascular Complications of T2DM

*Epidemiology.* The severity of cardiovascular complications in diabetes is demonstrated by the statistic that diabetics are 2 to 4 times more likely to have a stroke or die of heart disease than non-diabetics. Heart disease is the leading cause of diabetes-related deaths in the United States (48). In 2000, 4.4 million people over the age of 35 with diabetes reported development of a cardiovascular complication including coronary artery disease, stroke, or heart attack. Of these, 2.9 million reported diagnosis of coronary artery disease (CAD) (49). Further, CAD severity is increased in diabetics with these patients showing a higher prevalence of multivessel disease, three-vessel being the most prevalent, with more diseased segments per vessel than non-diabetic controls (78). Diabetics also have a poorer long-term prognosis following the incidence of a major cardiovascular event (180, 268). Therefore, the importance of research aimed at elucidating the mechanisms through which diabetes exerts its deleterious effects, especially those in the cardiovascular system is obvious.
Vascular Physiology. The following discussion presents current evidence concerning the onset of cardiovascular disease subsequent to T2DM. For clarity, a brief foundational description of vascular structure and function is presented here. The vascular tree, including the coronary circulation, contains each level of vessel from large arteries to capillaries. In the mouse coronary vasculature, the internal diameters of these vessels as defined by Laughlin et al (159) are as follows: large arteries, >300 μm; small arteries, 201-300 μm; resistance arteries, 151-200 μm; intermediate arterioles, 51-100 μm; and small arterioles, <50 μm. Conventionally, the three latter classifications are grouped as ‘resistance’ arteries and regulate blood flow and distribution by actively changing arterial diameter in response to metabolic, neural, humoral and physical stimuli. Studies in the hamster cheek pouch found that fifty percent of the total pressure drop across the vascular system occurs at the resistance artery level due to the high resistance conferred by these vessels on blood flow thereby supporting their role as primary blood flow regulators (66).

Large and small arteriole lumens are lined by a single endothelial cell layer. Once thought to be strictly a barrier between the vessel wall and lumen, vascular endothelial cells are now known to be integral components of normal vascular function. These cells contribute by secreting a number of chemical substances that regulate platelet aggregation, coagulation, fibrinolysis, and vessel tone. Vessel tone, and therefore local blood flow, is regulated through the balanced release of vasodilator and vasoconstrictor substances by the endothelium. With particular reference to the coronary circulation, the control of myocardial blood flow is also tightly coupled to cardiac oxygen demand.
Metabolic mechanisms exist to monitor the oxygen balance in the heart and respond to changes therein by activating or deactivating coronary vasodilator or vasoconstrictor pathways, some involving endothelial cells.

Endothelial cells secrete many vasoactive substances including vasodilators such as nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor along with vasoconstrictors such as endothelin-1 (ET-1) and thromboxane A$_2$ (TXA$_2$) (176). Once released, these substances diffuse to and alter the contractile state of vascular smooth muscle cells (VSMC). A single VSMC layer surrounds small arterioles while multiple layers surround larger arteries. The morphology of individual vessel types is well conserved across species; however, vascular anatomy is less conserved, particularly when comparing human and mouse coronary anatomy. The human myocardium is supplied with blood by two main coronary arteries, left and right, arising from the aorta. In contrast, the mouse myocardium is supplied by as many as three main coronary arteries.

*Pathogenesis of T2DM Vascular Disease.* Cardiovascular consequences of T2DM include complications such as hypertension, atherosclerosis, and peripheral vascular disease. A primary defect underlying many of these conditions is vascular endothelial dysfunction. Endothelial dysfunction refers to an imbalance in the release of vasoactive substances from the endothelium which can lead to thrombosis, vessel occlusion, vasospasm, or impaired vasodilator responses. Functionally, endothelial dysfunction is characterized by a reduction in endothelium-dependent vasodilation indicating a reduced NO bioavailability (see section C). Unfortunately, the cause of this
defect in T2DM has yet to be fully elucidated and is thought to be multifactorial. It should be noted here that studies examining vascular control mechanisms in diabetes have much variability resulting from, but not limited to, differences in diabetes type, duration of the disease, magnitude of hyperglycemia, type of stimulus used to induce diabetes experimentally, and the portion of the vascular tree under investigation.

**Role of Hyperglycemia.** A prime candidate for T2DM-related endothelial dysfunction appears to be hyperglycemia. Human and animal studies have shown that acute and chronic hyperglycemia led to decrements in endothelium-dependent vascular responses. Results from the Northern Manhattan Study (NOMAS) showed a continuous linear relationship between fasting blood glucose and endothelial function such that elevations in blood glucose correlated with impaired flow-mediated vasodilation in the forearm circulation (248). The subjects in this study with IFG were considered pre-diabetic (as defined in Section A) and exhibited a 25% decrease in endothelial function, as determined by flow-mediated vasodilation during reactive hyperemia, within the blood glucose range of 110-125 mg/dl when compared to those with blood glucose levels below 100 mg/dl. This study is exceptionally significant because it was conducted in a large urban multiethnic nondiabetic population free of overt cardiovascular disease thereby indicating the onset of endothelial dysfunction prior to the development of frank T2DM.

Isolating the effects of T2DM-related hyperglycemia on vascular function remains difficult since it is typically coupled with insulin resistance and/or hyperinsulinemia both of which can also cause endothelial dysfunction. However, endothelial dysfunction exhibited in human (80, 130, 273) and animal models (242, 250,
271) of T1DM support the effect of chronic hyperglycemia on endothelial function in the absence of insulin effects. Acute hyperglycemia in animal models also supports the role of high glucose levels in endothelial dysfunction. Gomes et al (97) found in rabbit aortic rings and perfused kidneys that three hour exposure to high glucose concentrations reduced acetylcholine-induced, endothelium-dependent vasodilation in both preparations. The glucose concentrations used in this study were designed to simulate those seen in human daily clinical practice. Studies of vascular responses to acute hyperglycemia in humans have yielded conflicting results. Williams et al (329) showed an impairment in brachial artery vasodilation following six hours of local hyperglycemia whereas Houben et al (118), using similar methods and glucose concentrations, failed to note any change in endothelium-dependent vasodilation. Overall, however, it is suggested that hyperglycemia may play a significant role in T2DM-related endothelial dysfunction.

Role of Insulin Resistance/Hyperinsulinemia. As noted above, insulin resistance and hyperinsulinemia have also been shown to cause endothelial dysfunction. Apart from its metabolic effects, insulin acts in the vasculature to modulate vascular tone through the release of NO and ET-1. The dominant vascular effect of insulin is vasodilation which is believed to occur in order to augment tissue glucose delivery and therefore insulin-mediated glucose uptake (21). In vitro studies have definitively exhibited insulin-stimulated release of NO and ET-1 from human endothelial cells (85, 349). Additionally, insulin signaling plays a role in both nitric oxide synthase (NOS) and ET-1 expression as shown in endothelial cells isolated from mice deficient in vascular endothelial insulin receptors. However, the physiologic impact of these in vitro results
has not been fully substantiated *in vivo*. In fact, while insulin-mediated vasodilation due to NO release is well established, ET-1 release in response to physiologic hyperinsulinemia is seen in T2DM patients and is absent in healthy individuals (113, 166, 187, 260). This may be explained in part by evidence that insulin-mediated vasodilation is blunted in T2DM thereby suggesting reduced insulin-mediated NO release and the removal of NO inhibition of endothelial ET-1 release (discussed in Section F) (132, 142, 276). These findings specifically illustrate that the insulin resistance associated with T2DM is present not only in skeletal muscle and adipocytes but also in vascular endothelial cells.

While the relationship remains unclear, it is evident from the literature that insulin resistance plays a role in T2DM-related endothelial dysfunction. Across a range of worsening insulin resistance, Prior et al (235) found that endothelium-dependent vasodilation was reduced in insulin-resistant patients in the absence of hyperglycemia. The response was further attenuated with the onset of hyperglycemia and overt T2DM. Some large population analyses examining the role of insulin resistance in endothelial dysfunction and cardiovascular disease have found that insulin is a weak predictor of these events as it is not independent of other risk factors (119, 253). Conversely, a follow-up of the Helsinki Policeman Study showed that the level of insulin resistance independently predicted the risk of coronary heart disease and stroke (237). The early endothelial defect characteristic of coronary and peripheral vascular disease has also been attributed to insulin resistance or hyperinsulinemia in smaller human and animal studies
(125, 142, 233, 240). Overall, a link between insulin resistance, compensatory hyperinsulinemia and endothelial dysfunction has been established.

*Role of Oxidative Stress.* The etiology of endothelial dysfunction due to hyperglycemia, hyperinsulinemia and insulin resistance is not fully understood; however, the central precipitating factor appears to be oxidative stress induced by these abnormalities. Oxidative stress is enhanced by both hyperglycemia and insulin resistance/hyperinsulinemia. Briefly, oxidative stress refers to an enhanced production of reactive oxygen species (ROS), predominantly superoxide anion ($O_2^-$) along with peroxynitrite (ONOO$^-$), which are able to cause tissue damage when produced in excess of normal antioxidant capacity (167, 355). Nitric oxide, along with other natural antioxidants such as superoxide dismutase, catalase and glutathione, scavenge ROS thereby protecting surrounding biological tissues from oxidative damage. Superoxide anion is produced by a number of enzymes including cyclooxygenase, xanthine oxidase, mitochondrial NADH dehydrogenase, NOS, and NAD(P)H oxidase.

With respect to T2DM, *in vitro* exposure of coronary endothelial cells to high glucose increases NAD(P)H oxidase levels and reduced glutathione levels by 50% thereby negatively shifting the oxidative balance (320). Numerous studies in humans (67), dogs (102), rats (142, 147, 148) and mice (17, 18, 211) have revealed reduced NO-mediated, endothelium-dependent vasodilator responses in conjunction with enhanced oxidative stress. Similarly to *in vitro* data, NAD(P)H oxidase appears to be the predominant source of superoxide in many of these systems. This is suggested by
NAD(P)H oxidase upregulation as well as by the restoration of NO-dependent vasodilator responses following NAD(P)H oxidase inhibition (17, 109, 142, 147, 148).

In the spontaneously diabetic db/db mouse, apart from upregulated NAD(P)H oxidase, superoxide is also produced by NOS operating with inadequate levels of the cofactor tetrahydrobiopterin (BH₄). In this model, NO-dependent vasodilator responses were recovered by incubation of small mesenteric arteries with BH₄ and by in vitro administration of apocynin, a NAD(P)H oxidase inhibitor (17, 211). Deficiency of BH₄ is common in models of T2DM. This may be partly due to reduced insulin activation of enzymes necessary for BH₄ biosynthesis. In insulin resistant fructose-fed rats with endogenous hyperinsulinemia, BH₄ levels were reduced and corresponded to increased NOS O₂⁻ production. The increased NOS O₂⁻ production was eliminated by incubation of aortic strips with exogenous BH₄ (269). Therefore, the oxidative balance within the vasculature is shifted in T2DM toward enhanced oxidative stress and this likely plays a role in reducing NO bioavailability through several mechanisms.

Of importance to the present study, significant O₂⁻ production has been demonstrated in Krebs-Henseleit buffer (KHB), the most common buffer used in perfused isolated heart studies. Superoxide production in this buffer is attributed to glucose autooxidation (KHB glucose concentration is 11 mM), trace metal contamination and bubbling with 95% oxygen. In isolation, KHB O₂⁻ production is sufficient to eliminate the NO concentration needed to induce vascular relaxation (~5 μM) (23). However, since innumerable studies have demonstrated NO-mediated vascular responses
using KHB, it is possible that KHB $O_2^-$ activity is tempered in biological systems by endogenous antioxidant activity.

Recently, the systemic influence of vascular oxidative stress was examined in T2DM patients treated for 12 weeks with the second-generation sulfonylurea gliclazide, which has antioxidant properties. Following treatment, patients had higher plasma total radical trapping parameter, an inverse measure of systemic oxidative stress, and enhanced NO-dependent vasodilation indicated by a significant reduction in blood pressure following L-arginine infusion (67). Overall, oxidative stress and vascular consequences in T2DM have been elucidated. However, the full extent of oxidative damage to vascular regulation in T2DM remains unclear.

While hyperglycemia, insulin resistance and hyperinsulinemia contribute to T2DM-related vascular disease, other traditional cardiovascular risk factors such as obesity, dyslipidemia, hypertension and lifestyle also play a key role in many individuals. Collectively, these conditions significantly increase the risk of cardiovascular disease through changes in haemostatic and fibrinolytic factors, platelet function and endothelial function, as described above (305). Thus, T2DM patients are increasingly more likely to experience thrombus formation, vascular plaque deposition, chronic vasoconstriction, and vascular inflammation. The result of these alterations is especially noted in the coronary vasculature where myocardial blood flow is impaired potentially leading to myocardial ischemia and infarct.
C. Nitric Oxide

Nitric oxide was identified in the 1980’s as the true identity of what had previously been deemed ‘endothelium-derived relaxing factor’. Nobel laureates Louis Ignarro, Ferid Murad and Robert Furchgott are credited with independently characterizing NO as this relaxing factor. Since this surprising discovery, the biological activity of NO has been extensively studied revealing it as one of most ubiquitous substances in biological systems. Nitric oxide’s breadth of significance was recognized in 1992 when it was chosen as the “Molecule of the Year” by Science (61). In the vasculature, we now understand that, apart from its vasodilator activity, NO is also an important anti-thrombotic, anti-proliferative and anti-inflammatory agent.

Synthesis and Release. Released by vascular endothelium, NO is produced through the five-step oxidation of L-arginine to NO and L-citrulline by the action of the soluble enzyme, nitric oxide synthase (NOS) (124). Vascular tissues contain two isoenzymes of NOS: the constitutive endothelial NOS (eNOS) and an inducible form of NOS (iNOS). NO production by eNOS can be activated through receptor-dependent and –independent pathways leading to elevated intracellular calcium and calcium-calmodulin-dependent activation of eNOS (86). In contrast, iNOS-mediated NO production is not calcium-calmodulin dependent and iNOS expression is increased in some cardiovascular disease states. iNOS produces copious amounts of NO orders of magnitude greater than that produced by eNOS (278). Inconsistent results concerning iNOS expression in T2DM have been reported (45, 340). Necessary cofactors for NO production by either NOS include oxygen, NADPH, tetrahydrobiopterin, flavin adenine dinucleotide and
flavin mononucleotide (165, 311). For simplicity, eNOS is referred to as NOS in the remainder of this text.

Following endothelial release, NO diffuses to vascular smooth muscle where it binds to soluble guanylyl cyclase (GC) thereby increasing cyclic guanosine-3’5’-monophosphate (cGMP) levels, inducing smooth muscle relaxation, vascular dilation, and increased blood flow (224). At high concentrations, NO reacts with oxygen to form nitrogen dioxide that subsequently can cause tissue damage (193). However, since NO has a much higher affinity for hemoglobin than oxygen and is oxidized to nitrite by hemoglobin, vascular nitrogen dioxide formation by NO is limited (193).

Vascular Regulation by NO. Basal release of NO occurs through chronic physical activation of endothelial cells by pulsatile flow and shear stress and plays an integral role in the maintenance of basal coronary tone (193, 316). This action of NO counteracts basal vasoconstrictor effects produced by myogenic activity, sympathetic adrenergic activity, circulating angiotensin II, and endothelial-derived ET-1. Numerous studies have shown that removal of the endothelium augments vasoconstriction mediated by these substances (31, 139, 206). Nitric oxide also limits the extent of coronary vasoconstriction through increases in shear stress-mediated NO release (277). As vessels constrict, shear stress on the endothelium increases leading to increased NO synthesis.

Nitric oxide is the primary contributor to endothelium-dependent vasodilator responses. Thus, vascular responses to NO-dependent agonists, such as acetylcholine (ACh), have become clinical and experimental tools for the examination of endothelial dysfunction. Basal NO bioavailability can also be determined experimentally by
blocking NOS and monitoring the subsequent reduction in vascular diameter. Inhibition of NOS can be achieved experimentally by treatment of vascular preparations with L-arginine derivatives such as $N_\omega$-nitro-L-arginine methyl ester (L-NAME). Once treated, endogenous NO production is eliminated and the role of NO in the vasculature is typically assessed by measurement of changes in either vascular blood flow or resistance. For example, elimination of NO production reduces an integral vasodilator stimulus resulting in decreased blood flow and increased vascular resistance due to vasoconstriction. The magnitude of these changes is indicative of the level of NO bioavailability in the vasculature. Therefore, reduced NO bioavailability would result in an attenuated decrease in blood flow or increase in vascular resistance.

**T2DM Effects on NO Vascular Actions.** Impairment of NO production or bioavailability has been implicated in the pathogenesis of coronary vascular disease, including coronary pathologies associated with T2DM. As noted above, numerous studies demonstrate impaired NO-dependent vasodilation in human and animal T2DM. The primary role of oxidative stress in reducing NO bioavailability in T2DM has already been discussed (Section B). Briefly, under normal conditions, NO reacts with and scavenges superoxide anion effectively protecting vascular tissue from oxidative damage and limiting the inflammatory response to tissue injury. In T2DM, enhanced superoxide production leads to a shift in the biological activity of NO toward its role as an antioxidant and away from vasodilation. This enhanced oxidative stress also appears to play a role in the reduction of vascular responses to NO donors. Some studies have demonstrated reduced VSMC relaxation to exogenous NO that subsequently is recovered
following pretreatment of vessels with ROS scavengers (17, 18, 90, 205). This suggests that exogenous NO is also scavenged by superoxide in T2DM. Responses to endogenous NO are also recovered in many cases with antioxidant treatment (17, 18, 90, 211).

The reduced NO bioavailability and activity in T2DM may also result from heightened levels of endogenous NOS inhibitors, reduced levels of NOS cofactors, changes in L-arginine-NO kinetics or disruption of eNOS dimerization. Acute and chronic accumulation of the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) has been found in T2DM (79, 170). In humans, plasma ADMA levels also increased in response to a single high fat meal resulting in reduced NO-dependent vasodilation (79). Additionally, BH4 is oxidized in T2DM due to enhanced oxidative stress thereby uncoupling NOS and leading to enhanced superoxide and reduced NO production by NOS (29, 269). Impaired ACh-induced vasodilation in small mesenteric arteries of the T2DM db/db mouse was restored by incubation with BH4 (211). Reduced basal conversion of infused L-arginine to NO has also been exhibited in T2DM patients compared to controls (15). Lastly, enhanced peroxynitrite formation in T2DM mice has been demonstrated to disrupt normal eNOS dimer formation (192). Therefore, numerous factors can be involved in reducing NO production and bioavailability in T2DM.

The T2DM-related reduction in NO bioavailability also impairs the anti-proliferative properties of NO. In healthy individuals, NO attenuates VSMC proliferation through cGMP-dependent and -independent mechanisms. The increased production of cGMP through GC activation leads to an increase in cyclic adenosine-3′5′-monophosphate (cAMP) levels and protein kinase A (PKA) activation resulting in
reduced intracellular calcium flux and attenuation of the proliferative mitogen-activated protein kinase (MAPK) cascade (311). Independent of cGMP, NO has also been shown to indirectly limit the formation of polyamines necessary for DNA synthesis through inhibition of arginase and ornithine decarboxylase while also upregulating Fas expression in VSMC thereby inducing apoptosis (311). These anti-proliferative actions of NO are important for normal vascular function; however, interruptions in these pathways can occur and aid in the development of vascular hypertrophy.

Vascular proliferation associated with T2DM is partly due to unabated activation of the MAPK pathway by insulin (132, 323). Two branches of insulin signaling exist in the vasculature following insulin receptor activation. The first, previously discussed, is linked to NO production through NOS activation while the second activates the MAPK pathway. Insulin resistance and T2DM have been linked with the uncoupling of insulin-induced NO production through the former pathway with no alteration in the latter (132). Therefore, coupled with a generalized reduction in NO bioavailability in T2DM, insulin signaling in this state results in enhanced MAPK activation and vascular proliferation.

Nitric oxide is also anti-thrombogenic and prevents the development of a proatherosclerotic milieu under normal conditions. Nitric oxide, both from the endothelium and platelets, has been shown to limit platelet activation and aggregation through inhibition of phosphoinositide-3 kinase and reduced platelet calcium flux leading to suppression of adhesion molecule expression in the platelet membrane (174). Also, the formation of peroxynitrite from the interaction of NO with superoxide anions leads to
a reduced production of thrombogenic arachidonic acid metabolites including TXA$_2$ (174).

Nitric oxide also plays a regulatory role in leukocyte-endothelial cell interactions. Similar to its effects on platelets, NO inhibits factors that lead to upregulation of adhesion molecules on endothelial cells thereby reducing leukocyte recruitment, adhesion and migration and therefore the initiation of plaque formation (311). T2DM patients exhibit significant increases in the basal circulating levels of adhesion molecules (16). Barbarto et al (20) have shown an enhancement of the vascular injury response characterized by increased adhesion molecule expression and inflammation in the T2DM Zucker rat. Importantly, gene transfer for the inducible form of NOS inhibited this enhancement supporting the anti-inflammatory properties of NO. However, other factors contribute to the inflammatory state associated with T2DM including, as mentioned above, unabated insulin signaling through the MAPK pathway which also upregulates adhesion molecule expression (178, 207).

D. Endothelin-1

Endothelins are a group of similar peptides (ET-1, ET-2, and ET-3). ET-1, the most potent vasoconstrictor released from the endothelium, was first isolated from porcine aortic endothelial cells by Yanagisawa et al (342) in 1988. In action, ET-1 is the natural counterpart of NO exhibiting pro-thrombotic, pro-inflammatory, and proliferative properties. Specifically, ET-1 is a strong chemoattractant and activator of monocytes and macrophages stimulating the release of cytokines and the upregulation of adhesion
molecule expression (107, 126). Constitutive overexpression of ET-1 also promotes vascular smooth muscle cell proliferation in an autocrine fashion (5).

**Synthesis and Release.** ET-1 is produced from the inactive precursor Big-ET-1 by endothelin converting enzyme (ECE) located in vascular endothelial and smooth muscle cells (146, 256, 338). Endothelial ECE expression is pronounced throughout the vasculature while VSMC ECE expression is irregular and inconsistent even within single vessels; therefore, the bulk of vascular ET-1 is endothelium-derived (154). Approximately 80% of ET-1 is released to the abluminal side of the endothelium supporting the paracrine and autocrine actions of ET-1, especially in modulating contractile tone of the underlying smooth muscle (310).

Numerous factors including insulin, vasopressin, angiotensin II, cytokines, ROS, and acute elevations of endothelial shear stress stimulate ET-1 release (105, 194, 251, 313). Conversely, ET-1 secretion is inhibited by NO, prostacyclin and long-term elevations in shear stress (194, 319). Basal endothelial ET-1 release is constitutive in cultured microvascular endothelial cells (313). In addition, it has been suggested that endothelial ET-1 release may be augmented by the exocytotic release of ET-1 from endothelial cell-specific storage granules (255). Once released, ET-1 binds and activates endothelin type A (ET\textsubscript{A}) and endothelin type B (ET\textsubscript{B}) receptors in the vasculature. The significant number of factors able to modulate ET-1 release coupled with the ability of ET-1 to activate second messengers such as PKA and RhoA in aortic smooth muscle suggest a high level of vascular cellular and functional crosstalk involving ET-1 (discussed in Section F) (65, 257).
**Endothelin Type A Receptors.** The ET$_A$ receptor is a classic G-protein-coupled receptor localized to VSMC (169, 267). ET-1 binding to the Gq-protein coupled ET$_A$ receptor on VSMC results in increased intracellular calcium levels and vasoconstriction via phospholipase C (PLC)-mediated diacylglycerol (DAG) and inositol triphosphate (IP$_3$) production. Additionally, DAG activates protein kinase C (PKC) thereby stimulating myosin light chain kinase and causing VSMC contraction (200, 230). ET$_A$-mediated elevations in intracellular calcium are prolonged (e.g., 30-60 min) resulting in sustained contraction (230). ET$_A$ activation primarily accounts for the role of ET-1 in basal vascular tone in rodents, dogs and humans (99, 100, 112, 149, 181, 319). Moreover, in isolated vessel studies and *in vivo*, ET$_A$ receptors play an integral role in vasoconstriction to endogenous or exogenous ET-1. For example, human mammary and coronary arteries, dog coronaries and systemic rabbit vessels constrict to ET-1 via ET$_A$ receptor activation (101, 158, 179, 318). Therefore, ET$_A$ receptors appear to be the dominant receptor mediating ET-1 vasoconstriction.

**Endothelin Type B Receptors.** Vascular ET$_B$ receptors are located on VSMC and endothelial cells. ET$_B$ receptors are Gq-protein coupled and, in VSMC, induce contraction and vasoconstriction in a fashion similar to that of the ET$_A$ receptor (64). In rat small mesenteric arteries, vasoconstriction due to ET$_B$ activation was additive to that mediated by ET$_A$ activation (188). The relative role of ET$_B$ receptor activation in ET-1 vasoconstriction is species and vessel-specific (discussed below). Interestingly, ET$_B$ activation in cat iris sphincter smooth muscle activates pathways involved in muscular relaxation; however, this effect has not been demonstrated in VSMC (73).
Endothelial ET<sub>B</sub> receptors, also Gq-protein coupled, are linked to NOS, which may account for ET-1-induced transient vasodilation (286, 290). Incidentally, this transient vasodilation has been noted in all species except the mouse (64). Two pathways are proposed for ET<sub>B</sub>-NOS coupling. First, via G-protein-mediated protein kinase B/Akt phosphorylation and second through a tyrosine kinase and calcium-calmodulin dependent pathway thereby activating NOS resulting in NO release (171, 299). Systemic ET<sub>B</sub> inhibition in rabbits caused an elevation in blood pressure indicating basal ET<sub>B</sub>-mediated NO release (101).

The physiologic relevance of endothelial ET<sub>B</sub> receptors has been elucidated in ET<sub>B</sub> receptor knockout mice. Mean blood pressure was elevated ~20 mmHg in ET<sub>B</sub> knockout animals compared to controls and neointimal formation following vascular injury was substantially increased in these animals (195, 204). Thus, basal endothelial ET<sub>B</sub> activation and NO production appears to play a role in long-term blood pressure regulation and limits vascular remodeling mediated by elevated ET-1 following injury in mice. Moreover, ET<sub>B</sub> receptors act as ET-1 clearance receptors and have been shown to mediate a positive autocrine loop by which ET-1 stimulates expression of its own gene (35, 127).

The opposing actions mediated by endothelial and smooth muscle ET<sub>B</sub> activation was demonstrated in the dog coronary vasculature by Teerlink et al (293). This group found transient vasodilation upon coronary infusion of low dose (0.1 and 0.3 µg) sarafotoxin S6c, a highly selective ET<sub>B</sub> agonist, and vasoconstriction at high doses (1.0 and 3.0 µg). This suggests, and is supported by competition binding assays, that ET<sub>B</sub>
located on VSMC and endothelial cells have differential affinities to ET-1 (293). Therefore, in dogs, endothelial ET_B receptors maintain a higher affinity for ET-1 than VSMC ET_B receptors. Overall, ET_B receptors can limit ET-1 vasoconstriction through inducing NO release but also contribute to this vasoconstriction by contracting VSMC.

Vascular Regulation by ET-1. It is commonly accepted that stimulation of ETA and ET_B receptors on VSMC causes vasoconstriction while activation of endothelial ET_B receptors induces NO-dependent vasodilation. The dominant vascular effect of ET-1, however, is a slow-onset sustained vasoconstriction. This has been demonstrated in numerous studies. Systemically, ET-1-induced elevations in mean arterial pressure have been noted in human, dog, rat, and mouse studies (2, 27, 28, 220, 221, 314). ET-1-induced transient vasodilation, due to ET_B-mediated NO release, is less consistently reported and, as suggested in dogs, may be dependent on the mode of ET-1 delivery (e.g. bolus or infusion) (2, 101, 314). The role of ET_B-mediated NO release in this transient vasodilation was confirmed by a similar biphasic response (vasodilation followed by vasoconstriction) to systemic infusion of the ET_B selective agonist IRL-1620 in rats (210).

While the overall effect of vascular ET-1 is vasoconstriction, the relative contributions of VSMC ET_A and ET_B receptors to this response appear to vary across species and regionally with species. Lodge et al (172) demonstrated that ET-1-mediated vasoconstriction of the rat aorta occurs primarily through ET_A receptors whereas in hamster aorta this response occurs due to ET_A and ET_B activation. Likewise, within species, ET-1 vasoconstriction in rat thoracic aorta and carotid artery occurs due to ET_A
activation whereas ET\textsubscript{B} receptor activation accounts for this response in rat pulmonary artery (324). In the mouse, 70% of systemic vasoconstriction to ET-1 is estimated to be a result of ET\textsubscript{A} activation with the remainder due to ET\textsubscript{B} activation (27). The involvement of endothelial ET\textsubscript{B} receptors, linked to NO production, also seems to vary within species. In the rat, ET\textsubscript{B}-mediated NO release attenuates ET-1 vasoconstriction in some (kidney, intestines, mesentery) but not all vascular beds whereas systemic ET\textsubscript{B} inhibition in rabbits and mice increases mean arterial pressure (6, 101, 204). Therefore, the ET-1 receptor system is magnificently complex in form and function.

In the coronary vasculature, basal ET-1 release contributes to baseline coronary tone in rodents and humans (99, 175, 182). Intracoronary infusion of the ET\textsubscript{A} receptor antagonist BQ-123 revealed that endogenous ET-1 accounts for 39% of basal tone in human epicardial and coronary resistance vessels (156). Similarly, endogenous ET-1-mediated vasoconstriction accounts for 29% and 50% of basal coronary tone in isolated rat hearts and mouse intraventricular artery rings, respectively (99, 175). These findings are important because they confirm that endogenous ET-1 concentrations are high enough to cause coronary vasoconstriction. In normal and pathological states, circulating plasma ET-1 levels are well below the concentrations suggested necessary to incite ET-1 receptor binding. However, due to the predominant autocrine and paracrine activity of ET-1, plasma ET-1 levels likely do not provide an adequate measure of local ET-1 biological activity (88).

The relative contributions of ET\textsubscript{A} and ET\textsubscript{B} receptors to coronary vascular responses also appear to vary across species and regionally within the coronary circuit.
ET_A receptor responses predominate, with varying degrees of VSMC ET_B involvement, in ET-1-mediated coronary vasoconstriction (39, 226, 227, 267, 317). Varied involvement of endothelial ET_B receptors has also been noted by evidence that selective ET_B agonists can induce vasoconstriction or vasodilation. Specifically, the ET_B agonist sarafotoxin S6c induces porcine left anterior descending artery constriction and dilation of porcine small coronary arterioles (267, 280). Similar responses have been noted in the perfused rat heart (19, 317). Of note, little is known concerning the relative roles of ET_A and ET_B receptors in coronary ET-1 responses in the mouse. However, it has been established that the ET_A to ET_B receptor ratio, receptor affinities and densities in mouse heart are similar to those seen in rat and human hearts (326).

*Type 2 Diabetes and ET-1.* Vascular ET-1 production and activity is normally limited due to NO-mediated ECE inhibition and receptor effects (discussed below). Reduced NO bioavailability such as occurs in T2DM can therefore elicit significant changes in the ET-1 system. Dysfunction of the vascular ET-1 system in T2DM, thought to be driven by hyperinsulinemia and reduced NO bioavailability, has been described (259, 323).

Animal models of T2DM and hyperinsulinemia have revealed enhanced ET-1, ET_A, and ET_B receptor gene expression (136, 339), enhanced ET-1 receptor binding (117, 143), and increased peak intracellular calcium concentrations following ET-1 receptor activation (117). Increased tissue and plasma ET-1 levels have been noted in some, but not all, animal and human studies (16, 136, 206, 262, 307, 335, 339). Elevated plasma ET-1 in humans may result from the increased ECE activity, possibly due to
hyperglycemia via PKC activation (11, 145). Conversely, ECE activity was reduced by >50% by superoxide in bovine aortic endothelial cells indicating that ET-1 production is influenced by oxidative states such as T2DM (173). Therefore, significant alterations in ET-1 production and receptor expression can occur in T2DM.

ET-1 may play an enhanced role in basal vascular tone in T2DM humans (41, 181, 182, 185, 186). Animal models of T2DM and insulin resistance have revealed increased (136, 143, 298), decreased (141, 247, 308) and unchanged (206) ET-1-mediated vasoconstriction. Due to significant differences in experimental design, the variation in these responses is difficult to reconcile. However, it could be related to differences in one or more of the following: species, metabolic parameters (overt T2DM versus hyperinsulinemia), vessel size or type (aorta versus small arterioles), or duration/severity of T2DM or hyperinsulinemia. For example, two studies examining mesenteric arteries from the hyperinsulinemic, but not hyperglycemic, fructose-fed rat exhibit reduced and enhanced ET-1 vasoconstriction, respectively. Specifically, reduced responses were noted in the large conduit superior mesenteric artery from 16 week fructose fed rats whereas enhanced ET-1 reactivity was noted in small mesenteric resistance arterioles from 4 week fructose-fed rats (143, 308). Therefore, the presence of numerous conflicting design issues is evident thereby limiting comparison. The former study may suggest that 16 weeks of enhanced local ET-1 production leads to a downregulation of ET-1 receptors. Direct measurement of ET_A and ET_B mRNA expression in these vessels, however, shows upregulation that is closely related to the level of hyperinsulinemia (339). Long-term systemic ET_A or mixed ET_A/ET_B receptor
blockade with darusentan or bosentan, respectively, caused a reduction in blood pressure in T2DM and hyperinsulinemic rats (307, 333). Therefore, while regional differences in ET-1 dysfunction may exist, an ET-1-mediated systemic pressor effect appears to dominate in these states.

**Diabetic Metabolic Abnormalities and ET-1.** While numerous factors influence the vascular ET-1 system, the central mediators of altered ET-1 reactivity in T2DM appear to be hyperinsulinemia and oxidative stress. As presented previously, enhanced oxidative stress reduces NO bioavailability thereby reducing the inhibitory effect of NO on the ET-1 system. These interactions will be discussed further in Section F. Although not a central factor, hyperglycemia has been shown to induce ET-1 release from bovine endothelial cells, but not in human umbilical vein endothelial cells in culture (187, 215, 341). A correlation between hyperglycemia or hemoglobin A1c, a marker of prolonged hyperglycemia, and plasma ET-1 was not found in T2DM patients with poor glycemic control (258).

A distinct interplay between insulin and the ET-1 system in vascular tissue has been elucidated. In cell culture, physiologic doses of insulin stimulate ET-1 release from human endothelial cells while severely pathophysiologic doses of insulin upregulate rat VSMC ETA expression (85, 87, 117). Studies using isolated resistance vessels support insulin-stimulated ET-1 release and vasoconstriction following NOS inhibition. This is likely due to insulin-mediated NO release that inhibits ET-1 production (76). Therefore, insulin has the capacity to stimulate both NO and ET-1 release.
Clinically, plasma ET-1 in T2DM patients exhibits a strong correlation with total insulin exposure independent of glycemic control (335). However, exogenous hyperinsulinemia in healthy men did not induce endogenous ET-1 release, although dissenting findings exist suggesting the necessity for other T2DM symptoms and emphasizing the widespread implications represented by increased ET-1 levels (43, 187, 241). The authors fully recognize, however, the limitation that these results cannot be readily extrapolated to diabetic subjects. Interestingly, twenty-five day insulin infusion induced ET-1-dependent hypertension in rats (137). These results suggest that prolonged hyperinsulinemia may induce insulin resistance characterized by insulin receptor uncoupling and reduced insulin-mediated NO release with unabated insulin MAPK signaling thereby stimulating ET-1 production. Studies have also shown that insulin signaling is necessary for basal ET-1 expression in mouse endothelial cells (309). Conversely, high levels of ET-1 are known to negatively impact cellular insulin signaling thus exacerbating insulin resistance (133). Therefore, shifts in vascular insulin activity during insulin resistance, particularly insulin-mediated ET-1 system effects can amplify existing perturbations in the NO-ET-1 balance and contribute significantly to the pathogenesis of vascular disease. Clearly however, the biology of the ET-1 system in states of insulin resistance and T2DM needs to be further explored.

E. Alpha1-adrenoceptors

\( \alpha_1 \)-adrenoceptors (\( \alpha_1 \)-AR) are classic G-protein coupled receptors found on VSMC. These receptors are members of a larger population of adrenergic receptors that includes \( \alpha \)- and \( \beta \)-AR. Activation of vascular \( \alpha_1 \)-AR by the catecholamines,
norepinephrine (NE) and epinephrine (EPI), results in VSMC contraction and vasoconstriction. This occurs via Gq-protein-mediated PLC activation leading to DAG and IP₃ production, PKC activation and a subsequent rise in intracellular calcium (104). Basal α₁-AR activation has been demonstrated in human, dog and rat vasculature (72, 108, 115, 266, 327). Therefore α₁-AR activation is an important determinant of vascular tone. α₁-AR-mediated vasoconstriction has been documented in various resistance artery beds including hamster cheek pouch (184), rat mesentery (54, 216), dog (69, 337) and mouse (175) coronaries. Recently, α₁-ARs have been shown to activate numerous other cellular second messengers including the MAPK pathway (155). In contrast to the α₁-AR, β₂-adrenoceptors (β₂-AR) located on VSMC induce vasodilation upon activation by catecholamines (281). Therefore, analyses of pure α₁-AR-mediated vascular responses require the utilization of a selective α₁-AR agonist such as phenylephrine (PE).

Coronary α₁-AR. Classic studies examining adrenergic vascular reactivity were performed in the coronary vessels of large mammals. In a seminal paper prior to our present knowledge of adrenergic receptors, Berne described the effect of EPI and NE in the dog coronary circulation (26). He found that NE and EPI produced a transient decrease in coronary blood flow, due to vasoconstriction, followed by a prolonged increase in coronary blood flow, due to vasodilation. The immediate vasoconstriction resulted from a direct effect of the catecholamines on the coronary vessels whereas the prolonged vasodilation was due to metabolic factors resulting from myocardial stimulation. A later study found that VSMC from large and small coronary vessels differ in their response to catecholamines with small vessel VSMC relaxing and large vessel
VSMC contracting, some only transiently (356). The clear role of \(\alpha\)-AR in this vasoconstriction was subsequently proven by Feigl using \(\beta\)-AR blocking drugs in the presence of direct sympathetic stimulation of the coronary vasculature (82). This was subsequently validated by Woodman et al (297) in conscious dogs (337). It is now known that vasoconstriction to catecholamines is dependent partly on the relative distribution of \(\alpha\)-ARs throughout the vasculature and in individual vessels. Chilian found in dogs that larger coronary arteries (>100 \(\mu\)m) constrict in response to exogenous NE whereas small coronary arteries (<100 \(\mu\)m) dilate, possibly due to intrinsic autoregulatory mechanisms (52). These responses were duplicated using the \(\alpha_1\)-AR agonist PE (52, 53). Today it is established that vascular \(\alpha_1\)-AR activation, via sympathetic outflow or circulating catecholamines, results in vasoconstriction and that in the coronary circulation this is often overridden by dominant local metabolic and endothelial influences (287, 337).

*Vascular Regulation by \(\alpha_1\)-AR.* Modern technology has allowed for elegant evaluation of the role of \(\alpha_1\)-ARs and their modulating factors. Mouse knockouts of the \(\alpha_{1D}\)- and \(\alpha_{1A/C}\)-AR subtypes show that these receptors are necessary to regulate arterial blood pressure and are located in the resistance vasculature (249, 292). The dominant modulators of catecholamine-induced vasoconstriction appear to be NO and metabolic factors (68, 134, 149, 239). Research suggests that \(\alpha_1\)-AR-mediated vasoconstriction may result in increased endothelial shear stress increasing the release of NO thereby limiting vasoconstriction (277). Furthermore, recent evidence suggests the presence of \(\alpha\)-ARs on the endothelium that may increase endothelial cell intracellular calcium resulting...
in an increased release of NO (301). This \( \alpha_1 \)-AR-mediated release of NO is further supported by the proposition that, in VSMC, the increased intracellular calcium from \( \alpha \)-AR activation is able to diffuse through myoendothelial gap junctions into endothelial cells thereby activating NO production and release (31, 344). In fact, NO operates in numerous ways to limit and modulate sympathetically-mediated vascular responses (discussed in Section F). Overall, a natural competition exists between \( \alpha_1 \)-AR-mediated coronary vasoconstriction and locally-mediated vasodilation (81). In healthy individuals, \( \alpha_1 \)-AR coronary vasoconstriction is physiologically limited; however, the manifestation of endothelial dysfunction may shift the balance of these opposing vascular effectors.

*Type 2 Diabetes and \( \alpha_1 \)-AR.* Dysfunction in the sympathetic control of blood pressure has been implicated as a cause of hypertension in some T2DM patients. Approximately 73% of adult T2DM patients in the United States are hypertensive (48). The current understanding of the relationship between insulin resistance and hypertension is unclear and two potential explanations exist. First, it is thought that chronically high blood insulin levels activate the sympathetic nervous system centrally leading to sympathetic overactivity manifest as an elevated heart rate, cardiac output, peripheral vascular resistance, renal sodium retention and ultimately hypertension (218). The neural effects of insulin are well established (10). In a study of 45 hyperinsulinemic, normotensive, nondiabetic individuals, Facchini et al (77) found a significant association between elevated heart rate and insulin resistance/hyperinsulinemia. Heart rate was used as a measure of central sympathetic outflow. Further, Anderson et al (9) showed that an acute physiologic blood insulin elevation increases sympathetic neural outflow and
produces forearm vasodilation. The latter response is likely due to normal insulin-stimulated NO production, which counteracts the sympathetically-mediated vasoconstriction. Examination of sympathetic activity in T2DM has yielded conflicting results (122, 289). Some animal models of T2DM also exhibit hypertension driven by increased sympathetic activity (44, 190). The second theory proposes that hypertension may lead to insulin resistance through negative vascular remodeling (218).

While the effect of insulin on sympathetic outflow is established and sympathetic hyperactivity is noted in many T2DM patients, this can not be directly extrapolated to local blood flow regulation in T2DM. In fact, sympathetic overactivity is not present in all T2DM patients. Hogikyan et al (116) found elevated brachial artery NE responsiveness and increased α-adrenergic tone in the absence of increased systemic sympathetic activity. Therefore, local α-adrenergic machinery can be influenced independent of systemic neural input. Enhanced, reduced and unchanged vascular NE responses have been reported in human and animals models of insulin resistance and T2DM (138, 140, 183, 216, 263). Focused analyses of α₁-AR-mediated responses using PE have yielded similar results of enhanced or unchanged vasoconstriction in T2DM (183, 192, 206, 211, 300, 346). Therefore, significant alterations in systemic and local α₁-AR activity have been demonstrated in T2DM.

In isolated cremaster arterioles from the T2DM Otsuka-Long-Evans-Tokushima Fatty (OLETF) rat, Toshida et al (346) found that α₁-AR sensitivity increased only after the onset of insulin resistance. The OLETF rat develops insulin resistance spontaneously around 16 weeks of age at which time enhanced sensitivity to the vasoconstrictor effect
of PE is noted. This suggests that T2DM-related changes in $\alpha_1$-ARs are dependent on the onset of insulin resistance. Determining whether insulin or glucose primarily influences vascular $\alpha_1$-AR in T2DM remains difficult. However, a novel study by Turner and White may help in the understanding of these relationships. In this study, PE responses were determined in the aorta and mesenteric circulation of lean Zucker rats, the hyperinsulinemic obese Zucker rat and obese Zucker rats made hyperglycemic and hypoinsulinemic by injection with the beta-cell toxin streptozotocin (STZ). This study revealed enhanced and unchanged mesenteric PE responses in STZ-treated and obese Zucker rats, respectively. Aortic PE responses were enhanced in both groups. However, the mesenteric vascular results suggest that hyperglycemia and hyperinsulinemia may differentially affect $\alpha_1$-AR with the former increasing $\alpha_1$-AR responsiveness. This study also suggests regional vascular $\alpha_1$-AR effects of T2DM since aortic PE responses were unchanged (300).

The mechanisms responsible for high glucose or insulin-mediated alterations in vascular $\alpha_1$-AR responses are not clear. Hyperglycemia upregulates and activates PKC in vascular tissue and endothelial cells while increasing DAG synthesis (157, 162, 334). This may suggest enhanced $\alpha_1$-AR signaling due to PKC activation; however, in vitro studies have revealed that PKC activation using phorbol esters inhibits $\alpha_1$-AR activity and its coupling to inositol phospholipid metabolism in cultured smooth muscle cells and hepatocytes (58, 163, 177). Insulin has also been shown to influence $\alpha_1$-AR signaling. In cell culture, insulin-mediated phosphorylation and desensitization of $\alpha_{1B}$-ARs has been demonstrated (92). However, regional hyperinsulinemia in humans did not alter
adrenergic responsiveness (288). Therefore, it appears that both hyperglycemia and hyperinsulinemia can influence vascular $\alpha_1$-AR activity in T2DM.

In the presence of heightened sympathetic activity, it is plausible that $\alpha_1$-AR could become downregulated due to overstimulation. Examination, in cell culture, of human $\alpha_1$-AR subtype responses to overstimulation revealed that some subtypes are regulated as such; however, others were upregulated possibly in compensation (343). In the OLETF rat, $\alpha_{1B}$-AR upregulation was observed with no change in $\alpha_{1D}$-AR expression (346). However, $\alpha_1$-AR overstimulation may not be present in all models of T2DM, since unchanged or reduced cardiac or plasma NE concentrations have been noted in T2DM humans and rats (37, 151, 153). Regardless, a detrimental influence of T2DM on vascular $\alpha_1$-AR responses has been elucidated and warrants further examination, especially within the coronary vasculature of T2DM models.

**F. Functional NO, ET-1, and $\alpha_1$-AR Interactions**

An abundance of research has been conducted individually on each of the three systems described above (Sections C, D, and E) in healthy and T2DM humans and animals. Physiologically, however, these substances do not act alone in the vasculature and often share receptors or second messenger systems in exerting their effects (69, 213). The balance of vasoconstrictor and vasodilator influences is imperative for the maintenance of normal vascular tone and function. Therefore, the interactions between these systems and their subsequent modification in T2DM are critical in understanding vascular regulatory changes in this state, especially in the coronary circulation where dysregulation may lead to impaired vasodilator responses, coronary vasospasm, ischemic
heart disease and myocardial infarct. Current literature pertaining to these interactions in healthy and T2DM models is presented below.

**α_1-AR and NO.** An interaction between α_1-ARs and NO is well established and is principally demonstrated by numerous studies showing a potentiation of α_1-AR-mediated vasoconstriction following endothelial denudation or NOS inhibition (31, 134, 139, 201, 206, 216, 294, 301, 346, 350). Centrally stimulated sympathetic vasoconstriction is also augmented following NOS blockade and local NE release is attenuated by both endogenous and exogenous NO (98, 149, 265). Taken together, these results suggest that locally produced NO, regardless of stimulus, acts to limit α_1-AR- and sympathetically-mediated vasoconstriction.

The proposed mechanisms underlying this relationship were presented in part in Section E. Briefly, α_1-AR-mediated vasoconstriction is primarily limited by NO release due to elevated wall shear stress during vasoconstriction (277). It has also been demonstrated that α_1-AR activation leads to direct NO release from endothelial cells due to increased endothelial intracellular calcium and NOS activation (301). The elevation in intracellular calcium may result from calcium movement from VSMC to endothelial cells through gap junctions or by activation of a proposed α_1-AR on endothelial cells (31, 139, 301, 344). In hamster cremaster arterioles, PE-induced vasoconstriction was enhanced in the presence of endothelial cell intracellular calcium chelation supporting the role of α_1-AR stimulated endothelial NO release (344).

A similar, although altered, relationship between α_1-AR and NO exists in T2DM. While α_1-AR vasoconstriction is enhanced following NOS inhibition or endothelial
denudation, the magnitude of this change is often, but not always, reduced in T2DM (157, 205, 206, 216, 346). This can be attributed to $\alpha_1$-AR activation inducing a more near maximal vasoconstriction at any agonist dose prior to NO blockade due to reduced NO bioavailability in T2DM. Kanie and Kamata demonstrated in $db/db$ mouse aorta that treatment with superoxide dismutase (SOD) to alleviate oxidative stress attenuated NE vasoconstriction, whereas treatment of control aortas with an inhibitor of endogenous SOD resulted in elevated NE vasoconstriction (140). This reinforces the idea that reduced NO bioavailability is a primary cause of enhanced $\alpha_1$-AR vasoconstriction in T2DM. Increased $\alpha_1$-AR expression has also been noted in T2DM rats and may contribute to this elevated contractile state in arterioles (346). However, it should be noted that not all models of T2DM exhibit enhanced vascular $\alpha_1$-AR responses. This may point to a temporal change in $\alpha_1$-AR responsiveness as endothelial cell dysfunction becomes more pronounced.

*ET-1 and NO.* As discussed, NO and ET-1 appear to be biological counterparts within the vasculature. ET-1 release is inhibited directly by NO through a guanylyl cyclase-cGMP dependent mechanism, and is also regulated by endothelial shear stress within a narrow range of coronary flow rates (33, 144). Hence, coronary vasoconstriction to ET-1 is limited by shear stress dependent release of NO (277). Prior inhibition of NO production permits enhanced coronary vasoconstriction to exogenous ET-1 (277). Goligorsky et al (96) also demonstrated that NO directly attenuates the ET-1-induced rise in intracellular calcium in VSMC. This occurs through two NO actions: (1) by displacing bound ET-1 from the ET$_\Lambda$ receptor and (2) by interfering with post-receptor ET-1-
mediated calcium mobilization pathways. Conversely, ETA inhibition did not alter NO production in a human leg blood flow study (182).

Numerous studies have revealed this functional inhibition of NO on ET-1. It is commonly demonstrated that ET-1, acting through the ETA receptor, contributes to the vasoconstrictor response to NO inhibition (101, 201, 245, 295). In isolated rat hearts, Wang et al (316) demonstrated a 30% decrease in coronary flow after NOS blockade that was attenuated to 16% by pre-treatment with BQ-123, a selective ETA receptor antagonist. This may be accounted for by enhanced endothelial release of ET-1; however, this conclusion is limited since ECE inhibition did not reduce L-NAME vasoconstriction in one study (33, 101, 245). This study also found that ETB receptor-mediated NO production acts independently of basal endothelial NO production since ETB blockade in the presence of L-NAME resulted in an increased constrictor response (101). This is supported in the human forearm where ETB-mediated NO release has been demonstrated to play a role in basal vascular tone since ETB inhibition significantly reduced, but did not inhibit, vasoconstriction to NOS inhibition (42).

T2DM-mediated alterations in the interaction between NO and ET-1 are difficult to pinpoint. As mentioned previously, changes in the vascular responses to exogenous and endogenous ET-1 are variable in this state. Also, elevated plasma ET-1 levels have been noted in some T2DM studies. This may simply be the result of varying levels of endothelial dysfunction and therefore NO bioavailability. However, other mechanisms have been proposed. In coronary arteries of the insulin-resistant Zucker obese rat, it has been suggested that the reduction in ET-1 vasoconstriction is due to enhanced ETB-
stimulated NO production along with the uncoupling of ET\textsubscript{$\alpha$} receptors from activating calcium mobilization pathways (141). Impaired ET\textsubscript{$\alpha$} activity has also been noted in T2DM human coronary arteries (156). However, in the insulin-resistance fructose-fed rat, enhanced mesenteric ET-1 vasoconstriction has been attributed to increased ET\textsubscript{$\alpha$} receptor expression coupled with reduced NO bioavailability (143). Therefore, regional differences may exist; however, the role of insulin may be the linchpin in these alterations.

Numerous studies in normal and insulin-resistant states have demonstrated that insulin can stimulate the balanced endothelial release of both NO and ET-1 (76). Acute hyperinsulinemia in healthy subjects leads to NO and ET-1 release as determined by a vasodilator response to ET-1 receptor inhibition and a higher than baseline vasoconstrictor response to NOS inhibition (43). The balance of these actions is shifted by T2DM-related alterations in insulin signaling, however, resulting in impaired insulin-mediated NO release and enhanced insulin-mediated ET-1 release (233). Also, an enhanced local ET-1 production in T2DM or insulin resistance may exacerbate insulin resistance due to the inhibitory effect of ET-1 on insulin signaling. Therefore, alterations in the NO-ET-1 interaction in T2DM may be partly dependent on insulin signaling defects in T2DM coupled with direct alterations in the NO and ET-1 systems.

\textit{$\alpha_{1}$-AR and ET-1.} Research has shown that $\alpha_{1}$-AR and ET-1 receptors are capable of a significant amount of receptor crosstalk with other intracellular signaling systems. Therefore, recent literature describing functional interactions between these systems is not surprising. In rat-1 fibroblasts transfected with hamster $\alpha_{1B}$-ARs, ET\textsubscript{$\alpha$} activation led
to α1-AR phosphorylation, altered G-protein coupling and attenuation of NE-induced calcium mobilization (306). It is suggested that this phosphorylation may occur partly through the action of PKC for two reasons: (1) the temporal time course for ET-1-mediated α1-AR phosphorylation is similar to that found for PKC translocation and (2) the α1-AR phosphorylation was inhibited almost completely by staurosporine and genistein in combination suggesting the involvement of serine/threonine and tyrosine kinases.

Functionally, it was demonstrated in dog coronaries that pretreatment with an ECE inhibitor or ET\textsubscript{A} receptor blocker resulted in inhibition of the long-term component of α1-AR-mediated vasoconstriction (69). This suggests that α1-AR activation stimulates ET-1 release. It has also been found that ET-1 enhances sympathetic nerve-induced constriction for a period of 30 minutes at high concentrations in rabbit ear arteries (336). Recently, various studies exhibiting an inhibitory influence of ET-1 on α1-AR-mediated responses have been published. D’Angelo et al (62) have demonstrated using a genetic model of ET-1-dependent hypertension that elevated endogenous ET-1 suppresses the sympathetically-driven pressor response to acute environmental stress. The same group reported similar results \textit{in vivo} in Dahl salt-resistant rats showing a potentiated pressor response to acute environmental stress following ET\textsubscript{A} receptor inhibition (63). In contrast, other studies have reported no effect of ET-1 on sympathetically-mediated responses. In the rat mesenteric vasculature, exogenous ET-1 did not influence NE release from sympathetic terminals; however, it did decrease the release of the sympathetic cotransmitter neuropeptide Y (114). ET\textsubscript{A} receptor inhibition with BQ-123
did not alter sympathetically-mediated vasoconstriction in dog skeletal muscle (149). In addition, combined BQ-123 and BQ-788 pretreatment did not alter human forearm vasoconstriction to exogenous NE (42).

Little is known concerning the interaction between $\alpha_1$-AR and ET-1 in models of insulin resistance or T2DM. Okon et al (206) demonstrated in diabetic $ob/ob$ mice that mixed $ET_A/ET_B$ receptor inhibition had no effect on aortic contractile responses to PE. Therefore, since both $\alpha_1$-AR and ET-1-mediated vascular activities play a role in the determination of vascular tone, a shift in the interaction between these two systems could prove to be deleterious, particularly coupled with reduced NO bioavailability. Clearly, this interaction in these states needs to be more closely examined.

In summary, numerous interactions exist between the vascular NO, ET-1 and $\alpha_1$-AR systems. Furthermore, significant alterations in these systems occur in T2DM and may therefore influence the activity of other vascular regulatory systems. For clarity, the prominent interactions discussed above are presented graphically in Figure 1.

**Figure 1.** Mechanisms of interaction between NO, ET-1 and $\alpha_1$-AR. L-NAME, BQ-123 and BQ-788 are inhibitors for the noted enzymatic and receptor-ligand interactions. This figure is printed with permission from R.E. Klabunde.
G. Diet-induced Type 2 Diabetes in C57BL/6J Mice

Numerous mouse models are used today to analyze the pathological effects of T2DM. These include models with genetically, chemically and diet-induced forms of the disease. Currently, the most popular models are those that manifest obesity-related T2DM, such as the OLETF and Zucker rats, db/db mouse, ob/ob mouse and the diet-induced obese C57BL/6J mouse (51). Of note, each of these mouse models was developed using a C57BL mouse strain that has a genetic predisposition toward T2DM. Metabolically, all are known to develop hyperglycemia and hyperinsulinemia (168, 283). The diet-induced T2DM mouse has been shown to exhibit consistent levels of hyperglycemia under resting and stress conditions (95, 283, 284).

Diet-induced T2DM occurs in C57BL/6J in response to high-fat or high-fat, high-carbohydrate feeding. Various diets combining different percentages of dietary fat and carbohydrate are used throughout the literature. Initial characterization of this model of T2DM by Surwit et al (283) showed that fasting blood glucose levels were 248 mg/dl compared to 170 mg/dl in controls while blood insulin levels increased 10-fold and body weight close to doubled (~27 g versus ~50 g). Other studies have yielded similar results (34, 56, 243, 322). However, the metabolic adaptation of C57BL/6J to a high-fat diet is heterogenous. Burcelin et al (36) demonstrated that following nine months of high-fat feeding, obesity and T2DM was developed in 50% of the mice studied while 10% were lean and diabetic, 10% were lean and nondiabetic and 30% manifest an intermediate phenotype. Analysis of the genetics behind these adaptations revealed that the
hyperinsulinemia and hyperglycemia in this model are controlled by different genetic factors (284).

The physiological basis for insulin resistance in these mice remains unclear; however, it appears to involve pancreatic dysfunction exacerbated by obesity. Lean C57BL/6J mice exhibit a defect in the second-phase of glucose-stimulated insulin release that is magnified by high-fat, high-carbohydrate feeding. Following high-fat feeding, β-cell senescence and altered pancreatic gene expression in these animals contributes to impaired insulin release (238, 272). The compensatory increase in first phase insulin release in response to insulin resistance is also reduced in these animals (3, 321). Therefore, this underlying defect may join with other factors following the onset of obesity in inducing T2DM. However, it has been suggested that the insulin resistance seen in this model is not solely related to changes in body weight.

Obesity appears to be a central component of T2DM development in these mice. Correspondingly, significant alterations in fat distribution and fat cell number and size, have been demonstrated. Following seven weeks of high-fat, high-carbohydrate feeding, C57BL/6J mice exhibited a nearly 200% increase in total carcass lipid content related to significantly larger epididymal, retroperitoneal, mesenteric and inguinal fat depots (243, 282, 322). Nonadipose tissue triglyceride levels are also increased two- to five-fold in the pancreas, liver and skeletal muscle suggesting a role for lipotoxicity (252). Further, dramatic fat cell hypertrophy and hyperplasia were also noted in the inguinal and mesenteric fat pads following four and five months of high-fat, high-carbohydrate feeding (243, 282). Adipocyte lipoprotein lipase, the key enzyme for fat accumulation,
activity was also increased (243, 282). However, norepinephrine-stimulated lipolysis was unchanged (282). Total cholesterol, triglycerides and low-density lipoprotein (LDL) levels are all elevated in this model whereas high-density lipoprotein levels vary in the literature (111, 208, 214, 243, 264). These data support the role of fat in the development of T2DM in this model. In fact, data show that accumulation of fat, not caloric intake, is the critical stimulus for obesity and T2DM in these mice (223, 282). Reduced physical activity has also been ruled out as an inciting cause for obesity. Therefore, many have suggested that these animals have genetically determined alterations of lipid handling mechanisms. Of note, diet-induced obesity and T2DM in these animals is reversed by placing mice on a low-fat diet (212).

Other aspects of the human diabetic milieu are also present in this model of T2DM. These animals reproducibly exhibit impaired glucose tolerance (36, 120, 252). Glucose transport into adipose tissue was reduced whereas skeletal muscle glucose uptake was unchanged. However, membrane levels of the glucose transporter GLUT-4 were reduced in both tissues (252). Correspondingly, glycogen synthase activity is decreased in the skeletal muscle of high fat fed C57BL/6J mice (121). Skeletal muscle oxidative phosphorylation and mitochondrial biogenesis may also be impaired in these animals since three weeks of high fat feeding induced a downregulation and expression of genes necessary for these processes (274). In the liver, activity of the gluconeogenic enzyme PEPCK is enhanced (252). More recently, it has also been demonstrated that diet-induced obese C57BL/6J mice are resistant to the satiety hormone leptin (236). Immunocompetence is also reduced in this model (60).
Alterations in the endocrine, renal and cardiovascular systems are known to be associated with human T2DM (8, 48, 305). However, it is unclear whether these are primary defects of diabetes or secondary to altered insulin, carbohydrate, and lipid metabolism. Diet-induced T2DM mice do not exhibit marked hypothyroidism and hyperadrenocorticism, which is present in other mouse models of T2DM (95). Measures of renal function including urine flow, glomerular filtration and sodium, potassium and protein excretion rates are normal in these animals (202). A similar study with a higher dietary fat percentage (60% versus 35%) demonstrated renal lipid accumulation, proteinuria and glomerulosclerosis (131). Hypertension occurs in some, but not all, studies and is maintained by sympathetic nervous system hyperactivity (190, 202). However, in this model, when hypertension occurs it is not necessarily linked to insulin since obese controls developed higher insulin levels than the C57BL/6J high-fat fed mice without developing hypertension (190). Others have found hypertensive symptoms in these mice only when studied within their thermoneutral range (i.e., 30°C) (330). Other cardiovascular complications noted in this model include endothelial dysfunction, enhanced adrenergic responsiveness, atherosclerosis, enhanced myocardial contractility, and dilated cardiomyopathy (120, 192, 209, 347). Therefore, the diet-induced obesity-related T2DM model in C57BL/6J mice closely resembles the human condition. As such, it is becoming increasingly common in the study of the onset and complications of T2DM.
II. HYPOTHESIS AND SPECIFIC AIMS

The rationale for this study is that T2DM is known to be associated with vascular dysfunction characterized by altered responses to vasoactive substances. Specifically, responses to NO, ET-1 and $\alpha_1$-AR activation are known to be altered. In light of this, it can be suggested that the interactions between these substances will also be altered in T2DM, which may underlie associated vascular pathologies such as hypertension and coronary artery disease. The present study evaluated the hypothesis that type 2 diabetes, induced by a high-fat, high-carbohydrate diet, alters coronary vascular responses to PE, NO and ET-1 and their subsequent interaction in isolated C57BL/6J mouse hearts.

Specific Aims

1. To Determine Phenotypic Alterations Resulting from Diet-induced Obesity and T2DM in C57BL/6J Mice.

This specific aim is designed to validate the use of the diet-induced model of obesity and T2DM in our laboratory. This will be accomplished through measurement of mouse body weight, blood glucose, plasma insulin, plasma lipid profile (total cholesterol, triglycerides, HDL, LDL), and blood pressure. Our results will then be compared to those in the existing literature.

2. To Determine the Independent Actions of Nitric Oxide, Endothelin-1, and Phenylephrine in the Diabetic and Non-diabetic Mouse Coronary Circulation

This specific aim will quantify mouse coronary responses to PE, NO, and ET-1 in both diabetic and non-diabetic mice. This will allow for the characterization of basal
vascular responses to each of these drugs using doses in accordance with existing literature. Systematic analysis of C57BL/6J mouse coronary vascular reactivity has not been conducted. Therefore, these responses in non-diabetic animals, apart from acting as controls for the present study, will significantly add to our knowledge of coronary function in these mice.

3. To Determine the Interactions between Nitric Oxide, Endothelin-1, and Phenylephrine in the Diabetic and Non-diabetic Mouse Coronary Circulation.

This specific aim is put forth to determine, from a functional perspective, the potential role of NO, ET-1 and the selective $\alpha_1$-AR agonist PE in coronary responses elicited by each other. The intention of this specific aim is to elucidate the functional interactions between NO, ET-1 and PE in the mouse coronary circulation. The significance of this specific aim is two-fold. First, these experiments will expand our understanding of the basic science of vascular regulatory patterns and present a more physiologically relevant view of coronary vascular regulation in the mouse. Second, these experiments will serve as controls for subsequent studies aimed at elucidating alterations in vascular regulatory patterns in diabetic mouse hearts. This specific aim will examine ET-1 receptor involvement in vasoconstriction induced by NOS inhibition and $\alpha_1$-AR activation with PE. Additionally, we will examine the role of endogenous NO production in modulating ET-1 and PE-mediated coronary responses.
III. RESEARCH DESIGN AND METHODOLOGY

A. Experimental Procedure

*Animals.* The use of animals in this study was approved by the Institutional Animal Care and Use Committee of Ohio University. The diet-induced model of mouse obesity and T2DM has previously been described (283). Briefly, male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 3 wk of age. These animals were given ad libitum access to water and a high-fat, high-simple-carbohydrate, low-fiber diet containing 20.5% protein, 35.8% fat, 0.4% fiber, 3.6% ash, 3.1% moisture, and 36.8% carbohydrate (Bio-serve, Frenchtown, NJ; #F1850) for 15 wks. Control animals were obtained at 7 or 9 weeks of age and given ad libitum access to water and standard rodent chow containing 22.0% protein, 5.0% fat, 5.0% fiber, 6.0% ash, 2.5% added minerals, and 59.5% carbohydrate (LabDiet, Richmond, IN; Prolab RMH 3000). Control animals were maintained on this diet for 11 or 9 wks. Animals were housed 4 per cage in a temperature-controlled room with a 12-h light-dark cycle. All mice (25-50 g) were weighed and fasted (12 hours) on the day of the experiment. Laboratory handling of mice was kept to a minimum to minimize handling stress.

*Blood Glucose Determination.* On the experimental day, following an overnight fast (~12 h), blood glucose concentrations of conscious mice were measured using the tail snip method and a calibrated OneTouch Ultra® glucometer. Blood glucose was determined for all mice prior to any drug injections.

*Plasma Insulin Determination.* Plasma samples for insulin analysis were collected from a separate set of control (n=10) and T2DM (n=7) mice. These mice were anesthetized
with sodium pentothal (100 mg/kg, ip) and blood was collected from the chest cavity following cardiac puncture. Samples were placed in EDTA-coated tubes and centrifuged for 15 min at 2,000 rpm, the plasma removed, and analyzed by insulin radioimmunoassay (Diagnostic Systems Laboratory Inc., Webster, TX) at the New York State Animal Health Diagnostic Center at Cornell University College of Veterinary Medicine (Ithaca, NY).

**Blood Lipid Profile Determination.** Plasma lipid profiles were determined for six control and six T2DM mice. Each animal was administered heparin (300 IU, ip) and anesthetized with sodium pentothal (100 mg/kg, ip) prior to blood collection by decapitation. Blood was centrifuged for twenty minutes at 2000 rpm and the plasma isolated. Direct measurement of total cholesterol, triglycerides, high density lipoprotein (HDL), and low density lipoprotein (LDL) was determined by enzymatic analysis (Equal Diagnostics, Exton, PA; Roche Diagnostics, Indianapolis, IN) at IDEXX Laboratories (West Sacramento, CA).

**Intraperitoneal Glucose Tolerance Test.** Glucose tolerance tests were performed on six control and five T2DM mice by intraperitoneal injection of glucose. Immediately prior to injection, mice were weighed and baseline fasted blood glucose (T0) was determined with a calibrated OneTouch Ultra® glucometer. All blood samples were taken using the tail snip method. Mice were then injected with 2 g/kg glucose and blood glucose measurements taken at 15, 30, 60, and 120 minutes post-injection.

**Blood Pressure Determination.** Blood pressure was determined by carotid artery cannulation in six control and four T2DM anesthetized mice. Mice were anesthetized
with sodium pentothal (50 mg/kg, ip). The surgical site was shaved and the right carotid artery accessed by an incision lateral to the trachea. The right carotid was cleared of connective tissue, isolated from surrounding muscle and nerves, and cannulated with PE10 tubing filled with heparinized saline (100 IU/ml). Following cannulation, the cannula was immediately connected to a pressure transducer and blood pressure was measured for 10 minutes on a PowerLab® workstation. Blood pressure reported was averaged over this time.

**Isolated Mouse Heart Preparation.** Mice were administered heparin (300 IU, ip) and anesthetized with sodium pentothal (100 mg/kg, ip). Hearts were rapidly excised by bilateral thoracotomy, placed into ice-cold buffer, the aorta cannulated with a 20 gauge metal cannula and the left atrium removed for placement of a ventricular drain tube.

Each heart was mounted on the Langendorff perfusion apparatus and perfused under non-recirculating conditions at constant flow with 37°C Krebs-Henseleit buffer (KHB) consisting of (in mM): NaCl 118.5, NaHCO₃ 25, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 2.5, and glucose 11.1, gassed with 95% O₂ and 5% CO₂. The buffer was previously vacuum filtered to remove particulate with a 5.0 μ nitrocellulose filter. Once placed onto the perfusion apparatus, a ventricular drain tube was inserted through the mitral valve into the left ventricle. Hearts were submerged in 37°C KHB for the length of the experiment. The hearts equilibrated for 20 min on the perfusion apparatus before beginning the experimental protocol. Following equilibration, the pump flow rate was set to achieve an initial coronary perfusion pressure (CPP) of 60 mmHg, which was continuously monitored using a PowerLab® workstation. Coronary flow was held
constant during drug treatments; therefore, changes in coronary vascular resistance (CVR) were directly proportional to changes in CPP. The hearts were allowed to beat spontaneously; however, the hearts were not performing external work (i.e., no fluid ejection or pressure development) and there was no preload on the ventricles. At the end of the protocol, the hearts were removed, blotted, and weighed.

*Isolated Heart Experimental Protocols.*

**I. Nitric Oxide System Analysis**

*Protocol 1 - L-NAME.* This protocol was designed to determine the role of NO in the maintenance of basal coronary tone and as an indicator of endogenous NO bioavailability and addresses Specific Aim 2. We hypothesized that NO bioavailability would be reduced in T2DM hearts. Hearts were treated with the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; 10⁻⁵ M) for 30 minutes following equilibration. L-NAME was added directly to the buffer reservoir. Changes in CPP from the baseline of 60 mmHg were measured after 30 min. The concentration of L-NAME used in this study has been shown to be effective at inhibiting NOS in the isolated mouse heart by Castro et al (47).
**Protocol 2 - L-NAME + Tempol.** This protocol was designed to determine the effect of superoxide free radicals on NO bioavailability. We hypothesized that the removal of superoxide with Tempol would restore normal L-NAME vasoconstriction in T2DM due to reduced superoxide NO scavenging. The response to 30 min of L-NAME ($10^{-5}$ M) treatment was determined with and without a 15 min pretreatment with the superoxide dismutase mimetic 2,2,6,6-Tetramethyl-1-piperidinyloxy (Tempol, $10^{-4}$ M). L-NAME and Tempol were both added directly to the buffer reservoir. Changes in CPP from the baseline of 60 mmHg were measured after 15 min of Tempol treatment and after 30 min of Tempol plus L-NAME treatment. The concentration of Tempol used in this study has been shown to effectively reduce superoxide scavenging of NO in a model of enhanced oxidative stress in mouse mesenteric resistance vessels (312).

**Protocol 3 - L-NAME + BQ-123.** This protocol was designed to determine the role of endogenous ET-1 acting through the ET$_A$ receptor in L-NAME-induced vasoconstriction and addresses Specific Aim 3. We hypothesized that endogenous ET-1 acting via the ET$_A$ receptor contributes to L-NAME-mediated coronary vasoconstriction and that this involvement is elevated in
T2DM. Hearts were treated with a combination of L-NAME (10^{-5} M) plus the selective ET_{A} receptor antagonist BQ-123 (10^{-6} M) for 30 minutes following equilibration. L-NAME and BQ-123 were added directly to the buffer reservoir. Changes in CPP from the baseline of 60 mmHg were measured after 30 min. The concentration of BQ-123 used in this study has been shown to inhibit ET-1 vasoconstriction in mouse abdominal aorta and a lower concentration (10^{-7} M) inhibits this response in the mouse mesenteric and renal vasculatures (28, 351).

Protocol 4 - L-NAME + BQ-788. This protocol was designed to determine the role of endogenous ET-1 acting through the ET_{B} receptor in L-NAME-induced vasoconstriction and addresses Specific Aim 3. Specific hypotheses concerning vascular ET_{B} receptor effects are difficult to formulate due to little literature information and the opposing actions of VSMC and endothelial ET_{B} receptors. Hearts were treated with the combination of L-NAME (10^{-5} M) plus the selective ET_{B} receptor antagonist BQ-788 (10^{-6} M) for 30 minutes following equilibration. L-NAME and BQ-788 were added directly to the buffer reservoir. Changes in CPP from the baseline of 60 mmHg were measured after 30 min. A concentration of BQ-788 lower that that used in
this study ($10^{-7}$ vs. $10^{-6}$ M) has been shown to inhibit responses to the ETB selective agonist IRL-1620 and ET-1 in the mouse mesenteric and renal vasculatures (28).

Protocol 5 – SNAP following L-NAME. This protocol was designed to examine vascular smooth muscle responsiveness to NO system activation by exogenous NO and addresses Specific Aim 2. We hypothesized that vasodilation to exogenous NO would be reduced in T2DM hearts. Dose responses to the NO donor and potent guanylyl cyclase activator (±)-S-nitroso-N-acetylpenicillamine (SNAP, $10^{-11} – 10^{-5}$ M) were determined following a 30 min L-NAME ($10^{-5}$ M) pretreatment to eliminate endogenous NO production. SNAP was infused through a syringe infusion pump at 5% of coronary flow and was filtered for particulate during infusion with 0.22 μ syringe filters (Cameo 3N Nylon; Osmonics, Inc., Minnetonka, MN). Changes in CPP from the baseline of 60 mmHg were measured after 30 min for L-NAME responses. CPP changes due to the SNAP dose response were measured once a stable CPP was achieved at each dose from the baseline CPP achieved by maximal L-NAME-induced vasoconstriction. The doses of
SNAP used in this study are consistent with other mouse studies and $10^{-5}$ M induces maximal vasodilation in mouse carotid and femoral arteries (59).

Protocol 6 - 8-br-cGMP following L-NAME. This protocol was designed to examine the NO signaling cascade downstream of guanylyl cyclase. We hypothesized that vasodilation to activation of NO signaling would be similar in control and T2DM hearts. The vasodilator responses to the cell permeable cGMP analog 8-br-cGMP ($10^{-6} – 10^{-4}$ M) following 30 min of L-NAME pretreatment was measured. Similarly to SNAP, 8-br-cGMP was infused by a syringe infusion pump at 5% of the coronary flow and filtered. Changes in CPP from the baseline of 60 mmHg were measured after 30 min for L-NAME responses. CPP changes due to the 8-br-cGMP dose response were measured once a stable CPP was achieved at each dose (typically within 8 min) from the baseline CPP achieved by maximal L-NAME-induced vasoconstriction. The doses of 8-br-cGMP used in this study were shown to induce vasodilation in mouse aortic rings by Hussain et al with $10^{-4}$ M eliciting near maximal vasodilation (123).
II. \( \alpha_1 \)-Adrenoceptor System Analysis

Protocol 7 - PE. This protocol was designed to determine mouse coronary vascular responses to \( \alpha_1 \)-AR activation with the selective \( \alpha_1 \)-AR agonist phenylephrine (PE) and addresses Specific Aim 2. We hypothesized that coronary vasoconstriction to PE would be enhanced in T2DM hearts.

Following equilibration, CPP was set at 60 mmHg by adjusting coronary flow and hearts were perfused with vehicle for 30 min to account for drug pretreatments in other protocols. Following vehicle infusion, CPP was reset to 60 mmHg and dose response measurements to PE (\(10^{-9}\) to \(10^{-5}\) M) were performed. PE was infused through a syringe infusion pump at 5% of the constant flow pump rate and was filtered for particulate during infusion with 0.22 \( \mu \)m syringe filters. Responses to PE were measured when a stable CPP was achieved at each dose (typically within 8 min). The doses of PE used in this study are similar to those that have been demonstrated to induce dose-dependent vasoconstriction in mouse aorta, carotid, femoral and mesenteric resistance vessels (59, 157, 206, 211).
**Protocol 8 - PE + L-NAME.** This protocol was designed to determine the influence of endogenous NO on $\alpha_1$-AR-mediated coronary vasoconstriction induced by PE and addresses Specific Aim 3. We hypothesized that coronary vasoconstriction to PE would be enhanced in both control and T2DM following L-NAME pretreatment, but to a lesser degree in T2DM hearts. The time course of this protocol is identical to that above for PE alone except that hearts were perfused with L-NAME ($10^{-5}$ M) following equilibration for 30 min. L-NAME was added directly to the perfusate reservoir. As described above, CPP was reset to 60 mmHg after L-NAME pretreatment and CPP changes to PE dose response ($10^{-9} – 10^{-5}$ M) were measured from this baseline.

**Protocol 9 - PE + L-NAME + BQ-123.** This protocol was designed to examine the role of $\text{ET}_A$ receptors in $\alpha_1$-AR-mediated coronary responses to PE and addresses Specific Aim 3. We hypothesized that $\text{ET}_A$ receptor
inhibition would enhance vasoconstriction to PE in both groups but to a greater degree in T2DM hearts. All hearts in this protocol were pretreated with L-NAME (10^{-5} M) for 30 min. The selective ET_A receptor antagonist BQ-123 (10^{-6} M) was added in concert with L-NAME. Following L-NAME plus BQ-123 pretreatment, CPP was reset to 60 mmHg and CPP changes to PE dose response (10^{-9} – 10^{-5} M) were measured from this baseline.

Protocol 10 - PE + L-NAME + BQ-788. This protocol was designed to examine the role of ET_B receptors in α_1-AR-mediated coronary responses to PE and addresses Specific Aim 3. Specific hypotheses concerning this protocol are difficult due to the opposing actions of ET_B receptors and since the role of ET_B receptors in α_1-AR has not been previously examined. All hearts in this protocol were pretreated with L-NAME (10^{-5} M) for 30 min. The selective ET_B receptor antagonist BQ-788 (10^{-6} M) was added in concert with L-NAME. Following L-NAME plus BQ-788 pretreatment, CPP was reset to 60 mmHg and CPP changes to PE dose response (10^{-9} – 10^{-5} M) were measured from this baseline.
III. Endothelin-1 System Analysis

*Protocol 11 - ET-1.* This protocol was designed to determine mouse coronary vascular responses to the vasoactive peptide endothelin-1 (ET-1) and addresses Specific Aim 2. We hypothesized that coronary vasoconstriction to ET-1 would be enhanced in T2DM hearts. The time course of this protocol is identical to that described above for PE alone except that ET-1 (10^{-10} M) was added to the perfusate reservoir following the 30 min vehicle perfusion. As described above, CPP was reset to 60 mmHg following the 30 min vehicle infusion. Hearts were then perfused with ET-1 for 60 min and measurements were taken when a stable CPP was achieved. Pilot studies revealed that 10^{-10} M ET-1 induced pronounced vasoconstriction in isolated mouse hearts. This dose also elicits vasoconstriction in isolated mouse coronary arteries (222).
**Protocol 12 - ET-1 + L-NAME.** This protocol was designed to evaluate the influence of endogenous NO on ET-1-mediated coronary vasoconstriction and addresses Specific Aim 3. We hypothesized that ET-1-mediated coronary vasoconstriction would be enhanced in both groups following L-NAME pretreatment, but to a lesser degree in T2DM hearts. The time course of this protocol is identical to that described above for PE plus L-NAME except that ET-1 (10^{-10} M) is added to the perfusate reservoir following the 30 min L-NAME (10^{-5} M) pretreatment. As described above, CPP was reset to 60 mmHg following the 30 min L-NAME pretreatment. Hearts were then perfused with ET-1 for 60 min and measurements were taken when a stable CPP was achieved.

![Time Course Diagram](image)

**Protocol 13 - ET-1 + L-NAME + BQ-123.** This protocol was designed to examine the role of ETA receptors in ET-1-mediated coronary responses and addresses Specific Aim 2. We hypothesized that ETA receptor inhibition would reduce ET-1-mediated vasoconstriction in both groups. This protocol is identical to that described above for PE plus L-NAME and BQ-123 except that ET-1 (10^{-10} M) is added to the perfusate reservoir following the 30 min L-NAME (10^{-5} M) plus BQ-123 (10^{-6} M) pretreatment. As described above,
CPP was reset to 60 mmHg following the 30 min L-NAME plus BQ-123 pretreatment. Hearts were then perfused with ET-1 for 60 min and measurements were taken when a stable CPP was achieved.

Protocol 14 - ET-1 + L-NAME + BQ-788. This protocol was designed to examine the role of ET<sub>B</sub> receptors in ET-1-mediated coronary responses and addresses Specific Aim 2. Due to regional differences in ET<sub>B</sub> receptor activity and the opposing actions of vascular ET<sub>B</sub> receptors, specific hypotheses concerning ET<sub>B</sub> receptors are difficult. This protocol is identical to that described above for PE plus L-NAME and BQ-788 except that ET-1 (10<sup>-10</sup> M) is added to the perfusate reservoir following the 30 min L-NAME (10<sup>-5</sup> M) plus BQ-788 (10<sup>-6</sup> M) pretreatment. As described above, CPP was reset to 60 mmHg following the 30 min L-NAME plus BQ-788 pretreatment. Hearts were then perfused with ET-1 for 60 min and measurements were taken when a stable CPP was achieved.
**Protocol 15 - IRL-1620.** This protocol was designed to examine coronary responses to ET<sub>B</sub> receptor activation. We hypothesized that ET<sub>B</sub> receptor activation would cause vasodilation in control animals and vasoconstriction in T2DM animals. Dose response measurements to the selective ET<sub>B</sub> agonist IRL-1620 (10<sup>-11</sup> – 10<sup>-9</sup> M) were performed. Following equilibration, IRL-1620 was infused through a syringe infusion pump at 5% of the constant flow pump rate and was filtered for particulate during infusion with 0.22 μm syringe filters. Responses to IRL-1620 were measured when a stable CPP was achieved at each dose (typically within 10 min). It has been demonstrated by Berthiaume et al that the doses of IRL-1620 used in this study induce marked vasoconstriction in mouse mesenteric, renal and systemic circulation (28).
Protocol 16 - BQ-123 and BQ-788. This protocol was designed to examine the role of ET$_A$ and ET$_B$ receptor activation in the determination of basal coronary vascular tone and addresses Specific Aim 2. We hypothesized that ET$_A$ receptor inhibition would reduce basal coronary tone in both groups but to a greater extent in T2DM hearts. As previously noted, hypotheses concerning ET$_B$ receptors are difficult to formulate. The selective ET$_A$ and ET$_B$ receptor antagonists BQ-123 (10$^{-6}$ M) and BQ-788 (10$^{-6}$ M), respectively, were utilized. Following equilibration, either BQ-123 or BQ-788 was added directly to the perfusate reservoir and hearts were perfused for 30 minutes. Responses to BQ-123 or BQ-788 were measured after 30 min.

IV. Minimal Vascular Resistance Determination

Protocol 17 - Adenosine & Post-ischemic Vasodilation. This protocol was designed to determine the minimal vascular resistance of the mouse coronary vasculature. Minimal coronary resistance was determined for two reasons: 1) to examine for differences between our experimental groups as a functional measure of coronary structural alterations; and 2) to aid in the determination of the relative efficacy of different vasodilator drugs (i.e., SNAP and 8-Br-cGMP). We hypothesized that minimal coronary resistance would be elevated
in T2DM hearts compared to control. In this protocol, minimal coronary resistance was determined by maximal vasodilator responses to adenosine (ADO) and postischemic vasodilator (PIVD) responses to 15 sec of global ischemia. Following 20 min of equilibration, PIVD was induced by turning the perfusate roller pump off for 15 sec and the minimal coronary resistance was calculated using the CPP measured five seconds following the reestablishment of flow. Preliminary experiments showed that 15 sec of ischemia was sufficient to produce maximal vasodilation. Approximately 10 min later, a 5 μl bolus of ADO (10⁻³ M) was injected into the perfusate line and the minimal coronary resistance for ADO was determined. Previous studies have demonstrated that 10⁻³ M ADO given by bolus or infusion induces maximal coronary vasodilation in mice (24, 291).

**Drugs and chemicals.** ET-1, BQ-123, BQ-788 and IRL-1620 were obtained from American Peptide Company (Sunnyvale, CA). ET-1, BQ-123 and IRL-1620 were prepared in deionized water, frozen in aliquots, thawed and used on the day of the experiment. BQ-788 was prepared in deionized water with the addition of 0.1M NH₄OH to achieve a BQ-788 soluble pH of 8, frozen in aliquots, thawed and used on the day of the experiment.
the experiment. All other chemicals and buffer were obtained from Sigma-Aldrich (St.
Louis, MO). L-NAME, Tempol, PE, SNAP, 8-br-cGMP and ADO were freshly prepared
on the day of the experiment in KHB. KHB was prepared fresh on the day of the
experiment in deionized water.

B. Data and Statistical Analysis

Coronary vascular resistance was calculated by dividing CPP by the coronary
flow and normalized per gram of heart. Descriptive statistics (mean ± SE) were
computed and the data graphed using Excel. Data were expressed as percent change
from baseline CVR. Data were further analyzed using In Stat® and NCSS® software.
Dose-response curves for PE, SNAP, 8-br-cGMP and IRL-1620 and blood glucose
measurements for glucose tolerance tests were compared by two-way ANOVA for
repeated measures followed by Fisher’s Least Significant Difference post-hoc test.
Within group ET-1 and L-NAME results were compared by one-way ANOVA followed
by Fisher’s Least Significant Difference post-hoc test. All other data were compared by
Student’s unpaired t-test. A p-value less than 0.05 was considered significant. Data were
expressed and plotted as mean ± SE.

C. Experimental Limitations

The isolated mouse heart preparation provides a highly reproducible model that
can be studied quickly and in large numbers; however, it inherently has limitations as an
ex vivo preparation. Obviously, as with any isolated organ system, the isolated mouse
heart operates outside normal systemic and neurohumoral influences. This includes
eliminating the effects of circulating vasoactive substances such as angiotensin II and catecholamines, and the effects of the autonomic nervous system on the myocardium and coronary vessels. However, in our studies, this was a significant advantage in that it allowed examination of vascular responses to agents such as the $\alpha_1$-AR agonist PE independent of exogenous $\alpha_1$-AR activation.

As with any isolated tissue, function of an isolated heart perfused with oxygenated buffer deteriorates over time. This is manifest as a small increase in coronary vascular resistance and some loss of vascular responsiveness. However, many studies have shown this model to be a stable and accurate model through which to examine coronary vascular function for up to two hours following removal (285). To control for natural deterioration, time course control studies were conducted randomly in both control and T2DM mouse hearts to monitor and account for changes in coronary vascular resistance over the course of the presented studies.

It must also be recognized that our results may also have been influenced by changes in the mechanical or metabolic activity of the heart since we used contracting hearts with uncontrolled contractile responses. Mechanical and metabolic influences on vascular tone were minimized by keeping the ventricles unloaded throughout the experiment thereby reducing tension development and oxygen consumption. These hearts were also allowed to spontaneously beat and were not paced.

Another limitation of this setup is that buffer perfused hearts rather than blood perfused hearts were used. This presents the advantage of being able to control exactly what is being delivered to the heart; however, many essential components of blood (i.e.,
hemoglobin) are inherently excluded. This may especially influence the results of studies examining NO bioavailability since hemoglobin readily binds NO thus buffer-perfused hearts maintain an inherently higher NO bioavailability. Therefore, alterations noted using this system, particularly those related to NO bioavailability should eventually be assessed in blood-perfused hearts. Buffer perfusion also leads to artificially high flow rates that are necessary in this model to ensure adequate oxygen delivery to the tissue.

Our studies cannot determine alterations in specific branches of the coronary vascular tree because only total CVR is measured. However, since most of the coronary vascular resistance resides in precapillary resistance vessels, we are able to infer changes in the function of these vessels from our results. Previous studies have shown that T2DM differentially effects the microcirculation. However, the determination of alterations in overall coronary resistance is important since it ultimately determines myocardial blood flow and tissue oxygenation.

Finally, it should be emphasized that high-fat, high-simple carbohydrate feeding in C57BL/6J mice does not result in the development of obesity and T2DM in all animals. In the present studies, we did not screen the high-fat fed animals in order to use only those that had developed frank T2DM. In fact, the metabolic adaptations of the high-fat fed animals used in these studies was far from homogenous. Therefore, this may be a limitation for the applicability of our results to the relationship between frank T2DM and coronary regulation. This does, however, strengthen the severity or deleterious nature of alterations found herein since each experimental protocol contains animals across a range of metabolic dysregulation. It should also be noted that housing these
animals four per cage may have influenced access to food and individual mouse stress levels. Other studies have housed these animals similarly, however, without noting substantial social influences on the development of obesity and T2DM.
IV. RESULTS

A. Model Characterization

*C57BL/6J Mouse Model of Obesity-related Type 2 Diabetes Mellitus.* The development of obesity and T2DM in C57BL/6J mice and the resulting changes in blood glucose and plasma insulin are shown in Table 1. Ad libitum feeding of male C57BL/6J mice with a high-fat, high-simple carbohydrate, low-fiber diet for 15 weeks resulted in a 44% increase in body weight, a 39% increase in fasting blood glucose levels, and a 316% increase in plasma insulin levels versus controls. Heart weight was also significantly increased in high-fat fed mice, although the heart weight to body weight ratio decreased by 17%. Further analysis revealed a moderate correlation between body weight and fasting blood glucose concentrations (r=0.6335) and a strong correlation between body weight and fasting plasma insulin concentrations (r=0.9744; Figures 2 and 3).

*Blood Lipid Profile Determination.* Plasma levels of total cholesterol, triglycerides, HDL and LDL were determined to examine for diabetic dyslipidemia in six control and six T2DM mice. As shown in Table 2, 15 weeks of high-fat, high-simple carbohydrate, low-fiber feeding of male C57BL/6J mice resulted in an 80% increase in total cholesterol, a 204% increase in triglycerides, a 29% increase in HDL and a 293% increase in LDL levels compared to control mice.
Table 1. Body weight, heart weight, heart weight:body weight ratio, blood glucose and plasma insulin in control and diet-induced T2DM mice at 18 wk of age

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Weight (g)</th>
<th>Heart Weight (g)</th>
<th>HW:BW Ratio</th>
<th>Blood Glucose (mg/dl)</th>
<th>Plasma Insulin (μU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>190</td>
<td>28.5 ± 0.1</td>
<td>0.145 ± 0.002</td>
<td>0.0051 ± 0.0001</td>
<td>128 ± 2</td>
<td>1.9 ± 0.3 (10)</td>
</tr>
<tr>
<td>T2DM</td>
<td>165</td>
<td>41.0 ± 0.5*</td>
<td>0.165 ± 0.002*</td>
<td>0.0042 ± 0.0001*</td>
<td>177 ± 4*</td>
<td>7.9 ± 0.8 (7)*</td>
</tr>
</tbody>
</table>

HW:BW Ratio, heart weight:body weight ratio; n = number of animals; values represent mean ± SE. Sample size for plasma insulin from a separate group of animals is noted in parentheses. To convert blood glucose to mM, multiply by 0.055. To convert plasma insulin to pM, multiply by 6.95. *p<0.05 compared to control.

Table 2. Plasma cholesterol, triglycerides, HDL and LDL levels in control and diet-induced T2DM C57BL/6J mice

<table>
<thead>
<tr>
<th></th>
<th>Control Mice (n=6)</th>
<th>T2DM Mice (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>80.2 ± 3.0</td>
<td>144.5 ± 3.2 *</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>33.0 ± 2.5</td>
<td>100.2 ± 7.6 *</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>57.8 ± 2.4</td>
<td>74.7 ± 2.2 *</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>2.8 ± 0.2</td>
<td>11.0 ± 1.4 *</td>
</tr>
</tbody>
</table>

HDL, high-density lipoprotein; LDL, low-density lipoprotein. Values are mean ± SE. To convert cholesterol, HDL and LDL to mM, multiply by 0.026. To convert triglycerides to mM, multiply by 0.011. *p<0.05 compared to control.
Intraperitoneal Glucose Tolerance Test. Insulin resistance is characterized by impaired tissue glucose uptake following a glucose challenge leading to prolonged blood glucose elevations. Six control and five T2DM mice were challenged with an intraperitoneal injection of 2 g/kg glucose. Prior to injection, fasting blood glucose levels were 158.5 ± 11.6 and 232.6 ± 10.7 mg/dl in this subset of control and T2DM mice, respectively. The

Figure 2. Body weight versus fasting blood glucose in control and T2DM C57BL/6J mice. Fasting blood glucose is moderately correlated to body weight in these animals. Each point represents an individual animal.

Figure 3. Body weight versus fasting plasma insulin in control and T2DM C57BL/6J mice. These are highly correlated in control (n=10) and diet-induced T2DM (n=7) animals. Each point represents insulin and glucose measurements for one or two pooled animals.
temporal changes in blood glucose across the two hours post-injection are plotted in Figure 4. Peak blood glucoses in each group were $527.7 \pm 21.2$ mg/dl at 30 min post-injection in control mice and $>600$ mg/dl (the maximum limit of the glucometer) at 60 min post-injection in T2DM mice. At 120 min post-injection, blood glucose in control animals was similar to baseline at $192.8 \pm 22.8$ mg/dl while levels in T2DM mice remained elevated at $491.8 \pm 43.1$ mg/dl indicating impaired glucose tolerance and insulin resistance.

Blood Pressure Determination. Blood pressure was determined by carotid cannulation in six control and four T2DM mice under light anesthesia to examine for diabetes-related hypertension. Mean blood pressure in control mice was $93.5 \pm 3.9$ mmHg compared to $86.2 \pm 7.3$ mmHg in T2DM mice indicating no change in blood pressure in anesthetized T2DM from control.
B. Mouse Coronary Vasomotor Responses

I. Isolated Mouse Heart Preparation Stability

Random time course control experiments were conducted to monitor and quantify the natural deterioration of the isolated mouse heart, specifically pertaining to coronary function. Following equilibration, CVR increased in both control and T2DM hearts (Figure 5). Following 30 min of buffer perfusion, CVR increased $0.6 \pm 0.4$ and $0.1 \pm 0.5$ mmHg/ml/min/g from baselines of $4.93 \pm 0.42$ and $5.2 \pm 0.4$ mmHg/ml/min/g in control and T2DM hearts, respectively. These correspond to CVR increases of 14% and 10%, respectively. After 30 min of buffer perfusion, coronary flow was adjusted to reset CPP at 60 mmHg as noted in Protocol 7 and a new baseline CVR was established. Thirty minutes after this adjustment, CVR increases to buffer perfusion were $0.6 \pm 0.4$ and $1.1 \pm 1.0$ mmHg/ml/min/g from baselines of $6.8 \pm 0.9$ and $7.1 \pm 1.0$ mmHg/ml/min/g in control and T2DM hearts, respectively. These correspond to CVR increases of 8% and 14%, respectively. There were no significant differences in time-dependent increases in CVR.

![Figure 5. Vascular stability of isolated mouse hearts from control and T2DM mice. Time 0 begins following equilibration and a new baseline was achieved following 30 min of buffer perfusion due to adjustment of CPP to 60 mmHg. Values are percent change from preceding baseline. Vertical bars are SE.](image-url)
between control and T2DM hearts.

II. Nitric Oxide System

Baseline CVR was 7.4 ± 0.3 (n=168) and 5.2 ± 0.2 (n=147) mmHg/ml/min/g in control and T2DM hearts, respectively (p<0.05). Therefore, T2DM mouse heart CVR was significantly reduced 30% compared to control animals.

L-NAME treatment (Protocol 1) induced marked vasoconstriction in both control and T2DM animals. L-NAME responses were not correlated with blood glucose concentration in either experimental group (Figure 6). CVR increases to L-NAME (10^-5 M) treatment were 5.6 ± 0.6 and 3.4 ± 0.4 mmHg/ml/min/g from baselines of 5.5 ± 0.4 and 4.6 ± 0.2 mmHg/ml/min/g in control and T2DM hearts, respectively. Therefore, vasoconstriction to L-NAME increased CVR significantly more in control hearts (101%) than in T2DM hearts (77%; p<0.05; Figure 7).

![Figure 6. Coronary L-NAME responses versus fasting blood glucose in control and T2DM mouse hearts. Coronary vascular responses to L-NAME (10^-5 M) in both control (♦) and T2DM (■) hearts are not correlated with mouse blood glucose concentration. Each point represents an individual animal.](image-url)
In separate experiments (Protocol 2), pretreatment with Tempol prevented the decrease in L-NAME-induced vasoconstriction observed in Protocol 1 for T2DM hearts (Figure 8). Following Tempol treatment, CVR increases to L-NAME were 5.0 ± 1.5 and 5.6 ± 1.2 mmHg/ml/min/g from baselines of 10.4 ± 1.9 and 5.4 ± 0.6 mmHg/ml/min/g in control and T2DM hearts, respectively. These data correspond to CVR increases of 55% and 96% in control and T2DM hearts, respectively. Control L-NAME responses were unchanged by Tempol pretreatment. Administration of Tempol to control and T2DM hearts decreased CVR by 8% in both groups (control, p<0.054; T2DM, p<0.05 compared to no change; Figure 9).

The role of ETA and ETB receptors in coronary vasoconstriction to L-NAME was examined using the selective ETA and ETB receptor antagonists BQ-123 and BQ-788 (Protocols 3 and 4), respectively. In control hearts, L-NAME vasoconstriction (101%
increase in CVR) was attenuated by both BQ-123 and BQ-788 pretreatment (Figure 10). CVR increased $2.6 \pm 0.4$ mmHg/ml/min/g from a baseline of $5.8 \pm 0.6$ mmHg/ml/min/g; indicating a 46% increase to L-NAME following BQ-123. When treated with BQ-788, L-NAME increased CVR by $4.4 \pm 0.8$ mmHg/ml/min/g from a baseline of $7.5 \pm 0.5$ mmHg/ml/min/g, or 59%. Therefore, in control hearts, ET$_A$ and ET$_B$ receptor blockade reduce L-NAME vasoconstrictor responses by 55% and 42%, respectively. In T2DM hearts, L-NAME vasoconstriction was unchanged following pretreatment with BQ-123 or BQ-788 (Figure 11). L-NAME alone increased CVR by 77%. When treated with BQ-123, L-NAME increased CVR by $2.2 \pm 0.7$ mmHg/ml/min/g from a baseline of $4.9 \pm 0.6$ mmHg/ml/min/g, or 53%. Comparatively, CVR increased $3.1 \pm 0.7$ mmHg/ml/min/g from a baseline of $4.5 \pm 0.4$ mmHg/ml/min/g indicating a 72% increase to L-NAME following BQ-788 treatment.

Figure 9. Coronary vascular responses to superoxide anion scavenging with Tempol ($10^{-4}$ M) in control and T2DM mouse hearts. Changes are percent change from baseline vascular resistance. Vertical bars represent SE.
Figure 10. Coronary vascular responses in control mouse hearts to NOS inhibition with L-NAME (10^{-5} M) following ETA receptor blockade with BQ-123 (10^{-6} M) or ETB receptor blockade with BQ-788 (10^{-6} M). Changes are percent change from baseline vascular resistance. Vertical bars represent SE. *p<0.05 compared to L-NAME alone.

Figure 11. Coronary vascular responses in T2DM mouse hearts to NOS inhibition with L-NAME (10^{-5} M) following ETA receptor blockade with BQ-123 (10^{-6} M) or ETB receptor blockade with BQ-788 (10^{-6} M). Changes are percent change from baseline vascular resistance. Vertical bars represent SE.
Coronary vascular smooth muscle responses to exogenous NO were examined using the NO donor SNAP (Protocol 5). Vasodilator responses to SNAP were reduced in T2DM hearts pretreated with L-NAME (Figure 12, p<0.05). Maximal CVR decreases to SNAP (10^{-5} M) were -9.3 ± 1.3 and -3.4 ± 1.1 mmHg/ml/min/g from baselines (following L-NAME) of 18.1 ± 2.0 and 7.9 ± 1.4 mmHg/ml/min/g in control and T2DM hearts, respectively. These data correspond to a 56% and 39% CVR decrease in control and T2DM hearts, respectively.

Because exogenous NO donated by SNAP administration can be scavenged by superoxide anion, the NO signaling pathway was also examined by measuring the vasodilator responses to the cGMP analog 8-br-cGMP (Protocol 6). There was no difference in vasodilator responses to 8-br-cGMP in hearts from control and T2DM mouse hearts pretreated with L-NAME (Figure 13). Maximal CVR decreases to 8-br-
cGMP (10^{-4} M) were -8.1 ± 0.8 and -9.6 ± 2.3 mmHg/ml/min/g from baselines of 12.6 ± 1.3 and 13.8 ± 2.1 mmHg/ml/min/g in control and T2DM hearts, respectively. These data correspond to maximal CVR decreases of 66% and 60% in control and T2DM hearts, respectively.

Figure 13. Coronary vascular responses to 8-bromo-cGMP (10^{-6} – 10^{-4} M) following L-NAME (10^{-5} M) pretreatment in control and T2DM mouse hearts. Changes are percent change from baseline vascular resistance. Vertical bars represent SE.

III. \(\alpha_1\)-Adrenoceptor System

Phenylephrine-induced similar coronary constriction from baseline in both control and T2DM animals across the dose range (10^{-9} – 10^{-5} M; Protocol 7; Figure 14). Maximal CVR increases to PE (10^{-5} M) were 2.5 ± 0.7 and 2.4 ± 0.6 mmHg/ml/min/g from baselines of 8.0 ± 0.7 and 6.6 ± 0.6 mmHg/ml/min/g, in control and T2DM animals respectively. Therefore, PE increased CVR by 30% and 38% in control and T2DM hearts, respectively. These responses were not different between groups following NOS blockade with 30 min L-NAME pretreatment (Protocol 8; Figure 15). However, control,
but not T2DM, PE responses were enhanced in the presence of L-NAME (Figures 16 and 17). Following L-NAME (10⁻⁵ M) pretreatment, maximal CVR responses to PE were 7.6 ± 0.9 and 5.5 ± 0.9 mmHg/ml/min/g from baselines of 16.0 ± 2.2 and 11.3 ± 2.0 mmHg/ml/min/g in control and obese animals, respectively. These data correspond to a 49% and 51% CVR increase in control and T2DM hearts, respectively.

**Figure 14.** Coronary vascular responses to α₁-AR activation by PE (10⁻⁹ – 10⁻⁵ M) in control and T2DM mouse hearts. Changes are percent change from baseline vascular resistance. Vertical bars are SE.

**Figure 15.** Coronary vascular responses to α₁-AR activation by PE (10⁻⁹ – 10⁻⁵ M) following NOS inhibition by L-NAME (10⁻⁵ M) in control and T2DM mouse hearts. Changes are percent change from baseline vascular resistance. Vertical bars are SE.
The possible modulatory role of ET\(_A\) and ET\(_B\) receptors in \(\alpha_1\)-AR-mediated coronary vasoconstriction was examined using the receptor selective antagonists BQ-123 and BQ-788, respectively (Protocols 9 and 10). These blockers were added in concert with L-NAME. Following pretreatment with L-NAME plus BQ-123 and L-NAME plus BQ-788, PE responses were unchanged in control hearts (Figure 16). The maximal CVR increase to PE (10\(^{-5}\) M) following L-NAME plus BQ-123 was 3.7 ± 1.1 mmHg/ml/min/g from a baseline of 7.9 ± 1.2 mmHg/ml/min/g, a 44% increase. Comparatively, following L-NAME plus BQ-788, the maximal CVR increase was 5.1 ± 2.9 mmHg/ml/min/g from a baseline of 12.5 ± 2.9 mmHg/ml/min/g, a 30% increase. In T2DM hearts, \(\alpha_1\)-AR-mediated vasoconstriction was enhanced with NOS and ET\(_A\) antagonism while ET\(_B\) antagonism resulted in no change (Figure 17). The maximal CVR increase to PE following L-NAME and BQ-123 pretreatment was 6.6 ± 2.0 mmHg/ml/min/g from a baseline CVR of and 8.6 ± 2.3 mmHg/ml/min/g, an 86% increase. Following L-NAME followed by BQ-788, the maximal CVR increase was 7.3 ± 2.1 mmHg/ml/min/g from a baseline of 11.8 ± 2.5 mmHg/ml/min/g, a 44% increase.

**Figure 16.** Coronary vascular responses in control mouse hearts to \(\alpha_1\)-AR activation by PE (10\(^{-9}\) – 10\(^{-5}\) M) alone or following pretreatment with the NOS inhibitor L-NAME (10\(^{-5}\) M) alone or in combination with the ET\(_A\) receptor antagonist BQ-123 (10\(^{-6}\) M) or the ET\(_B\) receptor antagonist BQ-788 (10\(^{-6}\) M). Changes are percent change from baseline vascular resistance. Vertical bars are SE. *p<0.05 compared to PE alone.
plus BQ-788 pretreatment, the maximal CVR increase was 5.1 ± 2.8 mmHg/ml/min/g from a baseline of 9.8 ± 1.6 mmHg/ml/min/g, a 53% increase.

IV. ET-1 System

Coronary constriction to ET-1 was markedly enhanced in T2DM animals (Protocol 11; Figure 18). ET-1 (10^{-10} M) induced small but significant constriction in control animals with a CVR increase of 1.7 ± 0.7 mmHg/ml/min/g from a baseline of 6.9 ± 0.5 mmHg/ml/min/g. This response was enhanced (p<0.05) in T2DM hearts with a CVR increase of 6.4 ± 1.3 mmHg/ml/min/g from a baseline of 6.4 ± 0.7 mmHg/ml/min/g. ET-1-induced constriction was enhanced (p<0.05) in control, but not T2DM, hearts upon NOS blockade with L-NAME pretreatment (Protocol 12). Subsequently, ET-1 responses were not different between control and T2DM hearts following L-NAME pretreatment (Figure 19). Under these conditions, maximal ET-1-induced CVR increases in the
The individual roles of ET\textsubscript{A} and ET\textsubscript{B} receptors in eliciting coronary ET-1 responses were examined using the selective receptor antagonists BQ-123 and BQ-788, respectively (Protocols 13 and 14). In control hearts, ET\textsubscript{A} blockade attenuated ET-1-mediated vasoconstriction while ET\textsubscript{B} blockade completely inhibited this response in the presence of L-NAME (Figure 20). Following L-NAME plus BQ-123 pretreatment, ET-1 increased CVR by $3.6 \pm 0.9$ mmHg/ml/min/g from a baseline of $9.8 \pm 1.3$ mmHg/ml/min/g, a 52% increase. In the presence of L-NAME plus BQ-788, ET-1 decreased CVR by $0.5 \pm 0.6$ mmHg/ml/min/g from a baseline CVR of $7.8 \pm 0.9$ mmHg/ml/min/g, a 6% decrease that is not statistically different from no change in CVR. In T2DM hearts, ET-1-mediated vasoconstriction was reduced by BQ-123 and
unchanged by BQ-788, each in the presence of L-NAME (Figure 21). CVR increased 2.4 ± 0.7 mmHg/ml/min/g from a baseline of 6.4 ± 1.5 mmHg/ml/min/g, or 40% to ET-1, in the presence of L-NAME plus BQ-123. Following L-NAME plus BQ-788 pretreatment, ET-1 increased CVR by 4.5 ± 0.9 mmHg/ml/min/g from baselines of and 5.7 ± 1.4

**Figure 20.** Coronary vascular responses in control mouse hearts to exogenous ET-1 (10^{-10} M) alone or following pretreatment with the NOS inhibitor L-NAME (10^{-5} M) alone or in combination with the ET\textsubscript{A} receptor antagonist BQ-123 (10^{-6} M) or the ET\textsubscript{B} receptor antagonist BQ-788 (10^{-6} M). Changes are percent change from baseline vascular resistance. Vertical bars are SE. *p<0.05 from all other treatments; †p<0.05 from ET-1+L-NAME+BQ-

**Figure 21.** Coronary vascular responses in T2DM mouse hearts to exogenous ET-1 (10^{-10} M) alone or following pretreatment with the NOS inhibitor L-NAME (10^{-5} M) alone or in combination with the ET\textsubscript{A} receptor antagonist BQ-123 (10^{-6} M) or the ET\textsubscript{B} receptor antagonist BQ-788 (10^{-6} M). Changes are percent change from baseline vascular resistance. Vertical bars are SE. *p<0.05 from all other treatments.
mmHg/ml/min/g, a 100% increase.

ET\textsubscript{B} receptor activation with the selective ET\textsubscript{B} agonist IRL-1620 exhibited minimal coronary responses in control hearts and vasoconstriction in T2DM hearts (Protocol 15; Figure 22). In control hearts, IRL-1620 induced a peak vasoconstriction of $1.2 \pm 0.7$ mmHg/ml/min/g at $10^{-10}$ M. This corresponds to a CVR change of 18% at this dose. Coronary responses to IRL-1620 in T2DM hearts were not different from control at $10^{-11}$ and $10^{-10}$ M; however, IRL-1620-induced vasoconstriction was enhanced at $10^{-9}$ M (p<0.05). At this dose, CVR changed -1.1 $\pm$ 1.0 and 0.8 $\pm$ 0.9 mmHg/ml/min/g from baselines of 8.7 $\pm$ 0.9 and 4.7 $\pm$ 0.9 mmHg/ml/min/g in control and T2DM hearts, respectively. This corresponds to CVR percent changes of -8% and 40% in these groups.

![Figure 22. Coronary vascular responses to the ET\textsubscript{B} selective agonist IRL-1620 ($10^{-11} - 10^{-9}$ M) in control and T2DM mouse hearts. Changes are percent change from baseline vascular resistance. Vertical bars are SE. *p<0.05 from control.](image)

Studies investigating the role of ET-1 in determining basal vascular tone were performed by examining the individual effects of ET\textsubscript{A} and ET\textsubscript{B} inhibition in control and
T2DM hearts with BQ-123 and BQ-788 (Protocol 16). Thirty-minute BQ-123 treatment had no effect on baseline vascular resistance in either group (Figure 23). However, thirty-minute treatment with BQ-788 elicited no CVR change in control hearts and pronounced vasodilation in T2DM hearts (Figure 24). CVR decreased $1.8 \pm 0.7$ mmHg/ml/min/g from a baseline of $5.9 \pm 0.9$ mmHg/ml/min/g in T2DM hearts, a 27% decrease. Baseline CVR in the control group was $9.2 \pm 1.8$ mmHg/ml/min/g.

![Figure 23](image1.png)

Figure 23. Coronary vascular responses to the selective ETA receptor antagonist BQ-123 ($10^{-6}$ M) in control and T2DM mouse hearts. Changes are percent change from baseline vascular resistance. Vertical bars are SE.

![Figure 24](image2.png)

Figure 24. Coronary vascular responses to the selective ETB receptor antagonist BQ-788 ($10^{-6}$ M) in control and T2DM mouse hearts. Changes are percent change from baseline vascular resistance. Vertical bars are SE. *p<0.05 from control.

V. Minimal Vascular Resistance

Minimal coronary resistances determined by PIVD and ADO treatment were not different within or between groups (Protocol 17). Each treatment induced maximal coronary dilation in each group. PIVD resulted in CVR decreases of $4.5 \pm 0.5$ and $3.2 \pm 0.5$ mmHg/ml/min/g from baselines of $7.8 \pm 0.6$ and $6.4 \pm 0.7$ mmHg/ml/min/g in control and T2DM hearts, respectively. These correspond to 55% and 47% decreases in CVR to minimal vascular resistances of $3.4 \pm 0.1$ and $3.2 \pm 0.2$ mmHg/ml/min/g in control and
T2DM hearts (Figure 25). Bolus ADO treatment resulted in CVR decreases of 7.3 ± 1.2 and 4.1 ± 0.9 mmHg/ml/min/g from baselines of 11.3 ± 1.3 and 7.0 ± 1.1 in control and T2DM hearts, respectively. These correspond to 63% and 53% reductions in CVR to minimal vascular resistances of 3.9 ± 0.5 and 3.0 ± 0.2 mmHg/ml/min/g in control and T2DM hearts (Figure 26). Minimal coronary resistance was similar between control and T2DM groups and also within each group when measured by ADO and PIVD.

Figure 25. Maximal coronary post-ischemic vasodilator responses to 15 seconds of global ischemia in control and T2DM mouse hearts. Changes are percent change from baseline vascular resistance. Vertical bars are SE.

Figure 26. Maximal coronary vasodilator responses to adenosine (bolus 10^{-3} M) in control and T2DM mouse hearts. Changes are percent change from baseline vascular resistance. Vertical bars are SE.
V. DISCUSSION

The purpose of this study was to evaluate the NO, ET-1 and α₁-AR systems in the coronary vasculature of control and diet-induced T2DM C57BL/6J mice. This analysis included examination of each of these regulatory systems, determination of the interactions between these systems and any T2DM-related alterations in coronary regulation by these systems. The salient findings of this study were: 1) 15 weeks of high-fat, high-simple carbohydrate, low-fiber feeding in C57BL/6J mice induced T2DM; 2) coronary NO bioavailability was reduced in T2DM due to scavenging by superoxide anion; 3) endogenous ET-1 plays a reduced role in L-NAME-mediated vasoconstriction in T2DM; 4) ET-1-mediated coronary vasoconstriction was enhanced whereas vasoconstriction to PE was unchanged in T2DM; 5) endogenous ET-1 via the ET₄ receptor plays an enhanced role in basal vascular tone in T2DM; 6) ET₄ receptor activation is permissive for ET-1 vasoconstriction in control hearts but not T2DM hearts; and 7) endogenous ET₆ receptor activation attenuates PE-mediated vasoconstriction in T2DM hearts.

The following discussion is organized into seven sections. The first section discusses the phenotypic and metabolic alterations due to the induction of obesity and T2DM in C57BL/6J mice. The second section discusses T2DM-related alterations in the mouse coronary NO system. The third section discusses the effect of T2DM on coronary vasoconstrictor responses to ET-1. The fourth section discusses the role of ET₆ and ET₄ receptors in basal coronary tone, L-NAME-mediated vasoconstriction and ET-1-mediated vasoconstriction and alterations due to T2DM. The fifth section discusses the effect of
T2DM on coronary vasoconstrictor responses to $\alpha_1$-AR activation with PE. The sixth section discusses the role of $\text{ET}_A$ and $\text{ET}_B$ receptors in $\alpha_1$-AR-mediated coronary vasoconstriction and T2DM-related alterations in this interaction. Finally, the seventh section is reserved for general conclusions reached from this study.

A. Effect of Diabetogenic Diet in C57BL/6J Mice

The diet-induced model of obesity-related T2DM in C57BL/6J (B6) mice was chosen due to its similarity to the human T2DM condition. In these mice, ad libitum access to a high-fat, high-simple carbohydrate, low-fiber (HFHSC) diet for a minimum of eight weeks results in obesity, hyperglycemia, hyperinsulinemia and dyslipidemia (243, 252, 283, 322). Paralleling the human condition, the onset of T2DM is gradual. Hyperglycemia has been demonstrated in this model following one month of HFHSC feeding whereas hyperinsulinemia is not exhibited until approximately three months of HFHSC feeding (3, 36, 284). Following fifteen weeks of HFHSC feeding, the mice used in this study were obese, hyperglycemic, hyperinsulinemic, and dyslipidemic (Tables 1 and 2).

Diet-induced Obesity. Recently, the term ‘diabesity’ was coined to emphasize the significant relationship between obesity and type 2 diabetes. Today, 60-90% of T2DM patients are overweight or obese (13, 84). Our mice exhibited a 44% increase in body weight compared to controls and reconfirm the effect of HFHSC feeding in inducing obesity in B6 mice. Obesity in this strain, in response to HFHSC feeding, results from regional differences in fat distribution and is not simply due to hyperphagia or reduced physical activity (243, 282). While HFHSC fed B6 mice eat somewhat more than their
low fat fed controls, this hyperphagia alone does not account for the extent of excess weight gain in these animals (202, 212). In addition, obese B6 mice exhibit elevated spontaneous motor activity and an increased feed efficiency (weight gained/kcal consumed) for fat compared to the obesity-resistant A/J mouse strain (34). This means that without consuming more calories, B6 mice are able to store more fat than A/J mice. Previous investigations by Rebuffé-Scriva and Surwit have shown that, similar to obese human T2DM, the most significant fat deposition occurs in the mesenteric fat pad due to fat cell hyperplasia and hypertrophy (243, 282). However, epididymal, retroperitoneal, inguinal and nonadipose fat deposition is also increased in these animals suggesting a role for lipotoxicity in the onset of metabolic abnormalities (252, 322). It has become commonly accepted that an underlying genetic predisposition, likely related to fat storage, is a primary factor in HFHSC diet-induced obesity in the B6 mouse (282, 284).

**Insulin and Glucose.** Other metabolic abnormalities commonly accompany central obesity in B6 mice. Insulin resistance is characterized by elevated blood insulin levels, glucose intolerance and hyperglycemia. We have demonstrated significant elevations in blood glucose and plasma insulin along with impaired glucose tolerance by intraperitoneal glucose tolerance test. Previously, it has been shown that lean B6 mice have a blunted insulin response to glucose that is exacerbated by HFHSC feeding (321). A recent elegant study found that impaired β-cell compensation for insulin resistance is a primary cause of glucose intolerance in high fat fed B6 mice (3). It was demonstrated that a compensatory increase in first phase insulin secretion was absent early in insulin resistance; likewise, hyperglycemia was present at this point even though total insulin
secretion increased in proportion to the fall in insulin sensitivity. Following three months
of high fat feeding and worsening hyperglycemia; however, first phase compensation was
noted albeit at a magnitude lower than that required to account for the reduced insulin
sensitivity. Therefore, it has been demonstrated that early hyperglycemia in this model is
due to a reduction in glucose effectiveness, the ability of glucose to suppress endogenous
glucose production and stimulate glucose uptake, and later due to impaired β-cell
compensation (3).

In addition to impaired insulin secretion, tissue glucose transporter-4 (GLUT-4) content and glucose transport and gluconeogenesis contribute to hyperglycemia due to
insulin resistance. Membrane levels of the glucose transporter GLUT-4 are reduced in
skeletal muscle and adipose tissue with decreased glucose uptake in the latter (252).
Likewise, activity of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase is
also increased in the liver of HFHSC fed mice (252). Taken together, the end result of
these alterations is increased glucose intolerance characterized by reduced glucose
disposal with subsequent hyperglycemia and hyperinsulinemia as we have noted.

Comparatively, the mean blood glucose values we report for each group (127.8
versus 177.4 mg/dl) fall within the low end of the literature range for this model (34, 243,
282, 283). It should be noted; however, that the metabolic adaptations of HFHSC B6
mice, in our lab and others, are far from homogenous. For example, the blood glucose
concentrations we obtained in HFHSC B6 mice in this study ranged from 66 mg/dl to 311
mg/dl. Burcelin et al (36) found that approximately 50% of B6 mice fed a high-fat diet
developed overt T2DM, 30% exhibited an intermediate, likely pre-diabetic, phenotype,
10% were lean and diabetic and 10% were lean and nondiabetic. Therefore, it is likely that our study includes animals from each of these groups and may influence the interpretation of our results. Determining vascular responses within certain ranges of blood glucose would require more extensive sample sizes and was beyond the scope of this study.

Lipid Profile. Development of the ‘atherogenic lipid triad’ is characteristic of T2DM in humans and is a strong predictor of coronary artery disease in these patients (164, 199). Diabetic dyslipidemia in humans consists of elevated serum triglycerides, reduced serum HDL levels and increased total serum LDL levels. In HFHSC B6 mice, we have demonstrated dyslipidemia; however, plasma levels of all measured lipids (total cholesterol, triglycerides, HDL, LDL) are increased (Table 2).

Total cholesterol and triglycerides are elevated in most studies using high fat fed B6 mice (60, 131, 208, 214, 243, 252). Few studies report direct measurements of LDL cholesterol; however, those that do cite increased levels (152, 275). In high fat fed db/db mice, Kobayashi et al (152) report specific elevations in the atherogenic small, dense LDL particles. In contrast, varying effects of high fat feeding on HDL cholesterol have been reported (208, 214, 264). However, Schreyer et al (264) have reported increased HDL cholesterol levels in B6 mice fed the same diet utilized in this study thereby supporting our findings. Hayek et al (111) have suggested that elevated HDL cholesterol in response to high-fat feeding in mice results from increased transport rates and decreased fractional catabolic rates possibly due to increased HDL particle size. Interestingly, it has been noted that high fat diet-induced elevations in HDL cholesterol
are not seen in studies that use high fat diets containing the bile component cholic acid. Subsequently, the negative effect of cholic acid on HDL cholesterol levels was exposed in B6 mice fed high fat diets with or without cholic acid (275). Therefore, it is likely that the presence of cholic acid accounts for the conflicting literature with respect to HDL cholesterol levels. Overall, our findings support literature reports of diabetic dyslipidemia in HFHSC fed B6 mice. The presence of atherosclerosis resulting from this altered lipid profile is unknown in our model; however, others have shown B6 mice to be highly susceptible to lesion formation (209, 264).

**Arterial Blood Pressure.** Apart from metabolic abnormalities, obesity and T2DM are often coupled with hypertension. In fact, approximately 73% of T2DM patients in the United States are hypertensive (48). Previous studies have produced conflicting data concerning hypertension in the HFHSC B6 mouse model. Initial characterization of blood pressure in this model revealed significant hypertension (systolic BP = 162 versus 139 mmHg) following 4.5 months of HFHSC feeding (190). It was further revealed that the hypertension was a result of elevated sympathetic nervous system activity. Later studies, including ours, have been unable to replicate these results (202, 347). In fact, only one other study notes any change in mean blood pressure in this model albeit a slight increase (5-8 mmHg) (330).

Unlike the present study, blood pressure measurements in previous studies have been performed in conscious mice using either tail cuff or telemetry devices. Blood pressure in the present study was measured directly in anesthetized mice using a carotid artery catheter attached to a pressure transducer. The accuracy of catheter blood pressure
measurement in rodents has been validated; however, anesthetics are known to have significant depressant effects on blood pressure in mice (129, 303). Typical literature values for conscious B6 mouse mean arterial pressure (MAP) are around 103 mmHg (38, 304). We report MAPs of 94 mmHg and 86 mmHg in control and T2DM mice, respectively, under pentothal anesthesia. Therefore, while we observed no difference in MAP, our results are possibly confounded by the measurement of MAP in anesthetized animals. However, a significant contradiction exists in the literature concerning systemic blood pressure regulation in this model.

*Heart Weight to Body Weight Ratio.* Obesity-related T2DM can place an increased burden on the ability of the cardiovascular system to supply adequate oxygen and nutrients to the tissues. This is reflected by the decrease in the heart weight to body weight ratio, a finding that has been reported in other obesity-related T2DM models (55, 296, 315, 347). An increase in this ratio has been suggested to be an indicator of chronic hypertension resulting in cardiac hypertrophy. In the presence of obesity, the influence of elevated heart weight may be masked if the increase in body weight is disproportionally high compared to the enhanced cardiac mass thus reducing the ratio. Regardless, it has long been established that heart weight and body weight maintain a relatively constant relationship in healthy age-matched individuals of a species (135). Therefore, a decrease in this ratio indicates that the heart, even if hypertrophied, must provide disproportionately greater perfusion to a larger overall tissue bed.

*Baseline and Minimal Coronary Resistances and Basal Coronary Tone.* Morphologic changes in coronary vascular structure are evident as changes in baseline
and minimal CVR. Baseline CVR can be used as an overall determinant of vascular cross-sectional area under basal conditions. Baseline CVR, normalized to heart weight, at 60 mmHg perfusion pressure was reduced 30% in T2DM hearts compared to control hearts. This suggests that total vessel cross-sectional area per gram of tissue is increased in the hearts of T2DM animals.

Minimal coronary resistance and the degree of basal coronary tone in our model were examined using a bolus dose of the vasodilator adenosine (ADO) and to post-ischemic vasodilation (PIVD) due to 15 seconds of global ischemia (Figures 25 and 26). Each of these treatments induces maximal dilation of the coronary vasculature. We hypothesized that minimal coronary resistance and basal coronary tone would be increased in T2DM hearts due to structural stiffening of the coronary vessels and enhanced vasoconstrictor tone, respectively. Our results indicate that a reduced basal coronary tone is present in T2DM mouse hearts. These data indicate that the coronary vasculature of T2DM mice are more dilated under basal conditions. Therefore, contrary to our hypothesis, this indicates enhanced vasodilator influences on the coronary vasculature. Further studies are necessary, however, to determine specific alterations in basal coronary regulation leading to this change.

T2DM-related oxidative stress has been implicated in coronary remodeling thereby accelerating the onset of diabetic microangiopathy, cardiomyopathy, and heart failure (110). Functionally, coronary remodeling is manifest as a reduction in coronary vasodilator reserve and an increased minimal coronary resistance (279, 348). Our results indicate no effect of obesity-induced T2DM on minimal coronary resistance (Figures 25
and 26). This suggests, therefore, that pronounced changes in coronary vessel structure may not be a substantive part of the diabetic milieu in these animals following 15 weeks of high-fat, high-carbohydrate feeding. However, further structural studies are needed to determine the relationship between functional and structural alterations to T2DM in this model.

In summary, high-fat, high-simple carbohydrate, low-fiber feeding for 15 weeks in B6 mice induces T2DM characterized by obesity, hyperglycemia, hyperinsulinemia and hyperlipidemia but not hypertension. This model also exhibits decreased baseline coronary resistance with a similar degree of basal coronary tone and no change in minimal vascular resistance. The latter of these indicates no marked structural alterations in the coronary vasculature of T2DM hearts. Previous studies have demonstrated that similar dietary manipulations in B6 mice induce other symptoms of T2DM noted in the human condition. Therefore, since diet-induced obesity and T2DM in B6 mice develop in a manner similar to that seen in humans, this model is useful in examining the deleterious effects of T2DM on coronary vascular function.

B. Effect of Diet-induced T2DM on the Coronary Nitric Oxide System

One purpose of this study was to examine NO-mediated coronary vascular responses in control and obesity-related T2DM mice. These studies were important to conduct in isolated mouse hearts from animals placed on a high-fat, high-carbohydrate diet to induce obesity and T2DM for two reasons. First, although the C57BL/6J mouse is a common strain used in the production of transgenic mouse models of disease, coronary vascular regulation has not been comprehensively studied in this strain. Second, as noted
above, this is a suitable model for examining changes in coronary vascular control mechanisms resulting from obesity and T2DM due to the similarities in pathology between T2DM in this model and the human condition (34, 56, 60, 190, 209, 243, 283, 321).

Regarding the NO system, we hypothesized that 1) endogenous NO bioavailability would be reduced in T2DM mouse hearts; 2) enhanced superoxide anion production would account for this reduction; and 3) vascular smooth muscle cell NO responsiveness would be reduced by obesity-induced T2DM.

**Nitric Oxide Bioavailability.** NO is an important endothelial-derived vasodilator substance. L-arginine, the precursor of NO, is converted to NO and L-citrulline by the action of NOS in endothelial cells (311). NO diffuses to the vascular smooth muscle where it binds and activates guanylyl cyclase thereby increasing cGMP levels, which induces smooth muscle relaxation, vascular dilation and increased blood flow. Basal release of NO occurs through chronic physical activation of endothelial cells by pulsatile flow and shear stress, and therefore plays an important role in the maintenance of basal coronary tone (24, 197). The importance of NO in coronary vascular tone is supported by our results showing that NOS inhibition with L-NAME leads to a 101% increase in CVR in control animals (Figure 7).

The inhibition of NO synthesis by NOS using L-arginine derivatives such as L-NAME has become common practice as an instrument to determine NO bioavailability. It should be noted however, that the vasoconstriction due to NOS inhibition by L-NAME is not solely the result of the removal of the vasodilator influence of NO. NO is also
known to suppress the production of many endogenous vasoconstrictor influences such as ET-1 and the cyclooxygenase pathway products TXA₂ and prostaglandin H₂ (PGH₂). Therefore, decreasing NO can lead to enhancement of vasoconstrictor influences.

Numerous studies have demonstrated that L-NAME-mediated vasoconstriction is significantly attenuated in the absence of ET-1, TXA₂ or PGH₂ activity. In the isolated rat heart, Pomposiello et al (231) found that coronary L-NAME-mediated vasoconstriction was eliminated following cyclooxygenase and TXA₂ synthase inhibition as well as PGH₂/TXA₂ receptor antagonism. Superoxide anion is permissive for L-NAME-mediated vasoconstriction in this model since it is required for the conversion of PGH₂ to TXA₂ (231). In the same preparation, Wang et al (316) found that ETₐ receptor antagonism reduced vasoconstriction due to NOS inhibition thereby indicating ET-1 involvement. Apart from the coronary circulation, the involvement of PGH₂, TXA₂ and ET-1 in vasoconstriction due to NOS inhibition has been noted in rat kidney, middle cerebral artery and in the systemic circulation (25, 196, 354). Therefore, L-NAME-mediated vasoconstriction is due to the removal of all NO-mediated vascular effects including NO vasodilation and NO inhibition of endogenous vasoconstrictors, specifically ET-1, PGH₂ and TXA₂. The involvement of ET-1 in L-NAME-mediated coronary vasoconstriction in the present study in discussed in Section D.

Impairments in NO release and/or bioavailability in obesity and diabetes have been implicated in the pathogenesis of coronary vascular disease (224). The reduced vasoconstrictor response to L-NAME in T2DM mice suggests that NO bioavailability is attenuated in the coronary vessels of these animals (Figure 6). Known precipitating
factors of reduced NO bioavailability in obesity and T2DM include reduced L-arginine to NO conversion (15), elevated levels of the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) (170), reduced bioavailability of the NOS cofactor tetrahydrobiopterin (BH₄) (29, 211), and increased NO quenching by reactive oxygen species (ROS) such as superoxide anion (355).

*Superoxide Scavenging of NO.* In T2DM, ROS can be formed through NADPH oxidase activation or eNOS operating with inadequate cofactors (i.e., BH₄) (29). Katakam et al (142) found in Zucker obese rats that reduced NO-mediated vasodilation of small coronary arteries was attributed to increased NADPH oxidase ROS production accompanied by a compensatory increase in eNOS protein expression. NO bioavailability and endothelium-dependent vasodilation can be recovered in these states by treating with ROS scavengers and systemic L-arginine or BH₄ infusion (46, 225). In *db/db* mouse isolated coronary arteries, recovery of acetylcholine- and flow-induced dilation was achieved with intraluminal superoxide dismutase administration (18). Our results support the role of ROS, specifically superoxide anion, in attenuating NO bioavailability in the coronary vessels of T2DM mice. Pretreatment with the superoxide dismutase mimetic Tempol recovered normal vasoconstrictor responses to L-NAME in T2DM mice indicating enhanced superoxide NO scavenging (Figure 8). Our findings that Tempol alone did not induce different responses between groups can be attributed to the multiplicity of mechanisms that regulate coronary perfusion and thereby do not necessarily indicate similarity in superoxide production between these groups (Figure 9) (68).
Two mechanisms may account for ROS reducing NO bioavailability. First, ROS may react directly with NO to form peroxynitrite (355). Second, ROS may reduce the activity of the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which normally accounts for most ADMA clearance. This mechanism has been suggested since the hyperglycemia-induced reduction in DDAH activity seen in high-fat fed streptozotocin rats was reduced by treatment of cultured endothelial cells with superoxide dismutase (170). Further, it is also possible that high-fat, high-carbohydrate feeding influences this system. High-fat feeding, even a single high-fat meal, can reduce DDAH activity thereby increasing ADMA levels and inhibiting NO production in human T2DM patients (79). Therefore, while our results support reduced NO bioavailability due to increased superoxide formation, the experimental high-fat diet may also contribute to this defect by other mechanisms.

Close examination of our data revealed a substantial difference in baseline CVRs between control hearts pretreated with and without Tempol prior to L-NAME treatment. These baselines were 5.5 ± 0.4 and 10.4 ± 1.9 mmHg/ml/min/g, respectively. Therefore, our results and interpretation of these data may be confounded. This disparity may be a result of these sets of experiments being performed at different times many months apart. Therefore, due to a more constricted baseline, L-NAME vasoconstriction in the L-NAME plus Tempol group may be reduced since the vasoconstrictor ‘reserve’ of these vessels is less at baseline. The majority of studies indicate that Tempol increases NO bioavailability, however, it has been suggested in one study that Tempol reduces NO bioavailability in rat isolated aorta (234). This phenomenon may account for the
increased baseline resistance following Tempol pretreatment in our study, however, this seems unlikely since L-NAME vasoconstriction was augmented in T2DM hearts treated with Tempol indicating an elevated NO bioavailability. Overall, our results show that scavenging of superoxide with Tempol recovers normal L-NAME vasoconstrictor responses in T2DM hearts. This is noted quantitatively and especially qualitatively with similar L-NAME responses following Tempol pretreatment (Figure 8).

Vasodilation to Exogenous NO. We also examined the effect of obesity and T2DM on endothelium-independent vasodilation to the NO donor SNAP. Human and animal studies have revealed reduced vasodilation to a variety of NO donors (i.e., NONOate, sodium nitroprusside, SNAP) in the T2DM state (18, 328, 333). The reduction in SNAP-induced vasodilation in isolated hearts from T2DM mice supports these findings (Figure 12). The difference in baseline CVRs between control and T2DM mice prior to SNAP treatment is attributed to the reduced vasoconstriction to L-NAME pretreatment in T2DM mouse hearts.

Due to the increased production of ROS in T2DM, the functional relevance of these results pertaining to the direct effect of T2DM on vascular smooth muscle NO signaling is questionable. In db/db mice, reduced coronary vasodilation to exogenous NO was recovered with rosiglitazone or superoxide dismutase treatment owing to a reduction in oxidative stress and scavenging of exogenous NO, respectively (17, 18). Therefore, the attenuation of vasodilation to NO donors may be primarily due to ROS scavenging of exogenous NO. The significance of vascular responses to exogenous NO therefore may be difficult to interpret in states of enhanced oxidative stress.
*cGMP-Dependent Vasodilation.* To directly examine the effect of obesity-related T2DM on endothelium-independent vasodilation and vascular smooth muscle NO signaling, we performed dose response measurements in isolated hearts to the cell permeable cGMP analog 8-br-cGMP. Analysis of vasodilator responses to 8-br-cGMP allows for examination of NO signaling downstream of the NO stimulated guanylyl cyclase and is uncompromised by ROS. We found that 10^{-4} M 8-br-cGMP produced nearly maximal vasodilation, and that there was no difference between control and T2DM hearts (Figure 13). This suggests that the cGMP signaling pathway is not altered in the T2DM state in this model and that the reduced SNAP-induced vasodilation is likely due to NO scavenging by ROS rather than a direct defect in smooth muscle NO responsiveness.

Others however, have attributed similar alterations in vascular NO responsiveness to altered guanylyl cyclase activity. Witte et al (332) noted a reduction in maximal NO stimulated guanylyl cyclase activity in aortic tissue isolated from T2DM Goto-Kakizaki rats. However, it is important to note that these measurements were collected from *in vitro* homogenized tissue extracts devoid of normal guanylyl cyclase regulation. Therefore, the functional consequence of these experimental conditions may not be realized *in vivo* as the enzyme is likely operating at submaximal capacity. The same group also showed no change in guanylyl cyclase activity or NO sensitivity in internal mammary arteries from T2DM patients using similar methods (331). Our results support the presence of normal NO signaling downstream of guanylyl cyclase in the coronary vasculature of obesity-induced T2DM mice. Therefore, a defect in guanylyl cyclase
upstream of cGMP-mediated NO signaling cannot be ruled out as a possible complementary mechanism to superoxide scavenging accounting for the reduction in vasodilation to NO donors such as SNAP, and more detailed studies are required to examine this.

In summary, the significant changes in coronary NO function noted following 15 weeks of high-fat, high-carbohydrate feeding emphasize the speed and severity of the deleterious effects of this pathology on coronary function. Our investigation suggests that, in obesity-induced T2DM mice, superoxide anion reduces NO bioavailability. We have also shown that vasodilation to the NO donor SNAP is reduced while no alteration in smooth muscle NO signaling downstream of guanylyl cyclase is present. We conclude that the enhanced production or reduced clearance of ROS in the T2DM state is the primary mechanism through which endothelium-dependent and -independent vasodilation to NO are attenuated and that normal cGMP coupled vasodilator responses are maintained in this model.

C. Effect of Diet-induced T2DM on the Coronary ET-1 System

A proper balance between coronary vasodilator and vasoconstrictor influences is integral for normal coronary function. We have shown that the bioavailability of NO, an important vasodilator influence, is reduced in T2DM mouse hearts. Numerous studies have demonstrated the inhibitory action of NO on the vascular ET-1 system. Correspondingly, others have demonstrated enhanced ET-1 responses in models of T2DM. Therefore, examination of ET-1-mediated coronary vasoconstriction and its interaction with NO is important in understanding the deleterious effects of T2DM on
coronary regulation in the T2DM mouse heart. To this end, we examined coronary responses to the vasoconstrictor ET-1 in the presence and absence of endogenous NO. We hypothesized that 1) coronary vasoconstriction to ET-1 would be enhanced in T2DM hearts; and 2) coronary vasoconstriction to ET-1 would be enhanced in both groups following NOS inhibition with L-NAME, but to a lesser degree in T2DM hearts.

ET-1 induced vasoconstriction is mediated through activation of the Gq-coupled ETₐ and ETₐ receptors on VSMC and may be modulated by endothelial ETₐ receptors linked to NO production (267, 290). ETₐ and ETₐ activation on VSMC causes PLC-mediated production of IP₃ and DAG, PKC activation, increased intracellular calcium levels and vasoconstriction (200). The inhibitory effect of NO on ET-1-mediated vasoconstriction has been well described in both human and animal models. ET-1 production, receptor binding and downstream calcium mobilization pathways are interrupted by NO (33, 96, 144). Also, shear stress-induced NO release limits constriction to ET-1 (277).

Our findings indicate that in control hearts, ET-1 induces a mild but significant constriction that is enhanced by NOS blockade with L-NAME (Figure 20). It is not surprising that NOS inhibition potentiates ET-1 coronary vasoconstriction since NO normally acts to inhibit ET-1 activity. In T2DM animals, vasoconstriction to ET-1 is markedly enhanced compared to control hearts without L-NAME pretreatment. In addition, this response is unchanged by L-NAME pretreatment in T2DM hearts and is similar to that seen in control hearts following L-NAME pretreatment (Figures 18 and
Similar results have been found in the isolated aorta of control and T2DM B6 mice (298, 351).

It should be noted that the concentration of ET-1 used in the present study, while inducing pronounced vasoconstriction, falls at the low end of ET-1 concentrations reported in the literature. This dose also falls below the reported EC$_{50}$ of 1.9 nM reported for ET-1 in isolated mouse coronaries which is lower than EC$_{50}$ values reported for other mouse vessels (217, 222, 254, 298, 351). Therefore, the dose used in this study is not supramaximal and it appears that mouse coronary arteries are more sensitive to ET-1 than other mouse vessels.

In light of the established relationship between NO and ET-1, the enhanced ET-1-mediated coronary vasoconstriction in T2DM hearts is not surprising. This may be due to the reduced NO bioavailability in T2DM hearts that effectively relieves NO inhibition on coronary ET-1 activity resulting in enhanced vasoconstriction. Therefore, reduced NO bioavailability likely contributes to the enhancement of ET-1-mediated coronary constriction in T2DM animals. Subsequently, we noted no change in ET-1 vasoconstriction following NOS inhibition in T2DM hearts suggesting very little basal NO attenuation of ET-1 responses. This is not due to the absence of NO, however, since L-NAME produced marked vasoconstriction in T2DM hearts (Figure 7). Therefore, upregulation of VSMC ET-1 receptors or enhanced receptor activity may also contribute to enhanced ET-1-mediated vasoconstriction in this state.

Upregulation of ET$_A$ and ET$_B$ mRNA has been noted in various vascular beds of rat T2DM models (117, 136, 143, 339). Hopfner et al (117) also demonstrated that ET-1
binding to aortic VSMC ETA receptors was enhanced following insulin pretreatment resulting in an increased peak intracellular calcium response. We did not directly assess ET-1 receptor density or sensitivity; however, our studies examining the functional roles of individual ET-1 receptor subtypes support a possible role for upregulation of VSMC ETB receptor-mediated vasoconstriction in this response (see Section D). In addition to alterations in receptor dynamics, other endogenous vasoactive substances may play a role in ET-1 vasoconstriction. Traupe (298) demonstrated that aortic contraction to ET-1 in control B6 mice is significantly inhibited by the cyclooxygenase inhibitor meclofenamate. This study also revealed elevated thromboxane receptor and synthase gene expression in obese B6 mice. Unfortunately, the effect of meclofenamate on ET-1 contractions in obese B6 mice was not examined. Of importance, however, is that this study suggests the involvement of locally produced vasoconstrictor prostanoids (i.e., TXA2 and PGH2) in ET-1-mediated vasoconstriction and this involvement may be increased in obesity leading to enhanced ET-1 responses. We did not examine the roles of other endogenous vasoconstrictor substances in ET-1-mediated vasoconstriction; however, this is an area of possible future examination in this model.

The majority of rodent studies, including ours, show enhanced vasoconstriction to exogenous ET-1 in T2DM (117, 136, 143) whereas human studies exhibit impaired vasoconstriction to exogenous ET-1 (41, 185, 203). This difference may be explained by the increased ET-1-mediated vascular tone and elevated plasma ET-1 levels often found in human T2DM (182, 262, 335). In this case, basal ET-1 receptor occupancy may be elevated in human T2DM patients thereby reducing binding of exogenous ET-1.
Methodological differences may also account for the variance in the literature. Results from the rodent studies were obtained using isolated vascular preparations, whereas the human data were collected using forearm blood flow measurements. Therefore, systemic factors may be acting in the human studies to reduce exogenous ET-1 vasoconstriction that are absent in the isolated rodent vasculature.

Alterations in the ET-1 system in the T2DM state are likely heavily dependent on the extent of metabolic derangements such as hyperglycemia and hyperinsulinemia. Insulin has been shown to stimulate the release of both NO and ET-1, the latter of which is enhanced in T2DM (43, 189, 233). Also, plasma ET-1 levels in T2DM patients show a strong correlation to total insulin exposure (335). Therefore, the extent of insulin resistance and hyperinsulinemia is likely influential in ET-1 system responses in both human and animal models of T2DM. Overall, our results support others which indicate that the normal balance between NO and ET-1 is shifted in T2DM such that coronary vasoconstriction to ET-1 is enhanced possibly due to reduced NO bioavailability and enhanced ET-1 receptor activity.

D. Effect of Diet-induced T2DM on Coronary ET$_A$ and ET$_B$ Receptor Responses to Endogenous and Exogenous ET-1

Previous studies in models of T2DM have revealed that ET-1 receptors, primarily ET$_A$, have altered density, sensitivity and activity in this state (136, 339). Currently, there is a dearth of information concerning the effect of T2DM on vascular ET$_B$ receptors. Likewise, very little is known in either the normal or T2DM mouse concerning the individual roles of ET$_A$ and ET$_B$ receptors in coronary regulation. Our
study has found that coronary ET-1-mediated vasoconstriction is enhanced in diet-
induced T2DM B6 mice. In addition, the literature shows that ET-1 acting via its
receptors plays a role in vasoconstriction to NOS inhibition and also in mouse basal
coronary tone. Therefore, we examined the role of endogenous ET-1 acting via ETA and
ETB receptors in basal coronary tone and vasoconstriction to NOS inhibition with L-
NAME. In addition, the role of these receptors in coronary vasoconstriction to
exogenous ET-1 was examined.

With respect to the ETA receptor, we hypothesized that 1) endogenous ET-1 via
ETA receptors contributes to basal coronary tone and that this would be enhanced in
T2DM hearts; 2) coronary vasoconstriction to exogenous ET-1 is mediated through ETA
receptors in both groups; and 3) endogenous ET-1 acting via ETA receptor activation
contributes to L-NAME-mediated vasoconstriction in both groups. With respect to the
ETB receptor, responses are difficult to predict with specific hypotheses due to the
opposing actions of VSMC and endothelial ETB receptors and regional differences in ETB
involvement. Also, as previously noted, little is known about the effect of T2DM on ETB
receptors. Therefore, using a selective ETB agonist and antagonist we examined the role
of ETB receptors in basal coronary tone, vasoconstriction to L-NAME and
vasoconstriction to exogenous ET-1.

ET-1-mediated vascular responses occur by activation of ETA and ETB receptors
located on VSMC and VSMC and endothelial cells, respectively. Activation of VSMC
ETA and ETB receptors leads to vasoconstriction through Gq-protein coupled increases in
intracellular calcium whereas activation of endothelial ETB receptors results in
endothelial NO production (200, 230, 286, 290). The literature reveals that vascular responses to ET-1 are species and vessel dependent. It has previously been found that the distribution, affinity and maximum binding densities of ET-1 receptors in the mouse heart are similar to that found in humans (326). However, application of this data to vascular studies is limited since these measurements were taken in whole heart samples and thus include measurement of myocardial ET-1 receptors (228). Nonetheless, functional ET-1 receptors are present in the mouse coronary vasculature.

Numerous studies have revealed alterations in ET-1 receptor subtypes in T2DM. In human T2DM patients, variable alterations in \( \text{ET}_A \) receptor involvement in vascular tone have been noted. These studies have shown that ET-1 is more, less or similarly involved in vascular tone via the \( \text{ET}_A \) receptor compared to controls. For example, vasodilation of the forearm vasculature to BQ-123 infusion either appears or disappears in T2DM (41, 186). As noted, elevated \( \text{ET}_A \) mRNA expression, receptor binding and activity have also been found in models of T2DM (136, 143, 308, 339).

Much less is known about \( \text{ET}_B \) receptor involvement in coronary regulation. Functionally, in some model systems including humans, studies have indirectly suggested that VSMC and endothelial \( \text{ET}_B \) activity may be altered in T2DM (4, 141, 156). To our knowledge, only one study has directly examined \( \text{ET}_B \) receptor expression under these conditions. In the obese diabetic Zucker rat, Wu et al (339) found that both \( \text{ET}_A \) and \( \text{ET}_B \) receptor mRNA expression was elevated in the aorta and mesenteric vasculature. Unfortunately, the vessel samples used for analysis were not cleared of endothelial cells therefore the relative changes in VSMC and endothelial \( \text{ET}_B \) expression is unknown.
Taken together, T2DM induces alterations in ET-1 receptor systems and very little is known concerning coronary ET-1 receptors in normal and T2DM B6 mice, especially ET\textsubscript{B} receptors. These alterations in receptor expression may potentially infer significant changes in vascular responsiveness to ET-1 aiding in the vascular dysfunction associated with T2DM.

**Role of ET-1 Receptors in Basal Coronary Tone.** Lui et al (175) demonstrated that endogenous ET-1 activity accounts for 50% of basal vascular tone in isolated mouse ventricular septal coronary arteries. In contrast, our results suggest that ET-1 plays a negligible role in basal coronary tone in control isolated mouse hearts. We found no change in baseline CVR following ET\textsubscript{A} or ET\textsubscript{B} receptor inhibition in these animals (Figures 23 and 24). It should be noted however, that a trend toward vasoconstriction was observed following ET\textsubscript{B} inhibition suggestive of basal endothelial ET\textsubscript{B}-mediated NO production in these animals. A similar result was noted with systemic BQ-788 bolus suggesting that ET\textsubscript{B}-mediated NO release plays a role in mouse blood pressure regulation (204). To our knowledge, this is the first study to examine the role of ET-1 in basal coronary tone in the isolated mouse heart.

Our examination of ET-1 receptor involvement in coronary tone of T2DM mouse hearts resulted in the novel finding that ET-1 via VSMC ET\textsubscript{B} receptor activation is an integral component of basal vascular tone in this state. Smooth muscle, as opposed to endothelial, ET\textsubscript{B} receptors are implicated since ET\textsubscript{B} inhibition led to vasodilation indicative of the removal of a vasoconstrictor influence. We found that selective ET\textsubscript{B}
inhibition resulted in a 28% decrease in CVR (Figure 24). As in control hearts, selective ET\textsubscript{A} inhibition had no effect on CVR in T2DM mouse hearts.

Even though our study was strictly functional, the enhanced role of ET\textsubscript{B} receptors in basal coronary tone may suggest that VSMC ET\textsubscript{B} receptor density or activity are elevated in T2DM. To further examine ET\textsubscript{B} receptor activity, we determined the coronary vascular responses to the highly selective ET\textsubscript{B} agonist IRL-1620. Our results showed that in control hearts, selective ET\textsubscript{B} activation led to no significant change in CVR at any dose examined. However, in T2DM hearts, IRL-1620 induced significant vasoconstriction (Figure 22). A similar vasoconstriction would be expected in control hearts if IRL-1620 responses were determined following NOS inhibition with L-NAME thereby reducing basal and ET\textsubscript{B}-mediated NO production and its opposing influence on VSMC ET\textsubscript{B}-mediated vasoconstriction. Therefore, this supports the suggestion that VSMC ET\textsubscript{B} receptor density or activity is enhanced in T2DM mouse hearts and provides a possible explanation for the enhanced coronary vasoconstriction to exogenous ET-1 in T2DM hearts noted above.

Examination of the literature suggests that a balance may exist within the opposing actions of the coronary ET\textsubscript{B} receptor system and that this balance can be altered pharmacologically and in pathologic states. Coronary ET\textsubscript{B} activation would be expected to induce VSMC contraction and NO release concomitantly therefore each response limits the other. Our results support a shift in the overall action of coronary ET\textsubscript{B} receptors towards VSMC ET\textsubscript{B}-mediated vasoconstriction in T2DM. The absence of a substantive response to IRL-1620 in control hearts suggests that the actions of activated
VSMC and endothelial ET_B receptors are fairly balanced in our model. Evidence from human and dog studies support this as intracoronary infusion of the ET_B selective agonist sarafotoxin S6c induced mild changes in coronary tone (39, 40, 227, 246). However, pharmacologic manipulation of this balance by NOS inhibition in dog hearts lead to a significant sarafotoxin S6c-induced elevation in CVR similar to that induced by ET-1 (39). Our results support a pathology-induced shift in this balance possibly due to enhanced VSMC ET_B expression, coupling or signaling or reduced ET_B-mediated NO release or bioavailability. The former of these suggestions cannot be supported further by our study; however, the latter is supported by our finding that NO bioavailability is reduced in T2DM mouse hearts. Therefore, ET_B-mediated coronary vasoconstriction would be enhanced due to the reduced vasodilator influence.

The marked discrepancy concerning the role of ET-1 in basal coronary tone between Lui et al (175) and the present study may be due simply to methodological differences. Importantly, it should be noted that Lui et al examined isolated coronary vessels under no flow conditions. Given the role of endothelial shear stress in inducing NO release that typically inhibits ET-1 activity, it is likely that no flow conditions would exaggerate the role of ET-1 on vascular tone. Also, isolated coronary arterioles, unlike isolated heart coronary arteries, are removed from any metabolic vasodilator influences of the myocardium. Although we used unloaded and unpaced hearts, spontaneous myocardial contraction occurred in our study. This may cause the release of metabolic byproducts such as adenosine, lactate and potassium ions that induce coronary vasodilation thereby opposing any endogenous ET-1 action. Therefore, it seems unlikely
that ET-1 accounts for 50% of basal vascular tone in mouse coronary arteries of beating hearts.

Additionally, Lui et al (175) utilized the mixed ET\textsubscript{A/B} antagonist bosentan whereas we examined the role of each receptor individually with selective antagonists. This presents the possibility that during selective inhibition of one ET-1 receptor, enhanced activity of the other ET-1 receptor may compensate. Brunner et al (35) demonstrated in rat hearts that selective ET\textsubscript{B} receptor inhibition increases ET-1 present in coronary effluent; however, this is likely due to the role of ET\textsubscript{B} receptors as ET-1 clearance receptors and may not occur during selective ET\textsubscript{A} inhibition. Nevertheless, further studies examining coronary responses in isolated mouse hearts to nonselective ET-1 receptor blockade that would eliminate all ET-1-mediated vascular responses are needed.

As noted previously, the ET-1 system is incredibly complex and definite species and regional variations exist in receptor density and activity. Alterations in the ET-1 system induced by T2DM may also be sensitive to species, duration and severity of the disease and method of T2DM induction. Therefore, application and extrapolation of data gathered concerning the ET-1 system must be done judiciously. While we have used data from other species to support our findings, it must be noted that disparate results are recorded frequently throughout the literature. Regional differences in ET-1 receptor populations and activities have been demonstrated within the vasculature of many species (19, 74, 280, 293, 317). Although this is likely also the case in the mouse coronary circulation, our results provide an important whole organ perspective of the effects of
T2DM on the coronary ET-1 receptor involvement in basal coronary tone. Overall, our results support an enhanced role of ET-1 in basal tone of T2DM mouse coronary arteries mediated by either enhanced ET_B receptor expression and activity or reduced ET_B-mediated NO release or bioavailability.

*Role of ET-1 Receptors in L-NAME Vasoconstriction.* As discussed previously, vasoconstriction to NOS inhibition is not due solely to the removal of the vasodilator influence of NO. This response is also due to the production of endogenous vasoconstrictors such as ET-1 that are normally inhibited by NO. Therefore, we examined the role of endogenous ET-1 acting through ET_A and ET_B receptors in coronary vasoconstriction to L-NAME in control and T2DM hearts.

The present study has revealed a differential role of ET_A and ET_B receptors in L-NAME-mediated vasoconstriction in control and T2DM hearts. Our results demonstrate that endogenous ET-1 acting via both receptor subtypes are involved in control L-NAME vasoconstriction whereas no involvement of these receptors is noted in T2DM hearts (Figures 10 and 11). Thus, we have demonstrated that in control hearts L-NAME vasoconstriction is caused not only by the removal of the vasodilator effect of NO but also by ET-1-mediated vasoconstriction. Quantitatively, in this study, ET_A and ET_B activation account for 55% and 42% of the vasoconstriction due to NOS inhibition in control hearts.

The role of ET-1 in vasoconstriction due to NOS inhibition, particularly via the ET_A receptor, has been shown previously (99, 101, 245, 295, 316). This result is not unexpected since endogenous NO normally inhibits ET-1 production and activity.
Therefore, NOS inhibition removes this inhibitory influence on the ET-1 system (96, 144, 319). The role of ET_B receptors in L-NAME vasoconstriction of normal subjects has been examined previously by Cardillo et al (42) in the human forearm and by Wang et al (316) in the isolated rat heart. Similar to our results, L-NAME vasoconstriction was reduced by BQ-788 in human forearm but not in rat heart. Cardillo et al concluded that ET_B-mediated NO release and therefore overall NO bioavailability was reduced by BQ-788 pretreatment resulting in reduced L-NAME vasoconstriction. Our results support this conclusion; however, both studies may also suggest that L-NAME vasoconstriction is reduced due to inhibition by BQ-788 of VSMC ET_B receptors. This would reduce the role of ET-1-mediated vasoconstriction in L-NAME vasoconstriction thereby reducing this response. Neither Cardillo et al nor the present study provide evidence to differentiate the potential contribution of endothelial and VSMC ET_B receptors in this response. Taken together, our results show that endogenous ET-1 production and activity via ET_A and ET_B receptors contributes to coronary L-NAME-mediated vasoconstriction in control animals.

Inhibition of ET-1 receptors had no effect on L-NAME vasoconstriction in T2DM hearts. This may seem paradoxical as one might suspect, as we hypothesized, that ET-1 would account for a larger percentage of L-NAME vasoconstriction in states with reduced NO bioavailability such as T2DM. However, other alterations such as reduced ET-1 production or reductions in ET_A and ET_B receptor density, binding or coupling to intracellular signaling pathways may account for our results since each of these would decrease the role of endogenous ET-1 in L-NAME-mediated vasoconstriction.
With regard to ET-1 production, the decreased role of ET-1 in L-NAME vasoconstriction in T2DM hearts (Figure 11) may involve the enhanced ROS production in our model. Superoxide has been shown to inhibit ET-1 production by ECE in cultured endothelial cells (173). Therefore, since we have demonstrated a superoxide-mediated reduction in NO bioavailability in this model, superoxide may also inhibit ECE thereby reducing ET-1 production, especially following L-NAME treatment when ECE NO inhibition is removed. Therefore, the contribution of endogenous ET-1 to L-NAME vasoconstriction would be reduced. In addition, this may suggest that the increased ECE expression in human T2DM patients with coronary artery disease may occur to compensate for reduced ECE activity (11). However, conclusions from this study are limited since the study population had profound vascular disease and numerous medications, including angiotensin converting enzyme inhibitors, which can influence the ET-1 system.

Alterations in ET_A and ET_B receptor dynamics may also account for the reduced role of ET-1 in L-NAME vasoconstriction in T2DM hearts. As discussed previously, many studies demonstrate increased ET_A and ET_B receptor expression along with ET_A receptor binding and signaling (117, 143, 339). However, in small coronary arteries from obese Zucker rats, Katakam et al (141) demonstrated impaired ET-1 vasoconstriction due to enhanced ET_B-mediated NO release and receptor-calcium signaling uncoupling. Some human studies also suggest impaired ET_A-mediated vasoconstriction to endogenous ET-1 (186). Therefore, while we did not examine ET-1 receptor dynamics, the literature supports a possible role for ET-1 receptor subtype alterations that would reduce
vasoconstriction to endogenous ET-1 thus reducing its role in L-NAME-mediated vasoconstriction in T2DM.

Interestingly, analysis of the literature suggests potential differences in ET-1 release during NOS inhibition in humans and mice. Low dose ET-1 has been shown to preferentially bind ET<sub>B</sub> receptors inducing vasodilation and higher doses induce vasoconstriction via VSMC ET-1 receptor activation (290). In the human forearm, pretreatment with the ET<sub>A</sub> receptor antagonist BQ-123 did not affect L-NAME vasoconstriction whereas we report a significant reduction using a similar protocol. This suggests that L-NAME-mediated ET-1 release in mice is higher than in humans since ET<sub>A</sub> receptors are bound at higher ET-1 concentrations and blockade of these receptors reduced L-NAME vasoconstriction only in mice. This may also be due, however, to reduced activity of endothelial ET<sub>B</sub> receptors in the mouse. Further research is necessary to determine the role of endothelial ET<sub>B</sub> receptors in this species.

In summary, we conclude that endogenous ET-1 production contributes to L-NAME-mediated vasoconstriction via ET<sub>A</sub> and ET<sub>B</sub> receptor activation in control animals. Conversely, ET-1 does not contribute to L-NAME vasoconstriction in T2DM hearts possibly due to superoxide-mediated ECE inhibition thereby reducing endogenous ET-1 production.

*Role of ET-1 Receptors in ET-1 Vasoconstrictor Responses.* As already discussed, we demonstrated enhanced coronary vasoconstriction to exogenous ET-1 in T2DM mouse hearts. This finding was extended by examining the role of ET<sub>A</sub> and ET<sub>B</sub> receptors in coronary vasoconstriction to exogenous ET-1. These experiments were
important for two reasons: 1) they served to ensure that the $\text{ET}_A$ and $\text{ET}_B$ receptor antagonists were performing the desired action of blocking ET-1 receptors and 2) they provided data on the specific receptor populations through which ET-1 vasoconstriction is mediated and whether T2DM influences receptor subtype involvement in this response.

As previously discussed, coronary vasoconstriction to ET-1 was similar in control and T2DM hearts in the presence of NOS inhibition by L-NAME (Figure 19). Following $\text{ET}_A$ inhibition with BQ-123, exogenous ET-1-mediated coronary vasoconstriction was reduced similarly in both control and T2DM hearts (Figures 20 and 21). Control and T2DM ET-1 vasoconstriction was reduced 43% and 60%, respectively. In contrast, ET-1 vasoconstriction was abolished in control hearts and unchanged in T2DM hearts following $\text{ET}_B$ receptor inhibition with BQ-788 (Figures 20 and 21). Therefore, in agreement with our previous results, $\text{ET}_B$ receptor involvement is also altered in coronary responses to exogenous ET-1 in T2DM.

Our finding that coronary vasoconstriction to exogenous ET-1 is reduced by $\text{ET}_A$ receptor inhibition is not surprising. In most species and vascular beds, $\text{ET}_A$ receptors play a dominant role in ET-1-mediated vasoconstriction (19, 39, 101, 143, 226, 317, 351). $\text{ET}_A$ inhibition did not, however, completely eliminate ET-1 vasoconstriction suggesting VSMC $\text{ET}_B$ receptor involvement and/or, as others have suggested, that ET-1 may stimulate the release of local vasoconstrictor substances such as TXA$_2$ and PGH$_2$ (298). In control animals, our results support the former and additional experiments are needed to examine the latter.
The abolition of ET-1-mediated vasoconstriction with ETB receptor inhibition in control hearts suggests that ETB receptor activation is permissive for ETA-mediated coronary responses. In other words, basal ETB receptor activation is necessary for ETA receptor-mediated responses to occur. Previous investigations have yielded similar results in the mouse mesenteric, renal and systemic circulation. Specifically, Berthiaume et al (27, 28) found that ETA receptor inhibition only partially inhibited ET-1-mediated vasoconstrictor and pressor effects whereas ETB antagonism eliminated ET-1 responsiveness locally and systemically. Of note, these studies, unlike the present, were conducted in the presence of endogenous NO production. Therefore, the elimination of ET-1 vasoconstriction by ETB inhibition is likely not NO-dependent. This role for VSMC ETB receptors appears to be species specific in mice since ETB blockade in humans, rabbits and rats either enhances, due to reduced ETB NO production, or does not change ET-1-mediated vasoconstriction (4, 30, 32, 143, 261).

In T2DM mouse hearts, ETB inhibition did not alter ET-1-mediated coronary vasoconstriction. Therefore, if ETB activation is necessary for ET-1-mediated vasoconstriction in control animals, this requirement is lost in T2DM. These data complicate our previous suggestion that enhanced ETB-mediated vasoconstriction may play a role in the overall enhancement of ET-1-mediated vasoconstriction in T2DM hearts. Following this suggestion, if the permissive role of ETB receptors is lost in T2DM, ETB receptor inhibition would be expected to reduce ET-1-mediated vasoconstriction. However, our results may be accounted for by the ET-1 clearance role of ETB receptors and possible upregulation of other vasoconstrictor systems. In rat
hearts, ET<sub>B</sub> receptor inhibition led to increased ET-1 levels likely due to ET-1 dissociation from ET<sub>B</sub> receptors (35). If present in our study, this ET-1 would likely bind ET<sub>A</sub> receptors therefore enhancing ET<sub>A</sub>-mediated vasoconstriction and masking the loss of ET<sub>B</sub>-mediated vasoconstriction. Upregulation of other endogenous vasoconstrictor systems that are activated by ET-1, such as TXA<sub>2</sub> and PGH<sub>2</sub>, has been noted in obese mice and may also mask a loss of ET<sub>B</sub>-mediated vasoconstriction in T2DM hearts (298).

Biochemical coupling of ET<sub>A</sub> and ET<sub>B</sub> receptors has not been elucidated in vascular smooth muscle; however, cellular crosstalk between these receptors would not be surprising. Each of these receptors is able to activate and impinge on numerous cellular signaling systems (12, 75, 150, 160). Coupled with the extent of T2DM-related alterations in cellular signaling components such as PLC, DAG and PKC, it is possible that crosstalk between these receptors could be uncoupled in this state. Unfortunately, little is understood in this area. Functionally, it is clear that an interaction occurs; however, the mechanism of that interaction and how it is altered by T2DM remains unclear. Taken together, our results support the role of ET<sub>A</sub> receptors in ET-1-mediated coronary vasoconstriction; however, it appears that, in mice, the ET<sub>B</sub> receptor acts in a permissive fashion to allow ET<sub>A</sub>-mediated vasoconstriction and that this role for the ET<sub>B</sub> receptor may be eliminated in T2DM.

E. Effect of Diet-induced T2DM on the Coronary α<sub>1</sub>-AR System

Previous studies have revealed that α<sub>1</sub>-AR-mediated vasoconstriction is enhanced, depressed or unchanged by T2DM (138, 140, 183, 216, 263). In addition, it has been demonstrated that alterations in α<sub>1</sub>-AR function can occur in the absence of systemic
sympathetic dysfunction or overactivity (116). In T2DM, it is possible that α₁-AR responses are altered by direct changes in α₁-AR receptor density and sensitivity or by reduced NO attenuation of α₁-AR-mediated vasoconstriction (31, 139, 277, 301, 346).

Therefore, we examined the coronary vascular responses to α₁-AR activation with PE and the role of NO in this response in control and T2DM mouse hearts. We hypothesized that 1) coronary vasoconstriction to PE would be enhanced in T2DM hearts; and 2) coronary vasoconstriction to PE would be enhanced following L-NAME pretreatment.

Circulating catecholamines, as well as norepinephrine released from sympathetic nerve terminals, activate α₁-AR on VSMC inducing Gq-protein coupled PLC-mediated IP₃ and DAG production, PKC activation, increased intracellular calcium levels and vasoconstriction (104). The α₁-AR family of receptors is found throughout the mouse vascular tree as indicated by tissue-specific genomic analysis (7). Likewise, knockout studies have shown that these receptors are necessary for the maintenance of normal arterial blood pressure (50, 249). Sympathetically-mediated basal vascular tone acting via α₁-AR has also been demonstrated in human, dog and rat vasculature, including the coronary circulation where α₁-AR-mediated tone is minimal (72, 108, 115, 266, 327).

Dose-response studies in control isolated mouse hearts resulted in substantive coronary constriction to the selective α₁-AR agonist PE. Following NOS inhibition with L-NAME, PE responses were augmented in control but not T2DM hearts (Figures 16 and 17). This supports findings that vascular adrenergic responses are normally attenuated by local NO production. This may occur through several mechanisms. Increased endothelial shear stress, activation of endothelial α₁-AR and shared calcium transients
between VSMC and endothelial cells each can result in increased NO release thereby limiting $\alpha_1$-AR-mediated vasoconstriction (31, 134, 139, 265, 301). We are unable to determine in the present study whether the opposing NO influence is due to basal NO release or NO released subsequent to $\alpha_1$-AR activation. However, our results support the role of NO as a normal competitive influence against coronary vascular constriction due to $\alpha_1$-AR activation in control animals.

A majority of the literature supports either no change or enhanced $\alpha_1$-AR-mediated vasoconstriction in T2DM (89, 183, 192, 206, 211, 300, 346). Contrary to our hypothesis, vasoconstriction to PE was not changed in T2DM hearts and was similar to that seen in control animals (Figure 15). Our results are consistent with a reduction in NO bioavailability in T2DM hearts because, unlike control hearts, NOS inhibition did not augment PE vasoconstriction. This suggests a lack of NO attenuation of PE vasoconstriction in T2DM hearts.

It should be noted that while we found no functional change in T2DM $\alpha_1$-AR vasoconstriction our results do not suggest that T2DM has no effect on the coronary $\alpha_1$-AR system. Although not found in the present study, possibly due to anesthesia, hypertension due to sympathetic nervous system overactivity has been demonstrated in this model by some investigators (190). In cell culture it has been demonstrated that human $\alpha_1$-AR subtypes are differentially influenced (i.e., downregulated and upregulated) by overstimulation in a manner that may allow for the maintenance of normal $\alpha_1$-AR vasoconstriction (343). In the context of T2DM, OLETF rats have demonstrated upregulation of $\alpha_{1B}$-AR (346). In addition, ROS and insulin have been suggested to
negatively influence $\alpha_1$-AR (91, 92, 191). Therefore, the balance of $\alpha_1$-AR subtypes may be altered in our model in the absence of functional $\alpha_1$-AR alterations.

In summary, coronary dysfunction in diet-induced T2DM B6 mice is characterized by a functionally unaltered $\alpha_1$-AR system. Unchanged responses to $\alpha_1$-AR activation in T2DM hearts following NOS inhibition further supports the presence of reduced NO bioavailability in this model. Further studies need to be completed in order to determine if diet-induced T2DM leads to altered $\alpha_1$-AR subtype expression or activity in this model.

F. Effect of Diet-induced T2DM on $\text{ET}_A$ and $\text{ET}_B$ Receptor Involvement in Coronary $\alpha_1$-AR Vasoconstriction

Previous studies have demonstrated significant interactions between the ET-1 and $\alpha_1$-AR systems \textit{in vitro} and \textit{in vivo} (62, 63, 69, 306). For example, in cultured rat-1 fibroblasts, $\text{ET}_A$ receptor activation leads to $\alpha_1$-AR phosphorylation and desensitization (306). In contrast, ET-1 has been shown to enhance sympathetically-mediated vasoconstriction for a period of 30 minutes at high concentrations in rabbit ear arteries (336). In addition, some but not all, \textit{in vivo} studies have demonstrated that ET-1 can suppress $\alpha_1$-AR-mediated changes in blood pressure (62, 63, 94). Little is known concerning the interaction of these two systems in T2DM. Therefore, we examined the role of endogenous ET-1 acting via $\text{ET}_A$ and $\text{ET}_B$ receptors in the coronary vasoconstrictor response to $\alpha_1$-AR activation with PE. We hypothesized that endogenous ET-1 via $\text{ET}_A$ receptors acts to attenuate $\alpha_1$-AR-mediated vasoconstriction and this inhibition would be increased by T2DM. The role of $\text{ET}_B$ receptors in $\alpha_1$-AR-mediated
vasoconstriction has not been examined and predictions concerning this receptor are
difficult since ET\textsubscript{B} receptor involvement in \(\alpha_1\)-AR vasoconstriction, if present, depends
largely on the balance of VSMC and endothelial ET\textsubscript{B} receptor activities.

In control animals, our results do not support an interactive role between these
systems. Neither ET\textsubscript{A} or ET\textsubscript{B} receptor inhibition altered coronary vasoconstriction to the
selective \(\alpha_1\)-AR agonist PE (Figure 16). It may be that our experimental time course to
examine this interaction was not long enough to see an effect. DeFily et al (69)
demonstrated in dog coronary arteries \textit{in situ} that the long-term component
(approximately 120 minutes) of PE-induced vasoconstriction was inhibited by ECE or
ET\textsubscript{A} inhibition. Therefore, with the short PE infusions of the present study of no more
than 10 minutes, a role for ET-1 in this response would be unnoticed. Our results do
elucidate, however, that \(\alpha_1\)-AR activation does not acutely induce ET-1 release in the
isolated mouse heart.

As previously discussed, we demonstrated significant alterations of ET-1 receptor
activity in T2DM mouse hearts. With respect to the relationship between ET-1 and \(\alpha_1\)-
AR, it has been found that long-term exposure to elevated endogenous ET-1 levels
attenuates the sympathetically-driven pressor response to acute stress in ET\textsubscript{B} receptor
deficient rats (62). This is important since plasma ET-1 levels and local ET-1 expression
and production are enhanced in models of T2DM (16, 136, 262, 307). Results of the
present study support the role of endogenous ET-1 via the ET\textsubscript{A} receptor to attenuate \(\alpha_1\)-
AR-mediated coronary vasoconstriction in T2DM hearts. Following ET\textsubscript{A} inhibition with
BQ-123, CVR increases to PE infusion (\(10^{-8} – 10^{-5}\) M) were significantly enhanced.
However, ET\textsubscript{B} receptor inhibition did not change PE vasoconstrictor responses (Figure 17). To our knowledge, we are the first to report T2DM-mediated alterations in the coronary interaction between ET-1 and \(\alpha\textsubscript{1}-\text{AR}\).

The majority of studies examining the interactions between these systems are functional in nature. However, cell culture studies have elucidated one possible explanation for ET\textsubscript{A} suppression of \(\alpha\textsubscript{1}-\text{ARs}\). As mentioned above, ET\textsubscript{A} receptor activation by ET-1 in rat-1 fibroblasts stably expressing \(\alpha\textsubscript{1B}-\text{ARs}\) leads to \(\alpha\textsubscript{1B}-\text{AR}\) phosphorylation, altered G-protein coupling, and attenuation of norepinephrine-induced calcium mobilization (306). A later study by the same group revealed that ET-1 also induces phosphorylation of \(\alpha\textsubscript{1D}-\text{ARs}\) in the same cell line (93). Further analysis suggests that \(\alpha\textsubscript{1}-\text{AR}\) phosphorylation was mediated partly by PKC activation for three reasons: (1) the temporal time course for ET-1-mediated \(\alpha\textsubscript{1}-\text{AR}\) phosphorylation is similar to that for PKC translocation; (2) the \(\alpha\textsubscript{1}-\text{AR}\) phosphorylation was significantly attenuated by combined inhibition of serine/threonine and tyrosine kinases and (3) PKC activation with a phorbol ester induced a dose-dependent \(\alpha\textsubscript{1}-\text{AR}\) phosphorylation (93, 306).

Numerous studies have revealed elevated PKC activity in models of T2DM. Avignon et al (14) found increased DAG levels and PKC activation in soleus muscle of type 2 diabetic Goto-Kakizaki rat, Zucker fatty rats, and obese rats. Considine et al (57) also noted increased PKC activity in T2DM humans and Zucker rats. A recent study found increased PKC\(\beta\) mRNA expression and activity in the mesenteric vasculature of the T2DM C57BL/6J-db/db mouse (157). This study was the first to examine alterations in PKC activity in the resistance vasculature during T2DM. Therefore, our finding that
ETA receptor inhibition augments α1-AR-mediated vasoconstriction may be due to the disinhibition of α1-AR by the removal of ETA-mediated α1-AR phosphorylation likely involving PKC activation. It should be noted, however, that our results also suggest a generalized upregulation of α1-AR responsiveness following ETA inhibition. This may be due to α1-AR upregulation in T2DM that is functionally masked by active ETA receptors.

G. Conclusions

The present study has further defined the diet-induced model of obesity and type 2 diabetes in the C57BL/6J mouse. We have confirmed the findings of others that high-fat, high-simple carbohydrate feeding in these animals leads to the development of T2DM characterized by obesity, impaired glucose tolerance, hyperglycemia, hyperinsulinemia and dyslipidemia but not hypertension. Our isolated heart studies have also revealed significant alterations in coronary vascular function in this model. From our results, we conclude that:

1) defects in coronary NO responses in diet-induced T2DM are due to reduced NO bioavailability due to enhanced superoxide scavenging of NO and not alterations in VSMC NO signaling;

2) endogenous ET-1 activity partially accounts for coronary vasoconstriction to NOS inhibition in control hearts but not in T2DM hearts possibly due to superoxide inhibition of ET-1 production by ECE;

3) endogenous ET-1 does not play a substantive role in basal coronary tone in control hearts but contributes significantly in T2DM hearts due to VSMC ETB.
receptor activation and possible upregulation or enhanced signaling through this receptor;

4) vasoconstriction to exogenous ET-1 is enhanced in T2DM possibly due to reduced NO bioavailability or enhanced VSMC ET\textsubscript{B} receptor activity;

5) vasoconstriction to exogenous ET-1 is partially mediated by ET\textsubscript{A} receptors and, in control animals, ET\textsubscript{B} receptor activation may be permissive for ET-1-mediated coronary vasoconstriction;

6) the mouse coronary $\alpha\textsubscript{1}$-AR system is functionally unaltered by diet-induced T2DM, but

7) ET\textsubscript{A} receptors appear to attenuate $\alpha\textsubscript{1}$-AR-mediated coronary vasoconstriction in T2DM hearts suggesting a functionally masked upregulation of the $\alpha\textsubscript{1}$-AR system in this model.

In summary, diet-induced obesity and T2DM leads to shift in the balance of coronary vascular regulation mechanisms such that vasodilator influences by NO are reduced and vasoconstrictor influences by ET-1 are enhanced. Oxidative stress may play a central role in this process along with long-term changes in ET-1 receptor density and activity possibly due to elevated production of ET-1. Significant alterations in intracellular interactions between ET\textsubscript{A}, ET\textsubscript{B} and $\alpha\textsubscript{1}$-AR are also suggested and warrant further investigation. The primary findings of this study are presented graphically in Figure 27. Overall, this study emphasizes the speed and severity of the deleterious effects of T2DM on coronary function as well as the importance of appropriate interactions between regulatory mechanisms in the control of coronary function.
Figure 27. Summary of findings in control and T2DM mouse hearts. Control mice (Panel A) demonstrate inhibition of endogenous ET-1 production by NO. Endogenous NO attenuates ET-1 and $\alpha_1$-AR-mediated vasoconstriction via cGMP inhibition of contraction. Exogenous ET-1-mediated vasoconstriction occurs primarily through ETA receptor activation and basal ETB receptor activation is permissive for coronary ET-1 responses in these animals. T2DM mice (Panel B) demonstrate reduced NO bioavailability due to enhanced superoxide NO scavenging. Inhibition of endogenous ET-1 by NO is unclear in these animals. ET-1-mediated vasoconstriction is enhanced possibly due to enhanced VSMC ETB receptor-mediated responses. ET-1-mediated vasoconstriction occurs primarily through ETA receptor activation and the permissive role of ETB receptors is absent in T2DM. PE-induced vasoconstriction is unchanged from control; however, ETA receptor activation attenuates $\alpha_1$-AR-mediated vasoconstriction in T2DM hearts. Endogenous NO does not reduce exogenous ET-1 and $\alpha_1$-AR-mediated vasoconstriction. NO, nitric oxide; NOS, nitric oxide synthase; O$_2^-$, superoxide anion; ONOO$^-$, peroxynitrite; GC, guanylyl cyclase; GTP, guanosine triphosphate; cGMP, cyclic guanosine-3’5’-monophosphate; ET-1, endothelin-1; ECE, endothelin converting enzyme; ETA, endothelin type A receptor; ETB, endothelin type B receptor; $\alpha_1$, alpha(one)-adrenoceptor; [Ca$^{2+}$]$_i$, intracellular calcium concentration.
References


34. **Brownlow BS, Petro A, Feinglos MN, and Surwit RS.** The role of motor activity in diet-induced obesity in C57BL/6J mice. *Physiol Behav* 60: 37-41, 1996.


249. **Rokosh DG and Simpson PC.** Knockout of the α1A/C-adrenergic receptor subtype: the α1A/C is expressed in resistance arteries and is required to maintain arterial blood pressure. *Proc Natl Acad Sci USA* 99: 9474-9479, 2002.


