PTERIN METABOLISM AND DIABETIC VASCULOPATHY

By

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To Uncle Joe

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The number of diagnosed and un-diagnosed cases of diabetes in the United States is expected to double over the next twenty-five years. With the rise in diabetes comes a rise in cardiovascular disease and the occurrence of vascular complications in the form of macro- and micro-angiopathies. These vascular complications are the pathological manifestation of an impaired endothelium arising from reduced nitric oxide biosynthesis. Reduced NO bioavailability in the endothelium has been identified as a critical mediator in the increased cardiovascular disease susceptibility observed in diabetics and is believed to occur as a consequence of increased oxidative stress.

Endothelial dysfunction is apparent in diabetes despite the fact that eNOS expression is actually increased. The paradoxical finding of a concomitant increase in eNOS expression and reduced endothelium-dependent vasodilation has drawn attention to the fact that eNOS itself, in pathological states, may be a source of superoxide anions, a process which has been termed "eNOS uncoupling". It is our hypothesis that the increased cardiovascular disease risk observed in diabetics is a result of altered pterin metabolism and subsequent pathological NO and ROS signaling. In support, our results indicate that in the diabetic state, tetrahydrobiopterin salvage

enzymes are impaired leading to the accumulation of pterin oxidation products and subsequent eNOS uncoupling. Moreover, our studies identify dihydrofolate reductase as a therapeutic target for treating diabetic vascular dysfunction. Specifically, using a gene therapy approach we demonstrated that over-expression of DHFR in the aorta of diabetic mice significantly improved endothelial-dependent relaxation.

Using a combination of cellular, molecular and physiological approaches we characterized the mechanisms involved in the regulation of pterin metabolism and the consequences on endothelial NO and ROS signaling as it pertains to the pathogenesis of diabetic macro- and micro-angiopathies. In summary, studies carried out in this thesis provide fundamental mechanistic information regarding the pathways through which diabetes alters endothelial cell function and may lead to new approaches to treat or prevent diabetic vascular complications.

CHAPTER 1
INTRODUCTION

The number of diagnosed and un-diagnosed cases of diabetes in the United States is expected to double over the next twenty-five years. Currently there are 23.7 million Americans with diabetes with an estimated healthcare cost of $113 billion[1]. Approximately 6% of global mortality is due to diabetes with 50% of diabetic mortalities due to cardiovascular disease (CVD). Myocardial infarctions occur twice as often in individuals with diabetes than the general population. Men with diabetes have a two to three fold higher incidence of CVD and women have a 4 fold higher incidence of CVD than men and women without diabetes[2]. According to the National Health and Nutrition Survey in 1999-2000, the prevalence of patients categorized as overweight was up from 55.9% to 64.5% and obesity was 30.5% up from 22.9%. Furthermore, extreme obesity classified as a BMI>40 was increased to 4.7% from 2.9%[3]. The occurrence of diabetes is on the rise and is directly attributed to the rise in obesity. Among all ages, both sexes and all ethnic groups, the occurrence of diabetes rose 4.9% to 6.5% from 1990-1998[5]. With this rise in diabetes comes a rise in CVD risk among this patient population. The occurrence of vascular complications in the form of macro- and microangiopathies are expected to grow exponentially over the next several decades[6].

Microvascular disease is the leading cause of kidney failure, blindness and nerve damage in individuals with diabetes. Moreover, early stages of hyperglycaemia cause a decrease in activity of vasodilators such as nitric oxide leading to increased vascular permeability, blood flow abnormalities and predisposition to cardiovascular disease. The permeability changes result in irreversible microvascular cell loss due to programmed cell death. Furthermore, growth factors initiate proliferative responses in

the vascular wall in an attempt to replace damaged cells. This proliferative response manifests to progressive capillary occlusion. Individuals with diabetes also have a higher rate of atherosclerotic macrovascular disease affecting large vessels leading to the heart, brain, and lower extremities causing myocardial infarctions, stroke and limb amputations. A classical example of macroangiopathy is coronary atherosclerosis and vascular intimal hyperplasia. Microangiopathy is exemplified by diabetic foot disease, retinopathy and nephropathy. In patients suffering from diabetes, macroangiopathy manifests as atherosclerosis like in non-diabetic patients, characterized by formation of plaques that follows in stages, but with an accelerated course due to the different risk factors such as hyperglycemia and hyperlipidemia[7]. Thus, atherosclerosis in diabetes begins earlier, is more markedly pronounced and progresses more rapidly. The pathogenic concept behind the increased cardiovascular risk seen in diabetics has focused on altered endothelial function that occurs as a result of a diabetes-induced endothelial damage[8].

Individuals with diabetes mellitus exhibit accelerated atherosclerosis, more diffuse disease, concomitant co-morbidities and have an increased risk for restenosis and graft failure following revascularization procedures[9, 10]. In addition to these macrovascular complications, impairment of the microcirculation of diabetic patients contributes to secondary complications in the lower extremities, such as foot infections and ulcerations as a result of impaired wound healing. These microcirculatory changes, which are mainly functional rather than structural, are responsible for the impaired ability of the microvasculature to vasodilate in response to injury. Functional impairment of endothelial activity precedes the development of these morphological alterations[11].

Endothelial dysfunction results from reduced bioavailability of the vasodilator nitric oxide (NO), mainly due to decreased NO production and increased formation of reactive oxygen species (ROS) [12-14]. Although hyperglycemia, insulin resistance, hyperinsulinemia and dyslipidemia independently contribute to endothelial dysfunction via several distinct mechanisms, increased oxidative stress seems to be the first alteration triggering several others.

Several mechanisms have been proposed for the increase in oxidative stress observed in diabetes; among them are the depletion of tetrahydrobiopterin (BH_4) and the accumulation of its oxidation product dihydrobiopterin (BH_2). *In-vitro* studies have demonstrated that the depletion of BH_4, an essential cofactor for endothelial NO synthase (eNOS), causes eNOS to readily produce superoxide ($\dot{O}^-_2$)[15, 16]. Moreover, it is known that BH_4 can be readily oxidized to its inactive form BH_2, since it is highly redox sensitive. The oxidation of BH_4 becomes important in the production of NO since BH_4 and BH_2 have an equal affinity for eNOS. In this regard, BH_2 binding results in incomplete electron transfer resulting in eNOS oxidase activity[17]. In the endothelial cell, BH_4 is produced by two main pathways, the salvage pathway and the de novo synthesis pathway. Through the de novo pathway, biosynthesis is a NADPH, zinc and magnesium dependent process that first requires the conversion of GTP to 7,8-dyhydroneopterin triphosphate. The catalyst for this step is GTP cyclohydrolase I (GTPCH), and it is the rate limiting step in BH_4 biosynthesis[18]. Subsequently, pyruvoyl tetrahydropterin synthase (PTPS) converts 7,8 dihydroneopterin triphosphate into 6-pryuvoyl-5,6,7,8-tetrahydropterin. Alternatively, Dihydrofolate reductase (DHFR), an

NADPH dependent enzyme catalyzes the salvage pathway in the reduction of BH_2 to BH_4[17].

Impaired endothelial function is apparent in experimental diabetes and in diabetic patients despite the fact that eNOS expression is actually increased[19-22]. Based on these observations it has been proposed that in diabetes, eNOS is uncoupled and NOS oxidase activity contributes to the increased risk of cardiovascular disease seen in this population. This would be expected to result in a feed-forward cascade in which eNOS derived $^{\cdot}O^{-}_{2}$ results in further eNOS uncoupling and eventual obliteration of NO synthesis. Therefore, research efforts are needed to identify novel therapeutic targets to prevent eNOS uncoupling and reduce the micro and macrovascular complications associated with diabetes.

CHAPTER 2
REVIEW OF THE LITERATURE

The endothelium is known to play a critical role in the maintenance of vascular homeostasis through its anti-proliferative, anti-atherogenic and anti-thrombotic properties. One of the key pathways for regulating the biology of the endothelium is through the production of the gaseous free radical Nitric Oxide (NO). The identification of NO was elegantly identified by an acetycholine response in rabbit aortic rings by Furchgott and Zawadzi. These studies demonstrated that when the endothelial cell layer was removed not only was the acetylcholine response negated, but resulted in overt vasoconstriction[23]. The relaxation effect was described as the endothelial-derived relaxation factor (EDRF); which was later shown independently by both Moncada, and Ignarro to be NO[24, 25]. One year later, NO was found to be synthesized from the substrate L-Arginine by the enzyme nitric oxide sythase[26]. Since these findings, NO has been extensively studied and found to be a significant regulator in vascular homeostasis and decreased levels are implicated in the pathogenesis of endothelial dysfunction. Moreover, endothelial dysfunction has been observed in pre-diabetic stages of insulin resistance and has been shown to play a central role in vascular impairment and atherogenesis[27, 28].

Nitric Oxide

Endothelial Nitric Oxide Synthase

Endothelial derived nitric oxide is synthesized by the enzyme eNOS. This is carried out through the oxidation of the guanidino carbon of the amino acid L-Arginine, forming NO and L-Citrulline[26]. In the vasculature, one of the primary functions of NO is to cause vascular smooth muscle cell (VSMC) relaxation. Relaxation occurs by NO

freely diffusing from the endothelium into the VSMC where it binds to the heme group of the enzyme guanylate cyclase. Guanylate cyclase catalyzes the conversion of guanosine triphosphate (GTP) to cyclic guanosine 3'5'-monophosphate (cGMP) and inorganic phosphate[25, 29]. In return, cGMP activates protein kinase G (PKG) leading to phosphorylation of the myosin light chain phosphatase. The myosin light chain is then dephosphorylated by myosin light chain phosphatase, resulting in the relaxation of the vascular smooth muscle cell (figure 2-1). In addition to its effects on SMC contractility, NO also counteracts pro-proliferative agents involved in athero-proliferative disorders and helps maintain smooth muscle cell quiescence[30].

Actions of Nitric Oxide

NO is important for maintaining vascular homeostasis by modulating vascular tone and through its anti-atherogenic, anti-proliferative and anti-thrombotic properties. Through animal models and human studies, it has been demonstrated that NO plays an important role in preventing platelet aggregation. In a cyclic GMP secondary mechanism, NO and prostaglandin work together to prevent platelet aggregation[31]. In this regard, human studies with healthy volunteers demonstrated a decrease in bleeding when the NOS inhibitor L-NNMA was administered intravenously[32]. In further support, using a rat model of common carotid artery thombosis, platelet aggregation was increased following administration of the NOS inhibitor L-NAME[33].

The endothelium plays a critical role in thrombosis through the actions of Weibel-palade bodies (WPBs). WPBs are endothelial granules that are released through exocytosis in response to physical damage, endogenous chemicals, proteins and lipid messengers. These factors include, but are not limited to: hypoxia, shear stress, thrombin, oxidized low-density lipoproteins and ROS. WPBs release von Willebrand

factor (VWF) into the blood, this increases platelet adhesion to the vessel wall and each other. Several families of proteins control the stages of exocytosis leading to the release of VWF. N-ethylmaleimide-sensitive factor (NSF) is the protein family that drives the molecular motor of WPBs endothelial exocytosis through vesicle trafficking. It has been demonstrated that exogenous NO can S-nitrosylate NSF rendering it inactive and inhibiting the exocytosis of WPBs[34]. This inhibition of WPBs showcases NO's anti-thrombotic capabilities.

Furthermore, the role of NO in maintaining vascular homeostasis can be seen in its anti-atherogenic properties. WPBs also releases P-Selectin, by exocytosis, to the outer cell surface where it interacts with ligands on leukocytes. This interaction initiates leukocyte rolling, microparticle production and recruitment which can lead to inflammation and atherogenesis. As previously stated, NO inhibits WPBs exocytosis by S-nitrosylation of NSF. Moreover, NO also exhibits anti-atherogenic properties by inhibiting the NF kappa B signaling pathway preventing inflammatory responses in the endothelial cell monolayer. NO does not inhibit NF kappa B directly, but through the induction and stabilization of the NF kappa B inhibitor, I kappa B alpha. This prevents NF kappa B from interacting with its DNA binding motifs on select genes. Inhibiting NF kappa B results in inhibition of pro-atherogenic adhesion molecules VCAM-1, E-selectin, and ICAM-1[35, 36].

In addition to anti-thrombotic and anti-atherogenic effects, NO along with several other signaling molecules maintains the anti-proliferative properties of the endothelium. For individuals with CAD, specifically coronary artery stenosis, balloon angioplasty is the standard treatment. Unfortunately, this intervention causes endothelial cell injury

and denudation which is a primary cause for lumen loss and late restenosis. Both human and animal studies of restenosis have concluded that reduced endothelial NO bioavailability contributes to the neointimal hyperplasia[37-39]. Until recently, the mechanism behind NO's anti-proliferative properties on the VSMC were unknown, however, it has now been recognized that NO inhibits cell progression in the S phase. Once a cell proceeds beyond the S phase, where DNA synthesis occurs, it will continue until the cell cycle is complete. Cyclin A and cyclin-dependent kinase 2 (cdk2) are both unregulated during S phase progression and are needed for the cell to complete this phase. NO halts the cell in S phase by inhibiting the phosphorylation of E2F, which is a gene family of transcription factors needed for progression of the S phase. Specifically, this is done by NO inducing down regulation of cyclin A gene transcription and inhibiting cdk2 phosphorylation of E2F during S phase[40].

NOS Regulation

The role of eNOS in the regulation of cardiovascular function and its contribution to disease pathogenesis has been well defined. Current research efforts are now focused on identifying the molecular mechanisms through which eNOS activity is controlled. In this regard, research has identified several critical pathways through which eNOS is regulated including: substrate/inhibitor bioavailability, protein-protein interactions and post translational modifications. Substrate bioavailability has been studied extensively and results demonstrate that although the Km for L-arginine is very low, L-arginine bioavailability can play a regulatory role in the control of eNOS catalytic activity.

Substrate Bioavailability: Arginine

Arginine is made available to the body from various sources including: protein turnover, dietary intake and endogenous biosynthesis. During fasting states, the

majority of circulating arginine is made available through protein turnover with a small amount derived from endogenous biosynthesis[41]. Endogenous biosynthesis of arginine, in a healthy adult human, is enough so that it is not considered an essential amino acid, however, in adults with kidney or intestinal dysfunction or children and infants endogenous, biosynthesis may not be sufficient[42]. The gut-kidney axis is where whole body synthesis of arginine occurs; this is an interaction between the small intestine and the kidney. The kidney takes up citrulline, which is produced from glutamine and proline in the small intestine, the citrulline is then converted to arginine. The liver also synthesizes arginine, however, the arginine is quickly hydrolyzed to urea and ornithine demonstrating that the liver is not a significant source of arginine[43]. The body receives its primary source of arginine from the kidney renal tubules, although the majority of cell types have the ability to synthesize arginine. Argininosuccinate synthase and argininocussinate lyase (ASL) have synergistic action and are responsible for the synthesis of arginine from citrulline. ASL is the rate limiting step in the synthesis[43]. Within the endothelium, the Citrulline-NO cycle is an alternative means of producing arginine, however, the Citrulline-NO cycle only recycles a fraction of the citrulline produced through eNOS oxidation of arginine[41]. In terms of the metabolic fate, almost half of the arginine that is consumed through diet is catabolized within the intestines before reaching the whole body[44]. Cellular arginine metabolism occurs through several pathways within the cell, however, it is predominately metabolized by the enzyme arginase and this has been shown to play and important role in the regulation of endothelial NO production (figure 2-2).

Substrate Bioavailability: Arginase

Arginase is the key enzyme for ariginine metabolism through the urea cycle and is responsible for the hydrolysis of arginine to ornithine and urea. The type I arginase isoform is expressed in the liver and is responsible for the majority of activity. The type II isoform of arginase is expressed predominately in the kidney as a mitochondrial protein with minimal expression in the liver[41].

Recent literature has shown that arginase is present in the vasculature and plays a role in vasomotor tone. Endothelial cells express both isoforms of arginase while VSMC only express type I. High arginase activity was identified in rat aortic smooth muscle cells and both isoforms have been found to be expressed in the pulmonary artery, carotid artery and the aorta[45].

Since arginine is a substrate for arginase, it has been suggested that arginase may inhibit the production of NO by competing with NOS for L-arginine. This has been shown to result in uncoupling of NOS and manifests in the production of superoxide. Zweier et al. demonstrated L-Arginine depletion in macrophages results in a reduction of NO produced by iNOS and an increase in NOS-derived $^{\cdot}O^{-}_{2}$ production[46]. Although arginine has a higher affinity for NOS than arginase, the activity of arginase is 1000 fold higher. Based on these biochemical kinetics it is evident that arginase can compete with arginine for substrate binding of NOS[47]. In support, microvascular endothelial cells from Dahl salt sensitive rats demonstrated a decrease in NO mediated relaxation with an increase in arginase activity. Furthermore, it has been demonstrated that over-expression of arginase in endothelial cells with either isoform of arginase can decrease

NO derived from eNOS[48]. Moreover, the inhibition of arginase activity in endothelial cells has been demonstrated to increase eNOS derived NO[49].

NOS Cofactor and Protein-Protein Interactions

Calmodulin

In addition to substrate regulation of eNOS, several critical protein-protein interactions have been identified for eNOS. Among these, calmodulin (CaM) was the first protein discovered to regulate eNOS activity. Calmodulin is a calcium dependent regulatory protein that includes four binding domains for calcium and regulates numerous different protein targets. Under resting calcium levels, eNOS is in an inactive state and upon calcium binding, calmodulin undergoes a conformational change enabling it to bind to eNOS[50]. Upon binding eNOS, the calcium/calmodulin complex confers an allosteric transition which facilitates electron flow from the reductase to the oxygenase domain. Specifically, this occurs through the CAM binding motif on eNOS which displaces an auto-inhibitory loop[51]. Formation of the eNOS/CAM complex is also critical for NOS activation by catalyzing the dissociation of eNOS from Caveolin-1, an interaction which has been shown to tonically suppress eNOS activity[52].

Caveolae

Caveolae are cholesterol rich invaginations located on the surface of the cell membrane. They are found in large amounts in VSMC, adipocytes and endothelial cells. Caveolae's major structural protein is Caveolin, which can be seen in 3 protein isoforms, Caveolin-1 (Cav-1), Caveolin-2 (Cav-2) and Caveolin-3 (Cav-3)[53]. Several studies have demonstrated that Cav-1 is a negative regulator of eNOS. Cav-1 directly associates with and inhibits eNOS activity in endothelial cells. As previously stated, eNOS is dependent on the binding of calcium/calmodulin complex for the production of

NO. As intracellular calcium rises, the calcium/calmodulin complex triggers the dissociation NOS from Cav-1 and when calcium levels subside Cav-1 sequesters eNOS rendering the enzyme inactive. In support, cellular studies have demonstrated that over-expression of Cav-1 reduces eNOS activity while Cav-1 KO mice exhibit increased NO production from the endothelium and enhanced endothelial dependent relaxation[54] [55]. It is important to state that calmodulin and caveolin are not the only NOS associated proteins capable of influencing the activity of eNOS. Heat shock protein 90 has also been shown to enhance the activity of eNOS.

Hsp90-eNOS

Hsp90 is a chaperone protein which accounts for 1-2 percent of all cytosolic proteins within the eukaryotic cell. It is mainly localized in the cytoplasm and exists as two isoforms, Hsp90 alpha and Hp90 beta[56]. Hsp90 participates in the maturation of proteins, when binding to proteins it influences the folding of the protein to its native state and prevents protein aggregation of unfolded proteins[57]. Hsp90 has also been shown to be important in signal transduction in cells. Several signaling proteins have been shown to interact with Hsp90, including MEK, v-Src and Raf-1[58]. With regards to eNOS, Hsp90 can allosterically enhance the activity of the enzyme following histamine, VEGF, and fluid shear stress stimulation[59]. Much of the data implicating Hsp90 involvement in NOS function comes from pharmacological studies using the ansamycin antibiotic and HSP90 inhibitor, Geldanamycin (GA). GA binds to the ATP binding site of Hsp90 inhibiting the ATP/ADP cycle which is required for protein-protein interaction[60]. Using this approach, Hsp90 was shown to be critical for eNOS activity in isolated mesenteric arteries and aortas of rats[61]. More recent molecular studies using site directed mutagenesis has revealed a Hsp90 binding domain on eNOS which yields a

mutant eNOS with a low affinity for Hsp90 reduced endothelial NO production and increased endothelial derived $^{\cdot}O^{-}_{2}$[62]. These findings suggest that Hsp90-eNOS interaction not only increases the activity of the enzyme but may play a role in regulating eNOS uncoupling. In addition to protein-protein interaction, eNOS post-translational modifications have also been shown to play a critical role in the regulation of both eNOS-derived NO and superoxide. Among these, eNOS phosphorylation is the most widely studied and its role in the regulation of endothelial function will be discussed in the next section.

eNOS Post-translational Modifications

eNOS Phosphorylation

Five phosphorylation sites on eNOS have currently been identified as targets, Ser 1177 (human)/Ser1179 (bovine), Ser114 (H)/Ser116 (B), Ser633 (H)/Ser635 (B), Ser 615(H)/Ser617 (B) and Thr495 (H)/Thr497 (B). However, evidence indicates that phosphorylation primarily occurs at serine (SER) residues and less frequently at tyrosine (Tyr) and threonine (Thr) residues.

Ser 1177/1179

The activation of eNOS through direct phosphorylation at Ser 1179 by Akt was first identified by Fulton et al. and later confirmed by Dimmeler[63,64]. Using pharmacological approaches, the upstream signaling pathways involved in eNOS Ser1177 phosphorylation have been identified. Specifically, pharmacological inhibitors of phosphatidylinositol 3-kinase (PI3K) pathway have been shown to inhibit NO release following stimulation by VEGF and insulin[65,66]. Sequence analysis subsequently revealed that eNOS is a target for phosphorylation by protein kinase Akt which is activated by PI3K[67]. Various stimuli can stimulate the phosphorylation site Ser1179 of

eNOS such as the depletion of BH_4, as seen in hyperglycemic states, insulin, shear stress and bradykinin[68].

Thr 495/497

The PKC pathway mediates phosphorylation of Thr495. Thr495 is located at the Ca2+/CaM binding domain of eNOS and it has been demonstrated that the phosphorylation interferes with the formation of the Ca2+/CaM-eNOS complex[69]. As a consequence, Thr495 phosphorylation is implicated in the pathogenesis of endothelial dysfunction given the propensity of this modification to suppress NO production. Several agonists have been shown to dephosphorylate Thr495 such as calcium ionophore, VEGF and bradykinin, agents which stimulate NO production. Both Ser1177 and Thr495 have also been shown to regulate eNOS oxidase activity with the former suppressing eNOS-derived ROS production and the latter inhibiting it. As such, these post-translation modifications are likely to play a critical role in NOS regulation under both normal physiological conditions as well as pathological conditions wherein eNOS "uncoupling" is likely to occur.

Generation of Oxidative Stress

eNOS Uncoupling

After the identification of NOS as the enzyme responsible for the production of the elusive endothelial derived relaxation factor, Xia et al. and Vasquez-Vivar et al. demonstrated the phenomenon of "eNOS uncoupling", in which pterin-depleted eNOS catalyzed the formation of $^{\cdot}O^{-}_{2}$ from the oxygenase domain of eNOS[15,16]. Subsequent studies demonstrated the pathological relevance of this *in vitro* phenomenon in animal models of hypertension, diabetes, and hypercholesterolemia. The first of these studies by Landmesser et al. demonstrated the presence of increased L-NAME inhibitable

vascular superoxide production in a desoxycorticosterone acetate (DOCA) salt induced model of hypertension[22]. This pioneering study laid the foundation for the hypothesis that endothelial dysfunction is manifested not only as impaired NO bioavailability but also increased eNOS oxidase activity. Similar findings have been reported in the diabetic state wherein endothelial dysfunction is apparent despite the fact that eNOS expression is actually increased[70]. Early studies using endothelial cells derived from diabetic mice demonstrated evidence of altered NO production as a result of insufficient BH_4. These conclusions were largely based on the observation that increases in $^{\cdot}O^{-}_2$ formation occurred in parallel with a decrease in BH_4 following a 48 hour exposure to high glucose[71]. More direct evidence for BH_4 involvement in eNOS uncoupling was provided by Juul et al. whose work demonstrated that over-expression of the rate limiting enzyme in BH_4 synthesis (GTPCH1) increased pterin levels and restored endothelial dependent relaxation[72].

eNOS Uncoupling and Tetrahydrobioterin (BH_4)

First described as an essential cofactor for the aromatic amino acid hydroxylases, tetrahydrobipterin (BH_4) is also an essential cofactor for all three NOS isoforms[73-75]. The role that BH_4 plays in NOS regulation has only recently become more defined. Located within each domain of eNOS is a binding site for a BH_4 molecule. *In vitro* studies demonstrate that BH_4 stabilizes and donates electrons to the ferrous-dioxygen complex in the oxygenase domain to help initiate the oxidation of L-Arginine[76-78]. BH_4 depletion leads to the dissociation of the ferrous-dioxygen complex and electrons from the flavin domain are donated to molecular oxygen instead, leading to the production of superoxide from the oxygenase domain[79,80]. This altered electron transfer in the

absence of BH_4 leads to the phenomenon of “NOS uncoupling” which has been documented in a variety of cardiovascular related diseases including diabetes[71,81,82] (figure 2-3).

As previously stated, BH_4 is an essential cofactor for the aromatic amino acid hydroxylases and NOS. The synthesis of BH_4 occurs via three pathways in the cell, the de novo pathway, the salvage pathway, and recycling pathway. In the recycling pathway, the oxidized product of BH_4, tetrhydrobiopterin-4alpha-carbinolamine, is recycled back to BH_4 in a two step enzymatic process. First Pterin-4alpha-carbionolamine dehydratase (PCD) reduces tetrhydrobiopterin-4alpha-carbinolamine to a quinonoid dihydrobiopterin intermediate which is then further reduced by Dihydropteridine Reductase (DHRP) to BH_4[83,84](figure 2-4).

De novo biosynthesis of BH_4 is a magnesium, zinc and NADPH dependent pathway. The first step requires the conversion of GTP to 7,8-dyhydroneopterin triphosphate. This reaction is catalyzed by the enzyme GTP cyclohydrolase I (GTPCH), and it is the rate limiting step in BH_4 biosynthesis[85]. GTPCH can be regulated at both the gene and protein level. Cytokines such as Tumor Necrosis Factor Alpha (TNF-α) and Interferon-ɣ (IFN-ɣ) increase GTPCH activity and result in increased levels of endothelial BH_4[86-88].

Following the GTPCH enzyme reaction, pyruvoyl tetrahydropterin synthanse (PTPS) converts 7,8 dihydroneopterin triphosphate into 6-pryuvoyl-5,6,7,8-tetrahydropterin. In macrophages, induction by cytokines leads to increased GTPCH activity, however, the activity of PTPS remains unchanged[89,90]. Under these conditions, PTPS becomes the rate limiting enzyme for BH_4 synthesis, and as a result the 7, 8

dihydroneopterin triphosphate intermediate accumulates and can become oxidized to neopterin. Neopterin is a stable metabolite that can be detected in the plasma and used clinically as a marker of inflammation in CAD[91]. The final step in the de novo synthesis pathway involves the NADPH dependent sepiapterin reductase enzyme catalyzing the reaction of 6-pyruvoyl-5,6,7,8-tetrahydropterin to the final product of de novo synthesis, BH_4[92]. A mouse SPR KO model has been generated and this model shows impaired synthesis of BH_4. To date however, no studies have been done to assess the effects on vascular endothelial function in these mice[65].

The salvage pathway is another in which BH_4 can be synthesized. One mechanism through which the salvage pathway regulates BH_4 is through the conversion of exogenous sepiapterin. Sepiapterin is metabolized to BH_2 by sepiapterin reductase and subsequently to BH_4 by the enzyme Dihydrofolate Redutase (DHFR). Alternatively, when BH_4 is non-enyzmatically oxidized to qBH_2 and then further oxidized to BH_2, DHFR can reduce BH_2 back to BH_4[93]. Recently, the role of endothelial DHFR in BAECs as it relates to NO regulation has been investigated. Results demonstrated that a loss in DHFR expression resulted in reduced endothelial NO production and decreased BH_4 bioavailability[94]. These results provide strong evidence that DHFR may serve a critical role in maintaining endothelial BH_4 and subsequent NO production. Moreover, under conditions of oxidative stress, the salavage and recycling pathways maybe critical in maintaining endothelial BH_4/BH_2 and NO production.

Recent studies from Gross et al. and Vazques-Vivar et al. have independently demonstrated that increased levels of the BH_4 oxidation product BH_2, rather than BH_4 depletion alone, is the molecular trigger for NO insufficiency[17,76]. Specifically, the Gross

group has demonstrated that BH_4 and BH_2 bind eNOS with equal affinity and BH_2 can rapidly and efficiently replace BH_4 in preformed eNOS-BH_4 complexes. This group has further shown that exposure of murine endothelial cells (ECs) to high glucose results in an increase in BH_2 levels from undetectable to 40% of the total biopterin pool. This BH_2 accumulation was associated with diminished NO activity and accelerated superoxide production. Since superoxide production was suppressed by NOS inhibitor treatment, eNOS was implicated as the principal superoxide source[17]. These studies suggest that endothelial dysfunction and eNOS uncoupling in the setting of oxidative stress involves both BH_4 depletion and oxidation and thus implicates impairment in both the de novo and recycling pathways, respectively.

Pathophysiology of Diabetic Endothelial Dysfunction

Maintaining vascular homeostasis is essential for preventing vascular disease and obtaining normal vascular function. It is known that diabetes can cause a devastating disruption of the endothelial environment; this disruption causes a loss of the endothelium's benefactors of anti-proliferative, anti-atherogenic and anti-thrombotic properties. As previously stated, eNOS and its product NO are needed to maintain a healthy endothelium. There is a paradoxical finding within diabetic patients; namely, eNOS expression is increased despite a reduction in endothelium-dependent vasodilation. There is growing evidence that the loss of NO is a result of diabetes-related oxidative stress. A variety of ROS generating enzymes cause oxidative stress and have been shown to be upregulated in diabetes. Moreover, ROS has been implicated in the uncoupling of eNOS by oxidation of the essential cofactor, BH_4. In diabetic patients, analysis of pterin levels reflects reduced BH_4 and elevated BH_2. It is our hypothesis that disruption of pterin balance contributes to the increased endothelial

$^{\cdot}O^{-}_2$ production and reduced NO bioavailability observed in diabetes as a result of uncoupling. The shift in NO and $^{\cdot}O^{-}_2$ renders the endothelium unable to maintain its homeostasic mechanisms manifesting in impaired vascular relaxation, endothelial barrier disruption and vascular remodeling.

In an attempt to "recouple" NOS activity, several groups have undertaken studies using pterin supplementation. In rat and mouse models of hypercholesterolemia and diabetes, oral BH_4 supplementation has been shown to significantly improve endothelial function[95-97]. The doses used in these studies followed human dosing regimens (10-20 mg/kg) used for the treatment of phenyl ketonuria (PKU). However, these rodent studies failed to correct for body surface area and thus yielded pharmacological levels 10 times less than the minimum therapeutic dose for treating PKU. In this regard, it is surprising that therapeutic benefit was achieved given that the doses used in the animal studies would not be expected to augment cellular BH_4. Moreover, BH_4 supplementation in a disease state associated with increased endothelial ROS production would be expected to worsen the outcome as a consequence of increased formation of the BH_4 oxidation product, BH_2. Indeed, clinical studies evaluating BH_4 supplementation have reported little if any benefit. Recent studies by Worthley et al. reported no benefit from intracoronary BH_4 infusion in patients with atherosclerotic coronary artery disease[98]. In contrast, work by Mayahi et al. revealed beneficial effects of both 6R-BH_4 and 6S-BH_4 on reactive hyperemic forearm blood flow, this despite the fact that the 6S-BH_4 isomer is not a ligand for NOS[99]. The inconsistencies in the literature regarding BH_4 supplementation therapy are not surprising given the complexity of the pterin metabolic pathways and highlight the need for a more complete

and mechanistic understanding of endothelial pterin regulatory pathways. With the exception of GTPCH-1, little is known regarding the effects of diabetes on pterin metabolism or the consequences on endothelial function[21]. In this regard, studies aimed at understanding the molecular mechanisms involved in the regulation of pterin metabolism and eNOS uncoupling in diabetes will provide novel insight into the role of eNOS cofactor involvement in the increased cardiovascular disease risks associated with diabetes and is the focus of this thesis.

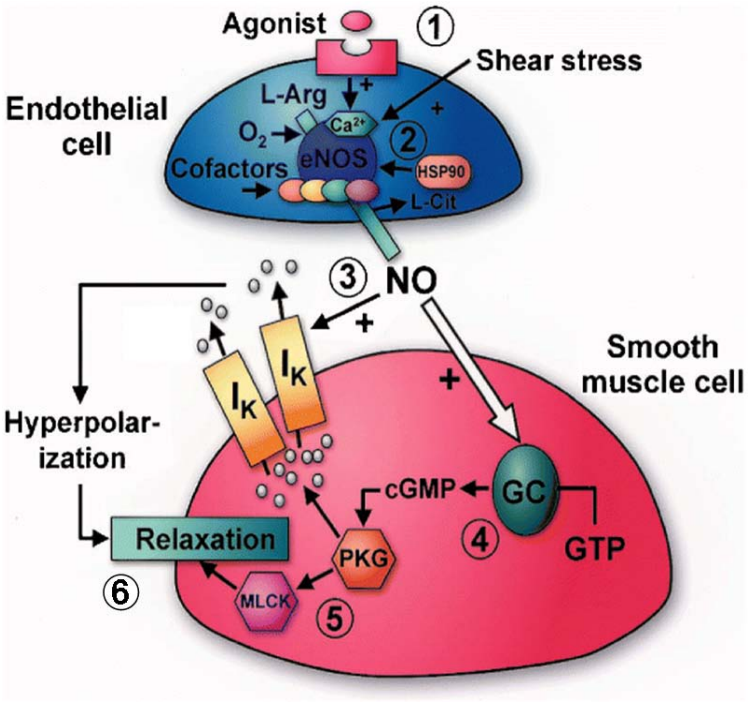

Figure 2-1. Mechanism of Nitric Oxide production and relaxation of smooth muscle cells. Khurana and Meyer Journal of Cerebral Blood Flow & Metabolism (2003) 23, 1251–1262

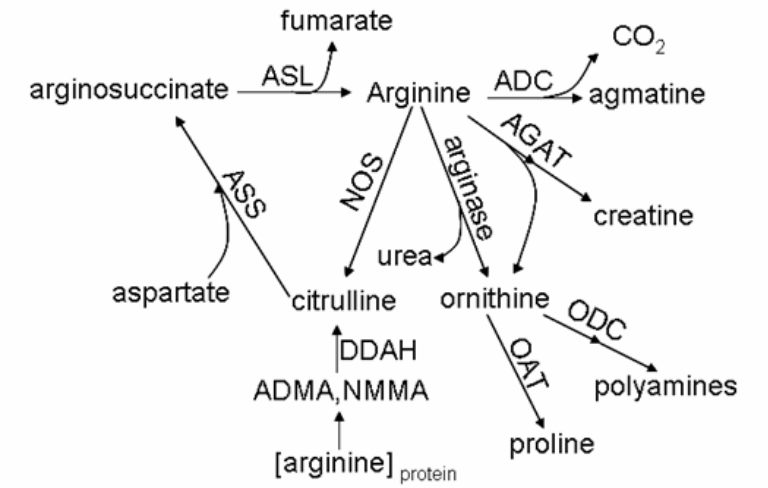

Figure 2-2. Cellular arginine metabolism occurs through several pathways within the cell, however, it is predominately metabolized by the enzyme arginase and this has been shown to play and important role in the regulation of endothelial NO production. ASL-arginosuccinate; ASS-arginosuccinate synthetase; ADC-arginine decarboxylase; AGAT-arginine glycine amidinotransferase; NOS-nitric oxide synthase; DDAH-dimethyl-arginine dimethylamino hydrolase; ODC-amithine decarboxylase; OAT-omithine aminotransferase.

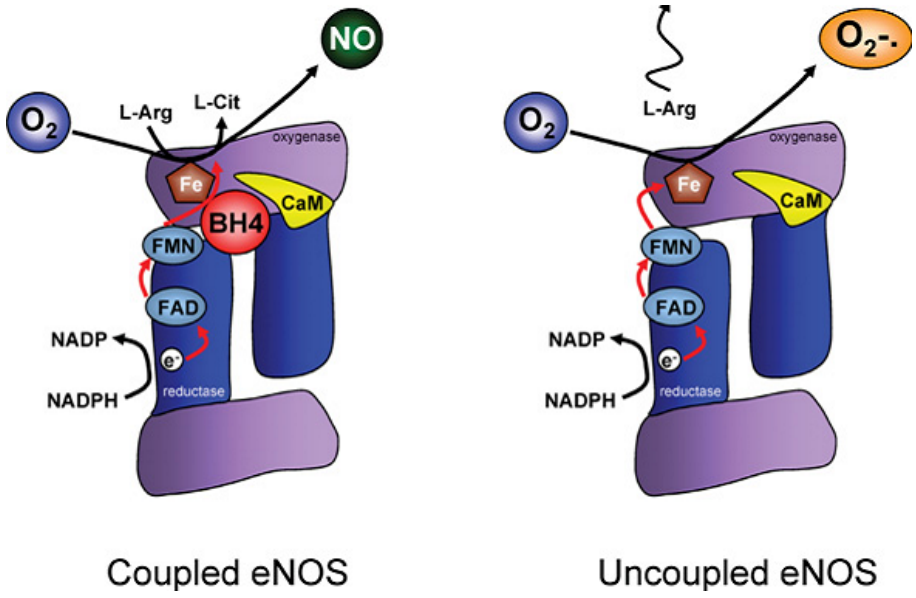

Figure 2-3. Mechanism of eNOS uncoupling and production of superoxide. Nicholas J. ALP et al. AJC 2007

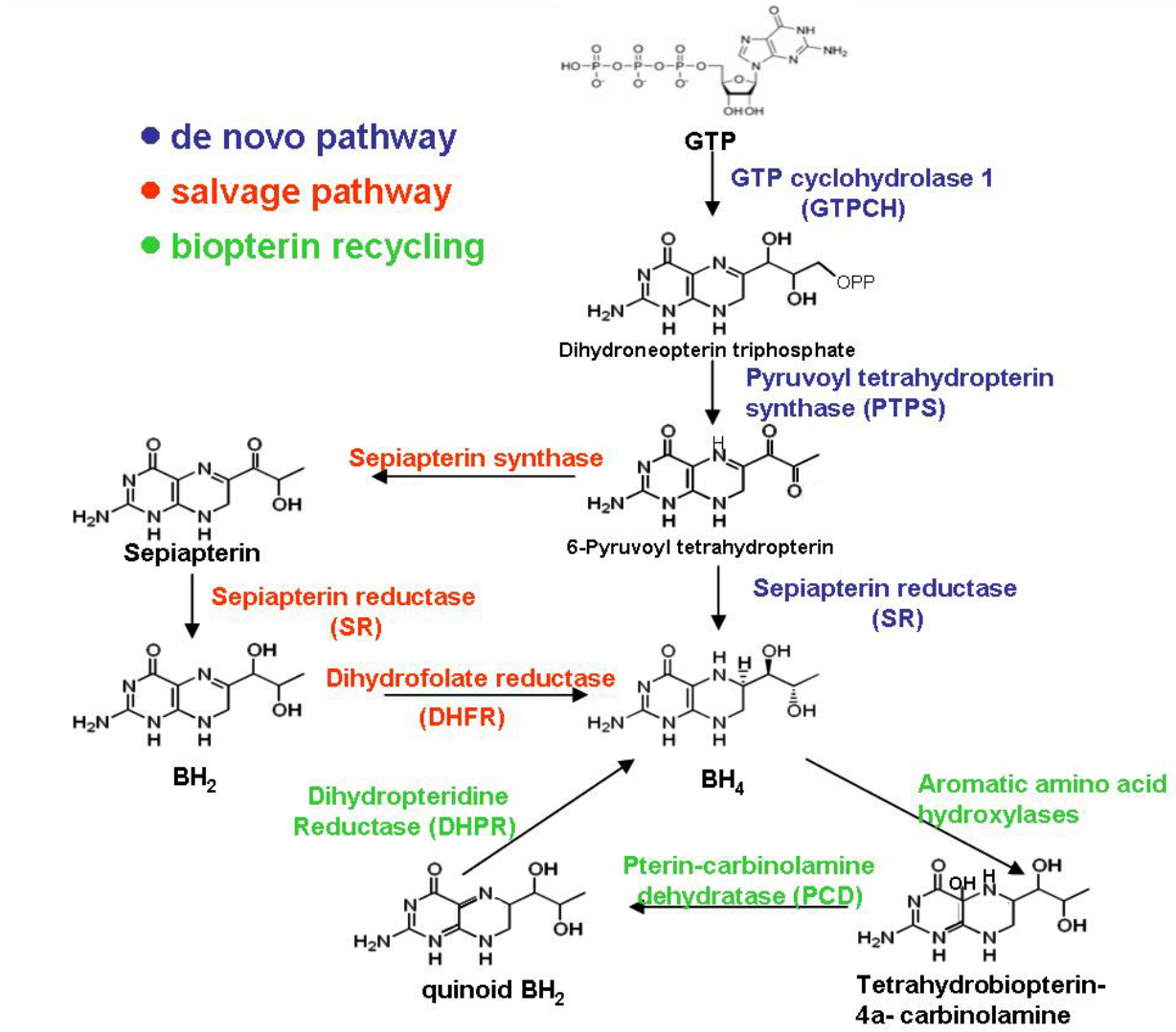

Figure 2-4. BH_4 biosynthesis pathway. The synthesis of BH_4 occurs via three pathways within the cell, the de novo pathway, the salvage pathway, and recycling pathway

CHAPTER 3
PTERIN METABOLISM AND DIABETIC VASCULOPATHY

Introduction

The risk of cardiovascular disease (CVD) is increased in diabetic patients, occurs earlier and is often more severe and diffuse. Impaired endothelial dysfunction and its sequence of events culminating in vascular smooth muscle cell proliferation, inflammation and a hypercoagulative state are the key factors contributing to the pathogenesis of diabetic vasculopathy. Although hyperglycemia, insulin resistance, hyperinsulinemia and dyslipidemia independently contribute to endothelial dysfunction via several distinct mechanisms, increased oxidative stress and reduced NO bioavailability seem to be the first insult triggering several others. Endothelial NO synthase (eNOS), present in the vascular endothelium, produces NO by oxidation of L-arginine to L-citrulline. NO has diverse anti-atherogenic and anti-proliferative properties which contribute to its role in the maintenance of vascular homeostasis. However, eNOS may be a source of superoxide production under certain conditions because of enzymatic "uncoupling" of L-arginine oxidation and oxygen reduction by the oxygenase and reductase domains of eNOS, respectively. Studies suggest that reduced availability of the cofactor tetrahydrobiopterin (BH_4) may result in eNOS uncoupling and that this may be an important contributor to the imbalance between production of NO and superoxide production in vascular disease. In support, hyperglycemia has been shown to increase NOS-dependent superoxide production in human endothelial cells[17], and recent data from animal studies suggest a possible role for BH_4 in mediating the eNOS dysfunction observed in diabetic vessels and endothelial cells[100].

Oxidant stress, such as that associated with diabetes, can potentially overwhelm the natural antioxidant defense mechanisms that serve to maintain BH_4 in its reduced form, resulting in endothelial dysfunction. Glutathione (GSH), Ascorbate and vitamin E are key cellular antioxidants that preserve BH_4, and diminished levels of these antioxidants are evident in diabetic patients[101]. Vitamin C treatment has been shown to increase eNOS activity in ECs specifically via chemical stabilization of BH_4[102]. Augmentation of endothelial BH_4 levels by adenovirus-mediated overexpression of the rate-limiting enzyme for BH_4 synthesis, GTP cyclohydrolase 1 (GTPCH), was also found to restore eNOS activity in hyperglycemic endothelial cultures and streptozotocin models of diabetes[21]. In aortas of mice with deoxycorticosterone acetate salt-induced (DOCA-salt) hypertension, production of NOS-derived ROS was markedly increased and BH_4 oxidation is evident[22]. Treatment of DOCA-salt mice with oral BH_4 attenuated vascular ROS production, increased NO levels and blunted hypertension compared with non-hypertensive control mice. However, translation of these findings into efficacious treatment strategies in human disease has been less successful. Indeed, clinical studies evaluating BH_4 supplementation have reported little if any benefit. Recent studies by Anderson et al. reported no benefit from intracoronary BH_4 infusion in patients with atherosclerotic coronary artery disease[98]. In contrast, work by Hingorani et al. has revealed beneficial effects of both 6R-BH_4 and 6S-BH_4 on reactive hyperemic forearm blood flow, this despite the fact that the 6S-BH_4 isomer is not a ligand for NOS[99]. The inconsistencies in literature regarding BH_4 supplementation therapy are not surprising given the complexity of the pterin metabolic pathways and highlight the need

for a more complete and mechanistic understanding of endothelial pterin regulatory pathways.

In this regard, recent studies from Gross et al. and Vaszquez-Vivar have independently identified the BH_4 oxidation product, BH_2, as the molecular trigger for eNOS uncoupling[17,76]. Experiments described in these studies demonstrate that BH_2 is required for eNOS uncoupling in the endothelium and that levels of this oxidized pterin are increased several fold by the oxidative stress that accompanies hyperglycemia. However, bioaccumulation of BH_2 following oxidation of BH_4 would not be expected to occur in the endothelium as dihydrofolate reductase activity should efficiently reduce this oxidized pterins back to BH_4.

Dihydrofolate reductase is an enzyme within the salvage pathway that catalyzes the NADPH dependent reaction, reduction of BH_2 to BH_4. As previously stated, DHFR may serve a critical role in maintaining the levels of endothelial BH_4 and reducing the level of BH_2. Thus, by normalizing BH_4/BH_2 ratios, DHFR may function to recouple eNOS and restore NO biovailability. Studies have shown that DHFR can be pharmacologically inhibited by methotrexate or genetically knocked down by RNA interference in cells and these interventions result in reduced endothelial BH_4 and increased BH_2 levels. In contrast, when GTPCH, the rate limiting step in the de novo pathway, was knocked down, total pterin levels were reduced, however, the BH_4/BH_2 ratio remained the same[103,104]. In summary, these findings suggest that the increased cardiovascular risk observed with diabetes may involve impaired pterin salvage pathways resulting in increased BH_4/BH_2 ratios, eNOS uncoupling and endothelial

dysfunction. The current study will examine pterin metabolism in diabetes and assess the vascular protective effects of DHFR gene therapy.

Materials and Methods

EPR: Materials

Diethyldithiocarbamate and Desferrioxamine were purchased from Sigma Aldrich. 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl pyrrolidine HCl (CMH hydrochloride) was from Axxora. A Benchtop ESR Spectrometer from Bruker Biospin was used for EPR measurements. C57BL/6J and db/db mice were purchased from Jackson Labs. All other chemicals were purchased from Sigma Aldrich.

Cell Culture- Bovine aortic endothelial cells (BAECs) were purchased from Cell-Systems and cultured in DMEM (Sigma, St Louis, MO) containing 10% FBS, 1% NEAA, 0.2% Endothelial Cell Growth Factor Supplement (ECGS) and 1% Antibotic-Antimyotic (Gibco, Carsbad, CA) and incubated at 37°C with air and 5% CO_2.

EPR: Methods

EPR spin trapping in cells- Spin-trapping measurements of NO were performed using a Bruker E-scan spectrometer with FE-MGD as the spin trap. For measurements of NO produced by BAECs, cells were cultured as described above and spin trapping experiments were performed on cells grown in 6 well plates. Attached cells were studied since scraping or enzymatic removal leads to injury and membrane damage with impaired NO generation. The media from approximately $1x10^6$ cells attached to the surface of the 6 well plates was removed and the cells were washed 3 times in KREBS and incubated at 37°C, 5% CO_2 in 0.2 ml of KRBES buffer containing the spin trap complex FE-MGD (0.5mM Fe^{2+}, 5.0mM) and the cells stimulated with calcium ionophore (1 µM). Subsequent measurements of NO production were performed

following a 30 min incubation period. Spectra recorded from cellular preparations were obtained using the following parameters: microwave power; 20mW, modulation amplitude 3.00 G and modulation frequency; 86 kHZ.

EPR spin trapping in vessels- Stock solution of CMH (10 mM) was dissolved in EPR buffer (PBS containing 20 µM Diethyldithiocarbamate and 50 µM Desferrioxamine) and bubbled with Nitrogen for 30 minutes on ice. Six aortic ring segments (2 mm) extracted from either wt or db/db mice were placed in a six well cell culture plate with EPR buffer. CMH at a concentration of 50 µM was then added and incubated at 37°C for 60 min with and without L-NAME (1 mM). Samples were then flash frozen with liquid nitrogen and loaded into a finger Dewar filled with liquid nitrogen. The Dewar was placed in the EPR with following settings: microwave frequency 9.7 GHz, microwave power 1.2 mW, modulation amplitude 6.7 G, conversion time 10.3 ms and time constant 40.96 ms.

Western Blot: Materials

Mouse tissues were harvested from C57BL/6J and db/db mice purchased from Jackson Labs. RIPA Lysis buffer kit for homogenization was purchased from Santa Cruz Biotechnology. The western blots were performed using 4-20% tris-glycine gels. The gels were labeled with a protein marker and magic mark western standard. The gels and markers were purchased from Invitrogen. Samples were run with SDS sample buffer from Biolabs. eNOS primary antibody was purchased from Cell Signaling and the corresponding secondary anti-rabbit antibody was purchased from Santa Cruz Biotechnology. DHFR primary antibody was purchased from Abcam and used an anti-mouse secondary (Santa Cruz). Western blots were developed on clear blue x-ray film

from Thermo Scientific using ECL western blot detection reagents from GE Healthcare. A Konica SRX developer was used.

Western Blot: Methods

Heart and kidney tissues were harvested from wt and age matched db/db mice. Tissues were homogenized using a motor and pestle under liquid nitrogen. 200 µL of RIPA buffer containing protease inhibitor cocktail was added to tissues and further homogenized in a glass homogenizer with a teflon grinder. Samples were then sonicated twice at two second intervals and immediately placed on ice. Next, samples were spun down at 3,000 rpm for 1 minute at 4°C and the supernatant was collected. Protein concentration was quantified using a Bradford assay. Samples (25-50 µg) were then loaded onto 4-20% SDS Tris-Glycine gradient gels and run at 150V for 1 hour and 45 minutes. Following electrophoresis, the protein was transferred onto a nitrocellulose membrane via a semi dry transfer blot system (Bio-rad). Following the protein transfer, the nitrocellulose membrane was blocked for 1 hour using 5% milk powder dissolved in Tris Buffered Saline and 0.05% Tween (TBST). Next, the membrane was washed 5x for 10 minutes with TBST and then the respective primary antibody was added and incubated overnight at 4°C. eNOS was detected by eNOS rabbit primary diluted 1:1000. DHFR was detected by DHFR mouse primary diluted 1:1000. Following the overnight incubation with primary antibody the membrane was washed with TBST 3x for 15 minutes and the respective secondary antibody was added (eNOS anti-rabbit, DHFR anti-mouse diluted 1:2000). After 1 hour incubation with secondary antibody at room temperature, detection was preformed using enhanced chemiluminescence kit and a Konica developing system.

Vessel Reactivity: Materials

Vascular reactivity studies were carried out using a four chamber wire myograph system from Danish myograph (Aaruhus, Denmark). All chemicals were purchased from Sigma-Aldrich. C57BL/6J and db/db mice were purchased from Jackson Labs.

Vessel Reactivity: Methods

*Mouse aorta***-** Constriction and relaxation of isolated mouse aortic rings were measured in an organ bath containing Krebs-Henseleit buffer (118 mM NaCl, 24 mM $NaHCO_3$, 4.6 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $CaCl_2$, 4.6 mM HEPES and 18 mM glucose). The bath was aerated with 95%O_2/5%CO_2 and kept at 37°C. The thoracic aorta was extracted from db/db and age matched wt control mice. The aorta was cut into 3 mm rings and mounted on a wire myograph. Force was measured via a force transducer interface with Chart software for data analysis. The segments were incubated for a 1 hour equilibration period during which the rings were stretched to generate a resting tension of 0.5 grams. The optimum resting force of the aortic rings was determined by comparing the force developed by 40 mM KCl under varying resting force. Aortic rings were preconstricted with 0.5 µM phenylephrine. The vascular relaxation response was determined using increasing concentrations of acetylcholine (10 nM to 1 µM). After the relaxation response, wells were washed out 3 times until vessels reached their original resting tension. For two consecutive trials, the constriction and relaxation response was performed. Additional groups consisted of vessels incubated with 10 µM BH_4, 300 units/ml superoxide dismutase, 150 units/ml of catalase and 300 µM arginine for 30 minutes followed by a constriction/relaxation dose response.

Human Internal Mammary Artery- Contraction and relaxation of human internal mammary artery rings were measured in an organ bath containing Krebs-Henseleit buffer. The bath was aerated with 95%O_2/5%CO_2 and kept at 37°C. The IMA was cut into 4 mM segments and mounted on a wire myograph. Force was measured via a force transducer interface with Chart software for data analysis. The segments were incubated for a 3 hours equilibration period, during which the rings were stretched to generate a resting tension of 2.0 grams. Aortic rings were preconstricted with 2 µM phenylephrine. Vascular relaxation response was determined using increasing concentrations of acetylcholine (500 nM to 10 µM). After the relaxation response, wells were washed out 3 times until vessels reached original resting tension. For two consecutive trials, the constriction and relaxation responses were performed. In separate trials, the vessels were incubated with 10 µM BH_4, 300 units/ml superoxide dismutase or 300µM arginine for 30 minutes followed by a constriction/relaxation dose response.

Adenovirus Transduction- Aorta's were dissected and mounted on a culture perfusion system (Danish Myo). The vessels were perfused with DMEM containing 10% FBS for 1 hour followed by static luminal transduction with adDHFR (1×10^{11} vp/mL of DMEM containing 0.3% FBS) for 1 hour. The perfusion buffer was returned to full media and perfusion continued (10 µL/min) for 24 hours. As previously stated, vessels underwent KCl, phenylphrine and acetylcholine dose response for two consecutive trials. On separate trials, the vessels were incubated with 10 µM BH_4, for 30 minutes followed by a constriction/relaxation dose response.

Adenoviral Vector Construction- Full-length human DHFR cloned into pcDNA 3.1 was purchased from Invitrogen. The vector was then subcloned into a pAd destination vector using Gateway® cloning technology. The pAd vector was transfected into HEK293 cells for viral amplification. The crude lysate was collected and sent to the Gene-therapy core at Nationwide Children's Hospital for purification.

PCR: Materials

RNAeasy Mini Kit was used from Qiagen (Valencia,CA). One Step RT-PCR kit was purchased from Invitrogen (Carsbad,CA). Bovine Primers for DHFR were purchased from Invitrogen. C57BL/6J and db/db mice were purchased from Jackson Labs.

PCR: Methods

The thoracic aorta was extracted from wt and db/db mice and cut into 3 mM segments. Post extraction, vessels receiving adenovirus were placed in a Petri dish and incubated in DMEM containing 10% FBS for 1 hour followed by transduction with adDHFR (1×10^{11} vp/mL of DMEM containing 0.3% FBS) for a 24 hour period at 37°C. The 3 mm vessel segments were then homogenized in lysis buffer from the RNAeasy Mini Kit. Following lysis, RNA was extracted using a RNAeasy Mini Kit. RNA concentration of the tissue sample was determined by absorbance of $A_{260/280}$. cDNA was then isolated using the One Step RT-PCR kit. Semiquantive PCR was preformed in order to detect changes in mRNA expression following DHFR transduction. Primers were, DHFR Forward (ACCTGGTTCTCCATTCCTGA) and DHFR Reverse (GTTTAAGATGGCCTGGGTGA). PCR product was run on a 2% agarose gel with a 1Kb marker for 1 hour at 90 volts.

High Pressure Liquid Chromatography: Materials

An ESA CoulArray high pressure liquid chromatography system was used for measurement of tissue pterin levels. Centricon centrifugal filter were purchased from Millipore. C57BL/6J and db/db mice were purchased from Jackson labs. 7,8-dihydro-L-biopterin (BH_2), NADPH, methotrexate (MTX) and KH_2PO_4 were purchased from Sigma Aldrich. EDTA and methanol were purchased from Fisher, while octyl sodium sulfate was from Fluka.

High Pressure Liquid Chromatography: Methods

For measurements of tissue DHFR activity, kidneys were extracted from wt and db/db mice. Samples were homogenized in deionized water with ascorbic acid (1 mg/ml) in a glass homogenizer and teflon grinder. After homogenization, protein concentration was measured via Bradford assay. 250 µg of protein was incubated with 100 µM BH_2, 100 µM NADPH with or without 10 µM Methotrexate (DHFR inhibitor) for 30 minutes in a 37°C water bath. Following the incubation, samples were loaded into a 3,000 molecular weight cut off Centricon filter and centrifuged at 10,000 rpm, 4°C for 60 minutes. DHFR activity was measured by the formatin of BH_4 using HPLC with electrochemical detection at 400 mV. The mobile phase consisted of Buffer B (100 mM KH_2PO_4, 25 mM octyl sodium sulfate and 0.6 mM EDTA) and Buffer A (100 mM KH_2PO_4, 25 mM octyl sodium sulfate, 0.6 mM EDTA and 2% MeOH) at pH of 2.5 and run at room temperature with a flow rate of 1.3 ml/min.

For measurements of endogenous levels of BH_4 and BH_2, kidney tissue was used. As previously stated, samples were homogenized in deionized water with ascorbic acid (1 mg/ml) in a glass homogenizer and teflon grinder. After

homogenization, protein concentration was measured via Bradford assay. 500 µg of protein was loaded into a 3,000 molecular weight cut off Centricon filter and centrifuged at 10,000 rpm, 4°C for 60 minutes. BH_4 and BH_2 were detected using HPLC with electrochemical detection at 400 mV and 800 mV.

Results

Although purified enzyme systems clearly demonstrate that loss of BH_4 results in eNOS uncoupling, the occurrence of this phenomenon in the cellular environment is less evident. Several recent studies have indicated that increased formation of the BH_4 oxidation product BH_2, is the molecular trigger of eNOS uncoupling and that BH_4 depletion alone is an insufficient insult to cause eNOS uncoupling. Therefore, to further examine the molecular mechanisms involved in endothelial eNOS "uncoupling", EPR studies were performed to measure both eNOS derived NO and $^{\cdot}O^{-}_{2}$ production from BAECs following either depletion or oxidation of BH_4. Intracellular BH_4 levels were depleted by pharmacological inhibition of GTP cyclohydrolase, the rate limiting enzyme in BH_4 synthesis, using DAHP (5 mM). Previous studies from our group and Gross et al. have demonstrated that 48 hour exposure to DAHP depletes cellular BH_4 to undetectable levels[17]. Hyperglycemia (30 mM, 48 hours) was used to induce BH_4 oxidation as previously demonstrated[17]. EPR measurements of endothelial cell NO production were then carried out in order to establish the effects of BH_4 depletion/oxidation on endothelial NO production. Results demonstrated that following stimulation with the calcium ionophore A23187, control (untreated) cells gave rise to a strong NO signal (fig. 3-1). In the presence of the GTPCH-1 inhibitor (DAHP, 5 mM), the NO signal was inhibited by almost 80%. Following 48 hour exposure to

hyperglycemic conditions (30 mM glucose), endothelial NO production was inhibited by 24 % (fig. 3-1).

These results indicate that BH_4 is essential for eNOS activity and that hyperglycemia impairs NOS activity. However, in order to determine whether NOS uncoupling is occurring, detection of eNOS derived $^{\cdot}O^{-}_{2}$ is required. Therefore, to assess if depletion/oxidation of BH_4 can in fact trigger eNOS oxidase activity, EPR spectroscopy was used to measure L-NAME inhibitable $^{\cdot}O^{-}_{2}$ production from BAEC's. L-NAME was used as it inhibits NOS derived superoxide generation. Results demonstrated that DAHP treatment alone resulted in increased eNOS derived (L-NAME inhibitable) $^{\cdot}O^{-}_{2}$ with a measured concentration of 0.07 nmols/10^6 cells. Hyperglycemia increased the amount of eNOS derived $^{\cdot}O^{-}_{2}$ production to 0.23 nmols/10^6 cells (fig. 3-2). These results demonstrate that although BH_4 depletion (DAHP group) inhibits NO generation almost completely, it does not result in significant eNOS derived $^{\cdot}O^{-}_{2}$ as oxidase activity is only a fraction of the total eNOS catalytic activity observed. In contrast, under hyperglycemic conditions, 25% of the total NOS catalytic activity was involved in $^{\cdot}O^{-}_{2}$ generation with the remaining 75% representing eNOS derived NO. These results demonstrate that BH_4 oxidation and not depletion is the critical molecular trigger for eNOS uncoupling. These results clearly demonstrate that loss of BH_4 results in inhibition of eNOS derived NO without a concomitant increase in superoxide. In contrast, under conditions of hyperglycemia, wherein BH_4 oxidation is increased, 50% of the total endothelial ROS production is eNOS derived (Figure.3-3). These results are consistent with a recent study from Channon et al. demonstrating eNOS uncoupling in

internal mammary arteries from diabetic patients[95]. In this study, eNOS derived superoxide accounted for 50% of the total ROS production in the vessel wall. Together, these results demonstrate a critical role of BH_4 and its oxidation products in the regulation of eNOS derived NO and $^{\cdot}O^{-}_{2}$.

eNOS Dysfunction in the Diabetic Mouse.

Subsequent *in-vivo* studies were carried out to validate our cellular studies demonstrating eNOS uncoupling under hyperglycemic conditions. The db/db diabetic mouse was used as a model to study diabetic vascular function and eNOS uncoupling *in vivo*. Diabetic db/db mice and age-matched wild type controls at 16 weeks of age were sacrificed and the aortas were removed for vascular reactivity and EPR spin trapping studies. Results demonstrated significant endothelial dysfunction in the db/db mice with a 47% reduction in endothelial dependent relaxation to Ach (10 nM-1 µM) following phenylephrine (0.5 µM) constriction (fig. 3-4). Additional studies were performed to determine whether the impaired vascular relaxation was associated with eNOS uncoupling. Aortic rings from wt and db/db mice were incubated with the ROS spin trap CM-H (20 µM) to measure NOS dependent ROS production. The L-NAME inhibitable component of the signal was interpreted as NOS-derived $^{\cdot}O_2$. Results demonstrate increased ROS production in the db/db mouse which was largely inhibited by L-NAME. These results provide clear evidence for NOS uncoupling in the diabetic endothelium (fig. 3-5). HPLC studies were then carried-out to assess the effects of diabetes on BH_4 and BH_2 levels from aortic homogenates. Results demonstrate a decrease in BH_4 from 22 pmols/mg protein to 7 pmols/mg protein in the diabetic group. The loss of BH_4 was accompanied by an increase in BH_2 in the db/db animals (fig 3-6).

BH_4 Supplementation in the Diabetic Mouse.

Preliminary data clearly demonstrate impaired endothelial function in the db/db mouse which is associated with eNOS uncoupling. Subsequent studies were performed to assess whether supplementation with the NOS cofactor, BH_4, could ameliorate the loss of endothelial function occurring in diabetes. Aortic rings from wt and db/db mice were placed on a wire myograph and the vascular relaxation response to Ach (10 nM – 1 µM) was evaluated in the presence and absence of BH_4 (10 µM) supplementation to the buffer (fig. 3-7). Initial relaxation responses were measured followed by 30 minute incubation in the presence of BH_4. Results demonstrated that BH_4 supplementation had no effect on endothelial dependent relaxation in the wt mice, however, in the db/db mice, the addition of BH_4 resulted in a 55% decrease in the relaxation response (fig 3-7). We hypothesized that the attenuation in relaxation observed in the db/db mice was a result of increased BH_4 oxidation resulting from increased oxidative stress in the diabetic vessels. To test this hypothesis, the BH_4 supplementation experiments were repeated in the presence of PEG-SOD (300 U/mL). The combination of BH_4 and PEG-SOD restored endothelial function in the diabetics mice to values above those observed in the wt mice (fig. 3-7). These results suggest that BH_4 oxidation is increased in the diabetic endothelium and plays a significant role in the loss of endothelial function observed in diabetes.

Bioaccumulation of oxidized pterins BH_2 or quinoid BH_2 would not be expected to occur in the endothelium as the combination of dihydrofolate reductase and dihydropteridine reductase should efficiently reduce these oxidized pterins back to BH_4. Therefore, subsequent studies were carried out to identify the cellular mechanisms responsible for the altered pterin levels. Activity assays were performed on tissue

homogenates from wt and db/db mice to identify disease associated changes in BH_4 metabolic pathways. Results clearly demonstrated reduced DHFR expression and activity in db/db mice (figs 3-8, 3-9). Specifically, western blot analysis of DHFR expression revealed a 2.5 fold decrease in DHFR protein expression in heart tissue from diabetic mice (fig. 3-8). This decrease in expression was associated with an 85 % decrease in DHFR activity (fig. 3-9). These results are consistent with our observation of increased BH_2 levels in the diabetic animals and provide a potential mechanism for BH_2 accumulation and NOS uncoupling in the diabetic endothelium.

Effects of DHFR Over-Expression on Endothelial Function in wt and db/db Mice

Upon identification of impaired DHFR activity in the diabetic mice, we carried out a series of studies aimed at assessing the effects of adenoviral-mediated over-expression of DHFR in aorta's from both wt and db/db mice. Aortas were dissected and mounted on a culture perfusion system (Danish Myo). The vessels were perfused with DMEM containing 10% FBS for 1 hour followed by static luminal transduction with adDHFR (1×10^{11} vp/mL of DMEM containing 0.3% FBS) for 1 hour. The perfusion buffer was returned to full media and perfusion continued (10 µL/min) for 24 hours. At the end of the 24 hour incubation, the vessels were removed and mounted on a wire myograph for assessment of endothelial dependent relaxation. Results demonstrated significantly impaired vascular relaxation responses to Ach in the diabetic group as compared to control (28% relaxation vs. 65% relaxation) (fig. 3-10). DHFR over-expression increased relaxation responses to Ach by 57% in the diabetic group, but had no effect on wt rings (fig. 3-10). In order to verify transduction of the adDHFR, PCR analysis was carried out to evaluate tissue expression of DHFR message. Results demonstrated the presence of increased DHFR mRNA in transduced tissues (fig. 3-11). Overall, these

results demonstrate that the impaired vascular function observed in db/db mice is at least in part manifested through increased BH_2 levels. The fact that DHFR over-expression was not able to fully restore vascular function suggests that other pterin metabolites, which are not amenable to reduction by DHFR, may also be involved in the observed endothelial dysfunction.

Effects of BH_4 Supplementation on Endothelial Dependent Relaxation of Internal Mammary Artery Segments from Non-Diabetic and Diabetic Humans.

Subsequent studies were performed to assess pterin metabolism and endothelial dysfunction in human diabetes. The IRB protocol inclusion criteria included all patients undergoing coronary artery bypass grafting between the ages of 18 and 80. Exclusion criteria included HIV or Hepatitis positive individuals as well as pregnant females. Because of the nature of the study, we are enrolling equal numbers of diabetic and non-diabetic patients regardless of age, sex, race or ethnicity. Clinically diagnosed diabetic patients and pre-diabetic patients identified by fasting glucose levels of >150 mg/dL and mild protein urea were placed in the diabetic cohort. Current recruitment efforts have yielded 8 patients with equal representation by diabetes status. Internal mammary artery (IMA) tissues were collected during coronary artery bypass surgery and vascular reactivity assessed immediately after collection using a wire myograph system. Briefly, the IMA tissue was dissected free of surrounding tissue and cut into 4 mm rings. The rings were allowed to equilibrate for a period of three hours while resting tension was increased to 2 grams. Upon stabilization of the tension, KCL responses were obtained followed by assessment of the endothelial-dependent relaxation response to Ach in vessels pre-constricted with PE (2 µM). Results demonstrated reduced endothelial dependent relaxation in the males as compared to the females with males exhibiting a

maximal relaxation response of 29% and females 54% (fig. 3-12). When the participants were stratified into diabetic and non-diabetic groups, results demonstrated a non-statistically significant trend toward worse maximal dilatory response in the diabetics (48% relaxation in non-diabetics vs. 40% relaxation in diabetics) (fig. 3-13). Regression analysis was then used to assess whether any correlation existed between endothelial function and age at surgery. As expected, those who presented with advanced coronary artery disease at an early age had worse endothelial-dependent reactivity as compared to older cohorts (fig. 3-14). To assess pterin involvement in the impaired vascular reactivity observed in these patients, we carried out a series of experiments using pharmacological BH_4 supplementation in the presence and absence of SOD. BH_4 supplementation resulted in a 15% reduction in endothelial dependent relaxation suggesting that BH_4 oxidation was occurring and the endothelium was unable to reduce the oxidized pterins (fig. 3-15). Combination therapy with BH_4 and SOD increased endothelial-dependent relaxation by 43% while SOD alone increased the response by only 21% (fig 3-15). Although statistical significance was not achieved in these studies, they provide some evidence that the redox environment of the diseased endothelium creates an oxidizing milieu which facilitates BH_4 oxidation leading to NOS impairment.

Discussion

While the mechanistic basis for the attenuated NO production observed in diabetic endothelial dysfunction is uncertain, both slowed NO synthesis and accelerated NO scavenging by ROS have been implicated as causative factors. Thus, increased production of reactive oxygen species and loss of endothelial NO bioactivity are key features of the vascular dysfunction associated with diabetes[105]. Among the current

hypothesis in the field is that diabetes is a chronic inflammatory state associated with increased oxidative stress. This oxidative stress is believed to result in increased oxidation and decreased bioavailability of the essential eNOS cofactor BH_4. This redox sensitive cofactor is required for NO synthesis and whereas fully reduced tetrahydrobiopterins support NOS catalysis, oxidized pterins are believed to be catalytically incompetent[76]. Biochemical studies using recombinant NOS have demonstrated that eNOS, in the absence of BH_4, has the potential to be a major source of superoxide with catalytic rates approaching those of NADPH Oxidase and Xanthine Oxidase[8].

BH_4 oxidation has been described in vascular cells under conditions of oxidative stress associated with hypertension, ischemia reperfusion injury and diabetes[21,22,106]. However, recent studies evaluating eNOS uncoupling suggest that BH_4 depletion alone does not significantly increase superoxide fluxes[76,17]. Instead, it appears that increased levels of the BH_4 oxidation product, BH_2, are required for eNOS uncoupling. Therefore, in the current study we examined the effects of both eNOS depletion and oxidation on eNOS oxidase activity. Results demonstrated that although BH_4 depletion inhibits NO generation almost completely, it does not induce significant eNOS oxidase activity. In contrast, hyperglycemia was associated with both reduced eNOS-derived NO and significantly increased eNOS oxidase activity. These results demonstrate that BH_4 oxidation and not depletion is the critical molecular trigger for eNOS uncoupling and are consistent with a recent publication by Gross et al. which reported that exposure of endothelial cells to diabetic glucose levels (30 mM) resulted in an increase in BH_2 levels

from undetectable to 40% of total biopterin[17]. This BH_2 accumulation was associated with diminished NO activity and accelerated superoxide production.

Subsequent studies were carried out to examine whether eNOS uncoupling is involved in the endothelial dysfunction associated with diabetes. Male db/db mice at 16 weeks of age were used as a model of diabetic vasculopathy as these animals exhibit significantly impaired endothelial-dependent relaxation. Results demonstrated significantly impaired relaxation responses to acetylcholine in the db/db mice which was associated with increased eNOS-derived superoxide production and a reduced BH_4/BH_2 ratio. These results were consistent with eNOS uncoupling and thus raised the question whether pharmacological supplementation of BH_4 could "recouple" eNOS activity. Therefore, we used pharmacological supplementation with authentic BH_4 and assessed the effects on endothelial-dependent relaxation. BH_4 supplementation had no effect on wt aortic rings, but elicited a marked reduction in the relaxation response of the diabetic vessels.

These results indicate that the pathology in diabetes appears quite different from that of other cardiovascular diseases in that BH_4 supplementation is not able to rescue the loss in eNOS function. In fact, our preliminary data clearly demonstrate that BH_4 supplementation worsens endothelial function in diabetes. In contrast, we have previously published that BH_4 infusion into the ischemia reperfused heart restores the coronary relaxation response to histamine[106]. We thus hypothesized that diabetes must be associated with both increased BH_4 oxidation and an inability to reduce oxidized pterins back to BH_4. However, it is unclear why BH_2 would accumulate in cellular and animal models of diabetes. Bioaccumulation of BH_2 or quinoid BH_2 following oxidation

of BH_4 would not be expected to occur in the endothelium as the combination of dihydrofolate reductase and dihydropteridine reductase should efficiently reduce these oxidized pterins back to BH_4. Given that numerous studies have clearly identified increased BH_2 formation in diabetes suggests that this condition is likely associated with impaired pterin salvage or recycling pathways. Therefore, we assessed the effects of diabetes on the enzymes involved in the BH_4 salvage/recycling pathways.

Using a combination of molecular and physiological approaches we evaluated the effects of diabetes on DHFR expression and activity. Results demonstrated that db/db mice have both reduced expression and activity of this enzyme which is consistent with the increased level of oxidized pterins observed. In order to assess whether reduced activity of this enzyme is directly involved in the pathogenesis of diabetic endothelial dysfunction, we used a gene therapy approach to over-express DHFR in the vascular endothelium of wt and db/db mice. Results demonstrated that over-expression of DHFR had no effect on relaxation responses in the wt group which would be expected since these animals do not accumulate oxidized pterins. In contrast, DHFR over-expression in the db/db mice increased endothelial-dependent relaxation by >50% thus demonstrating that accumulation of oxidized pterins is directly involved in eNOS dysfunction associated with diabetes. Overall, these data demonstrate that in murine models of diabetes, BH_4 oxidation is increased and the activity of the BH_4 salvage enzyme, Dihydrofolate Reductase, is inhibited.

Although these results demonstrate a clear role for eNOS uncoupling in the pathogenesis of diabetic endothelial dysfunction, we acknowledge that other pathogenic mechanisms are likely involved in diabetic vasculopathy. Nevertheless, we believe that

that NOS dysregulation may be central component regulating many others. For example, advanced glycation end product (AGE) formation is accelerated in diabetes due to the increase in available glucose and the pro-oxidative environment. AGEs have the propensity to form crosslink between proteins through cysteine residues which often result in loss of protein function and represents a process which has been implicated in diabetes related cellular pathology[107]. AGE can also interacts with several cell-surface AGE binding receptors including, receptor for advanced glycation endproducts (RAGE), which can lead to endocytosis or cellular activation resulting in pro-oxidant and pro-inflammatory events. The binding to RAGE activates NF-KappaB, which controls several genes involved in pro-inflammatory responses[108]. Over time, AGE can post-translationally modify proteins, contribute to atherosclerosis and cause inflammation leading to micro and macro-angiopathy[107]. These are pathological events which are also closely linked to impaired NO bioavailability and suggest that some inter-dependence may exist between AGE and NOS. Specifically, we hypothesize that cysteine may represent this link as it is a site for both NO signaling through the formation of S-nitrosyl complexes as well as AGE modification. Thus, we predict that loss of NO bioavailabilty secondary to diabetes may result in unmasking of cysteine residues and increased susceptibility to formation of AGE complexes on these proteins. Furthermore, oxidation of BH_4 and eNOS uncoupling may amplify the production of AGE by creating a more oxidizing environment. In this regard, we believe that NOS impairement is likely an early event in diabetes and thus contributes to many of the other related pathological processes. Future research efforts should examine these potential inter-relationships during disease development.

We are currently extending these observations to the human disease and have initiated a clinical study to assess the effects of diabetes on pterin metabolism and its role in diabetic vasculopathy. Although our current cohort is small, we have observed a trend toward worsened endothelial function among diabetics. Our current efforts will focus on delineating the pterin metabolic pathways in the endothelium and their role in the pathogensis of diabetic vasculopathy.

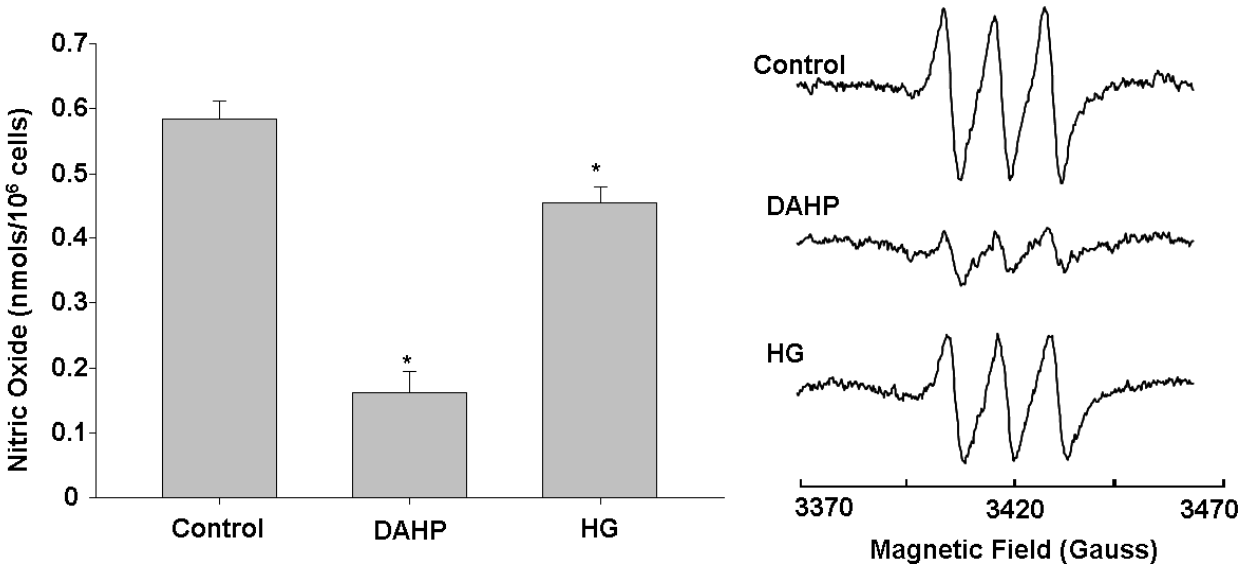

Figure 3-1. Effects of DAHP and HG on NO of BAECs. BAECs were treated with DAHP (10 mM) or under hyperglycemic (HG) conditions (30 mM) for 48 hrs to induce BH_4 depletion or oxidation, respectively. Left panel represents cellular NO production. Representative spectra are presented in the right panel. *represents statistical significance, $p<0.05$

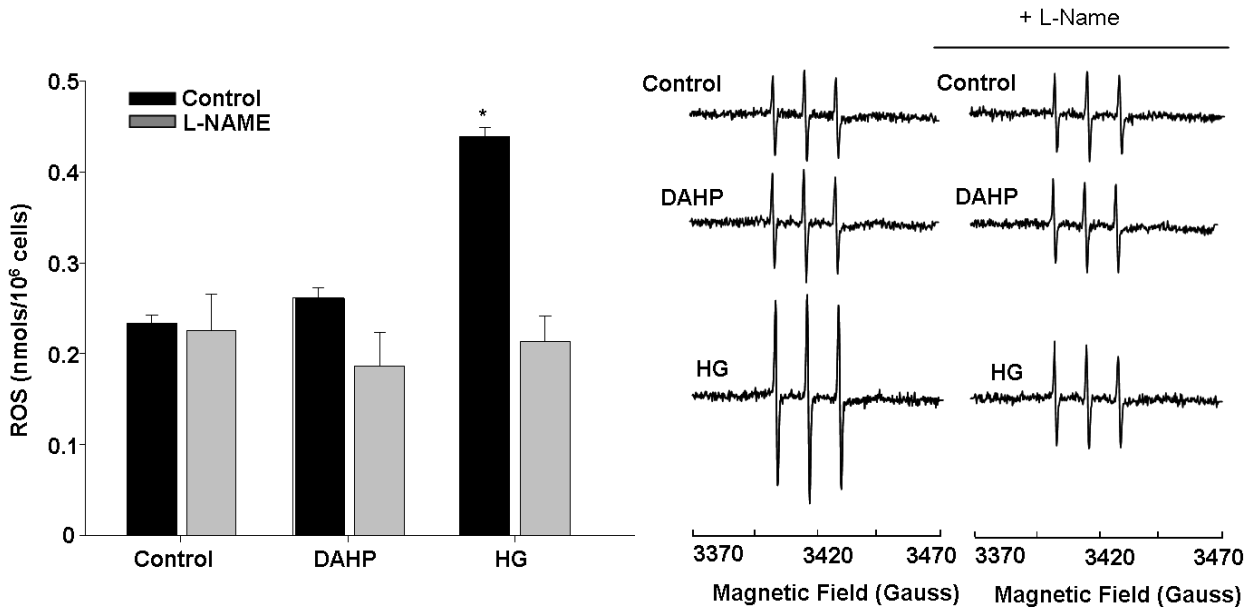

Figure 3-2. Effects of DAHP and HG on superoxide of BAECs. BAECs were treated with DAHP or under hyperglycemic (HG) conditions for 48 hrs to induce BH_4 depletion or oxidation, respectively. EPR spectroscopy was performed using the spin trap CMH (50 µM) to detect eNOS derived superoxide. Left panel represents cellular ROS production. Representative spectra are presented in the right panel. *represents statistical significance, $p<0.05$

Group	NO (nmols)	$^{\cdot}O_2^-$ (nmols)	Total-Activity (nmols)
Control	0.58	0	**0.58**
DAHP	0.16	0.07	0.23
HG	0.45	0.23	0.68

1.) Under control conditions total eNOS catalytic activity is 0.58 nmols/10^6 cells.
2.) DAHP (BH_4 depletion) inhibits NO by 73%, and total eNOS catalytic activity by 60%.
3.) NOS derived superoxide represents 44% of the total eNOS activity remaining following DAHP treatment. This represents 12% of the eNOS catalytic activity present in the control group.
4.) Hyperglycemia (BH_4 oxidation) inhibits NO by 24% while total eNOS activity is increased 17%
5.) Uncoupled NOS represents 34% of the total eNOS activity present in hyperglycemic cells. This represents 40% of the total eNOS catalytic activity present in the control group.

Figure 3-3. eNOS catalytic activity. BAECs were treated with DAHP (10 mM) or under hyperglycemic (HG) conditions (30 mM) for 48 hrs to induce BH_4 depletion or oxidation.

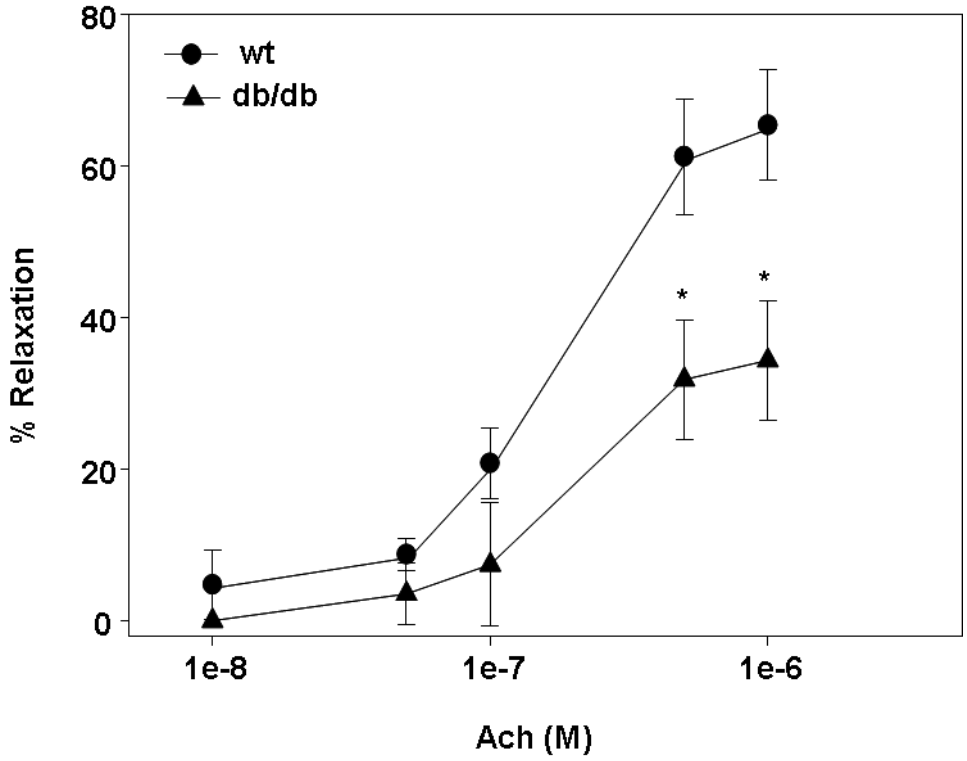

Figure 3-4. Endothelial dependent relaxation was measured in aorta from wt and db/db mice. Rings were constricted with PE (0.5 µM) and the relaxation response to Ach (10 nM-1 µM) was measured on a wire myograph. Data are presented as mean ± SEM of n=4. *represents statistical significance, $p<0.05$

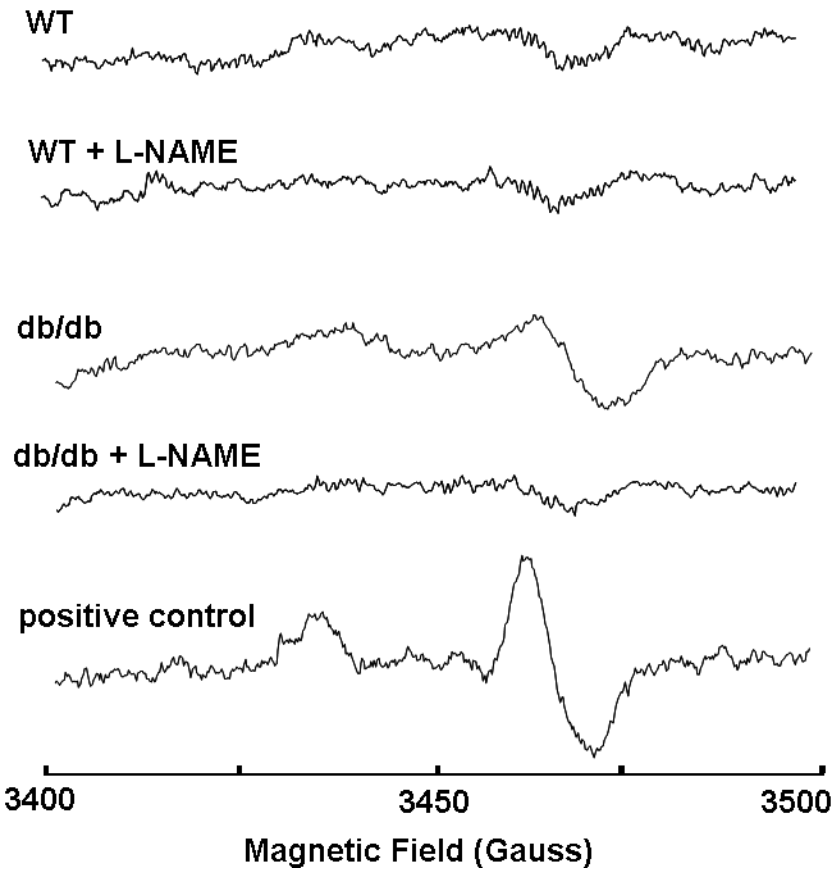

Figure 3-5. eNOS derived ROS production was measured using EPR spin trapping techniques. Studies were performed on wild type (wt) and diabetic (db/db) mice in the presence and absence of L-NAME (1 mM) The $\cdot O^-_2$ generator riboflavin/light was used as a positive control. The ROS signal can be observed between 3450-3470 gauss.

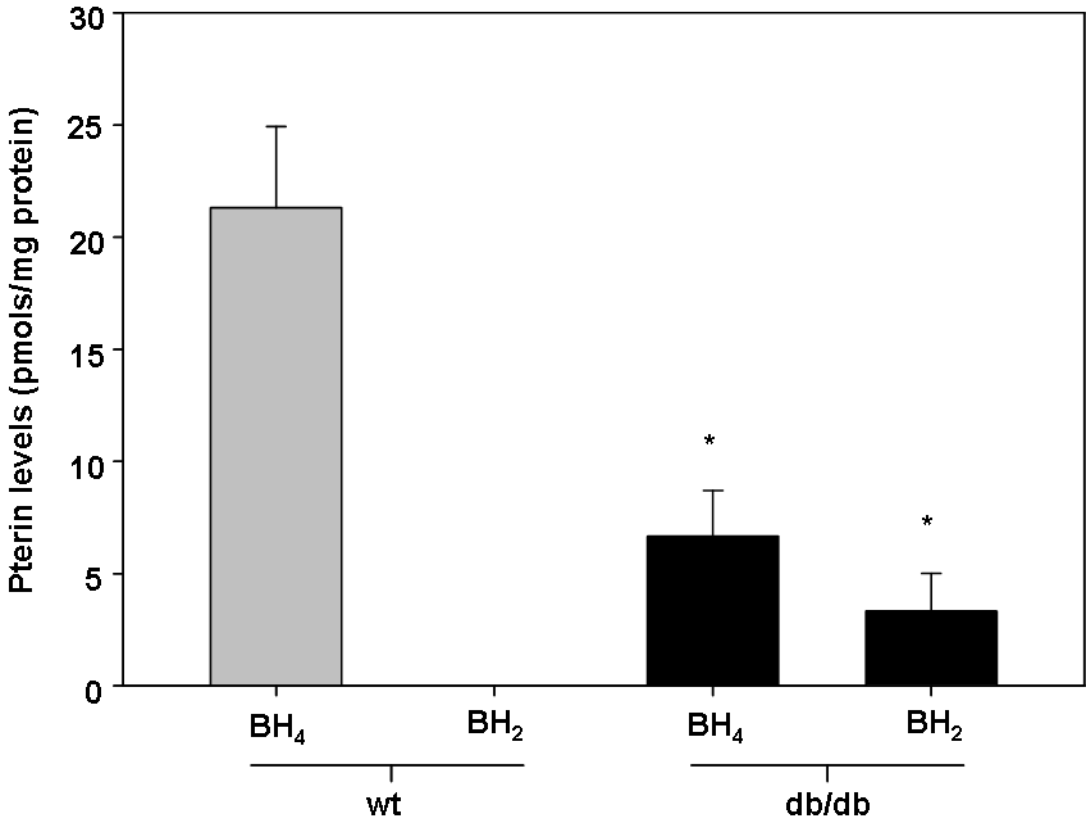

Figure 3-6. Effects of diabetes on vascular BH_4 and BH_2 levels. BH_4 was detected using HPLC with electrochemical detection at 400 mV. BH_2 was detected using flourescence detection at ex. 348, em. 444. B.) *represents statistical significance, $p<0.05$

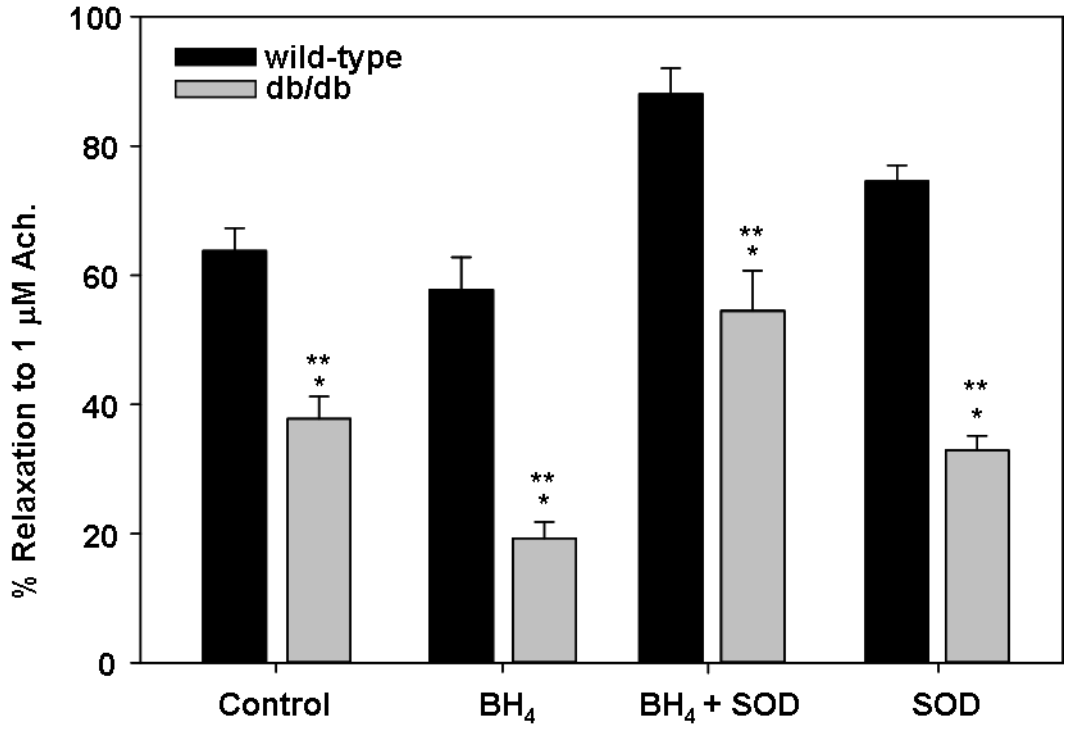

Figure 3-7. Effects of BH_4, and SOD on Aortic rings. Aortic rings from age matched control (wild-type) and diabetic (db/db) mice were isolated and endothelial dependent relaxation assessed with Ach. Rings were supplemented with BH_4 (10 μM) and SOD (150 U/mL). Using the Holm-Sidak method for comparison: * represents $p<0.05$ as compared to respective control, ** represents $p<0.05$ as compared to wt control.

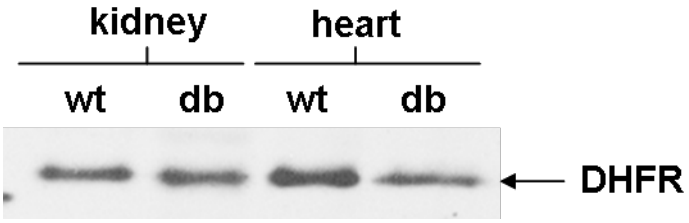

Figure 3-8. Effects of diabetes on DHFR expression. IP western blot (200 μg) of kidney and heart DHFR expression

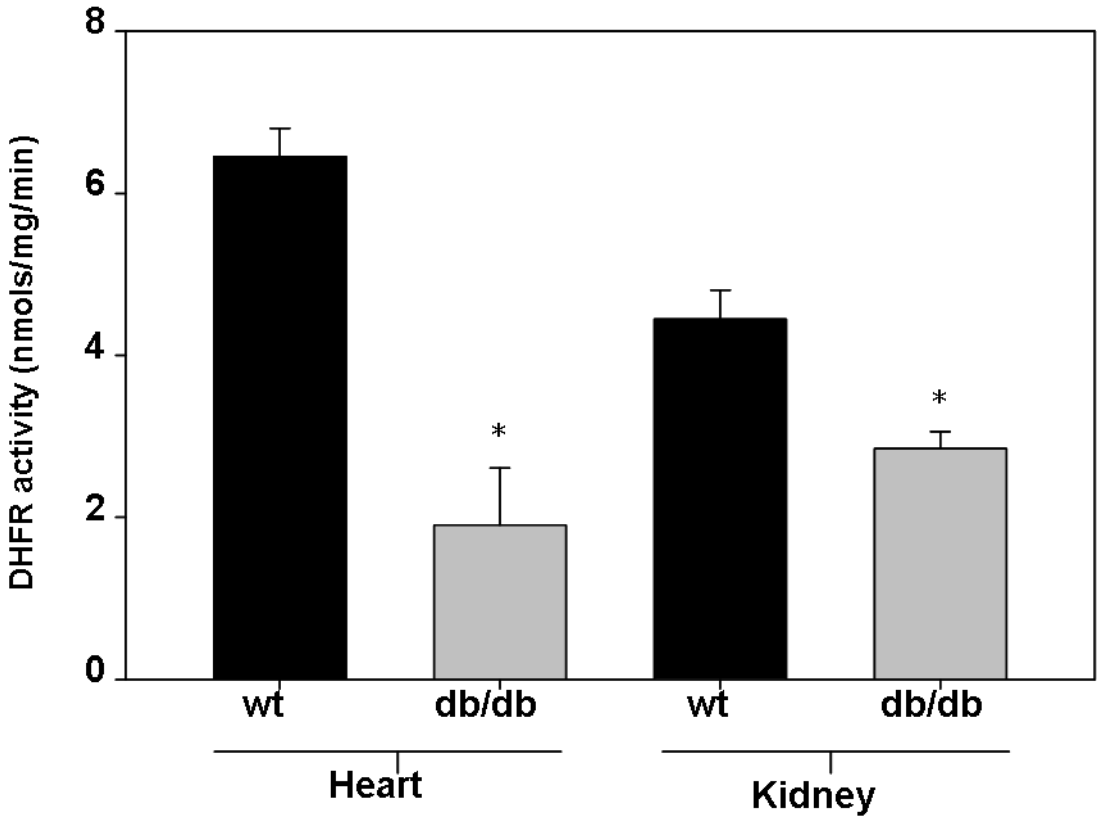

Figure 3-9. Effects of diabetes on DHFR activity. 200 μg of kidney and heart homogenate from wt and db/db were incubated in the presence of BH2 (100 μM) and the conversion to BH_4 measured by HPLC. * represents $p<0.05$.

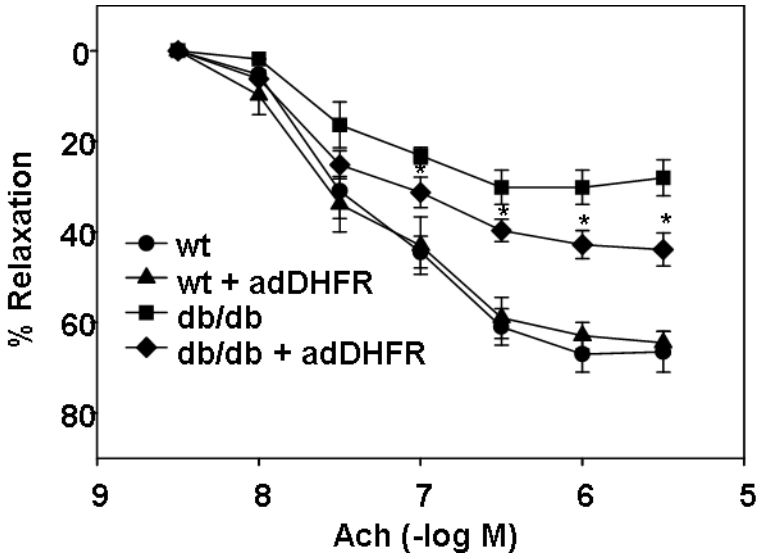

Figure 3-10. Effects of DHFR over-expression on vascular relaxation. Vascular reactivity to Ach (5 nM-5 µM) in aortas from wt and db/db mice with and without adDHFR (1 x 10^{11}vp/mL). n=4-6. * represents $p<0.05$ between db/db and db/db + adDHFR.

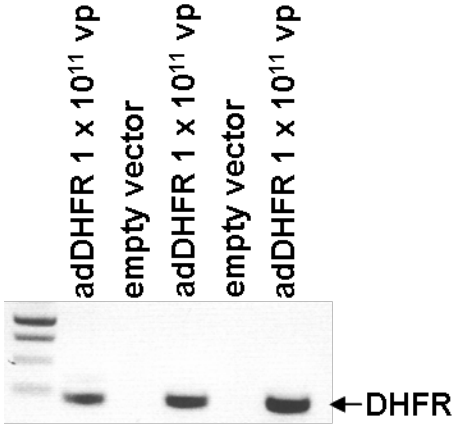

Figure 3-11. Effects of DHFR over-expression. Semi-quantitative PCR analysis of DHFR expression in mouse aorta following 24 hour transduction with adDHFR.

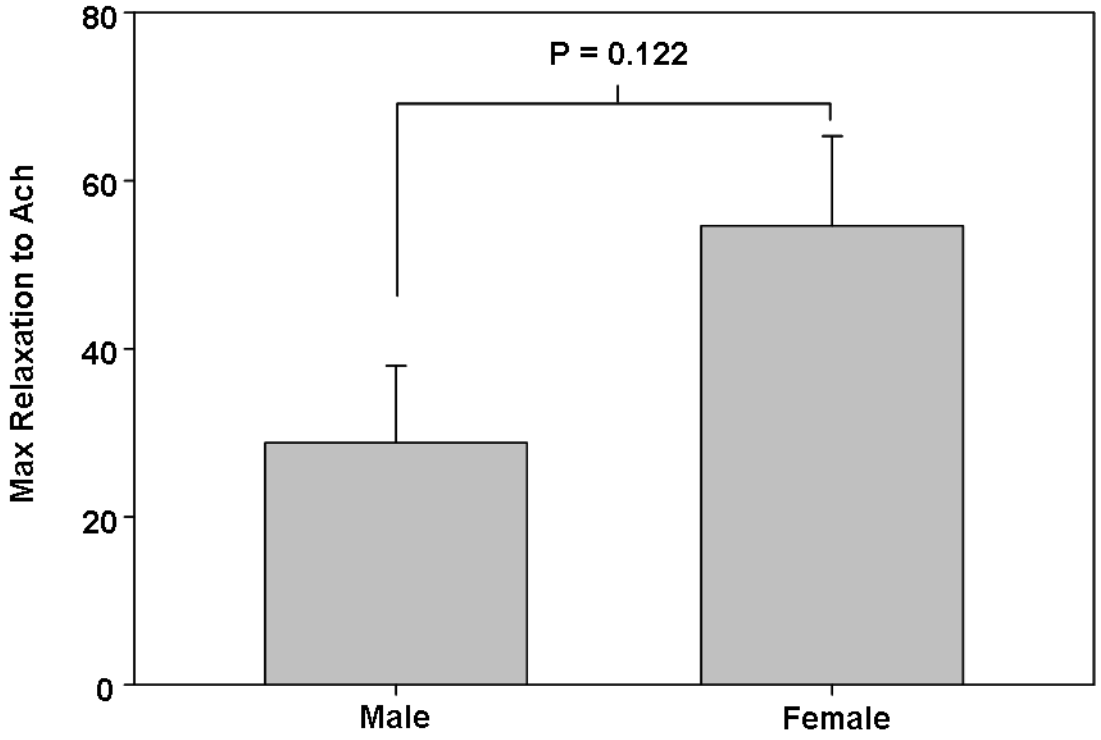

Figure 3-12. Endothelial dependent relaxation males vs. females. Relaxation was measured in Internal Mammary Artery of human patients with and without diabetes. All patients were undergoing coronary artery bypass grafting between the ages of 18 and 80. Comparing male vs. female, rings were constricted with PE (2 µM) and the relaxation response to Ach (500 nM-10 µM) was measured on a wire myograph.

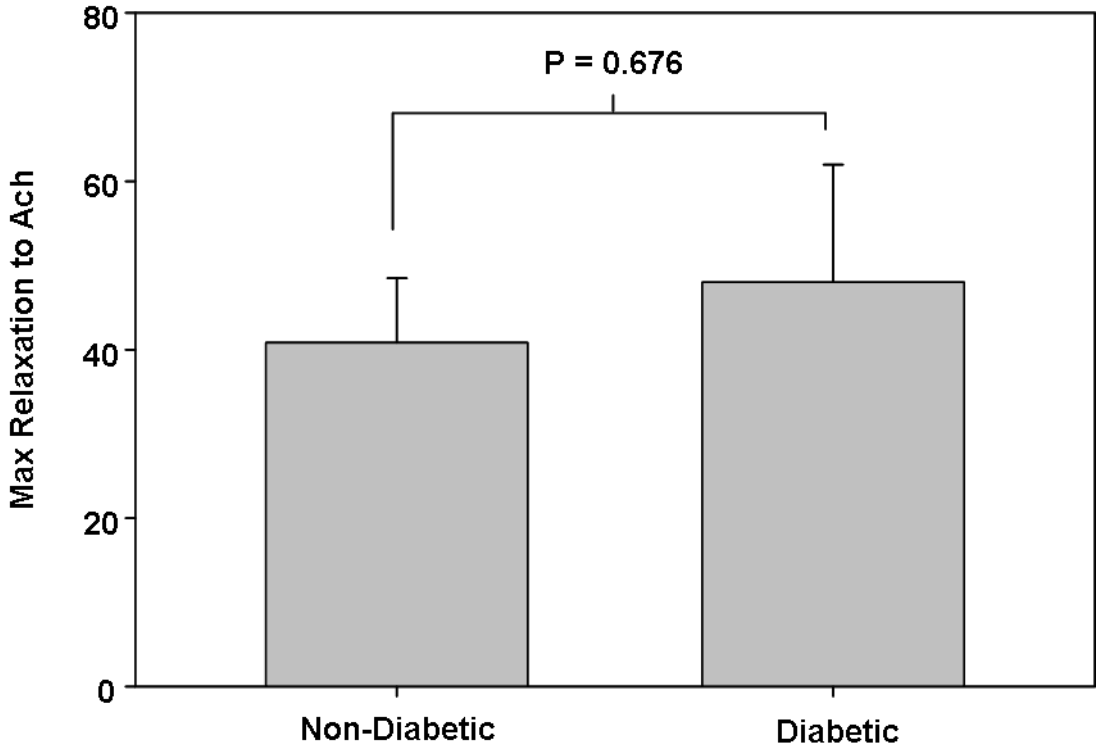

Figure 3-13. Endothelial dependent relaxation non-diabetic vs. diabetic. Relaxation was measured in Internal Mammary Artery of human patients with and without diabetes. All patients were undergoing coronary artery bypass grafting between the ages of 18 and 80. Comparing non-diabetic vs. diabetic for both female and male patients, rings were constricted with PE (2 µM) and the relaxation response to Ach (500 nM-10 µM) was measured on a wire myograph.

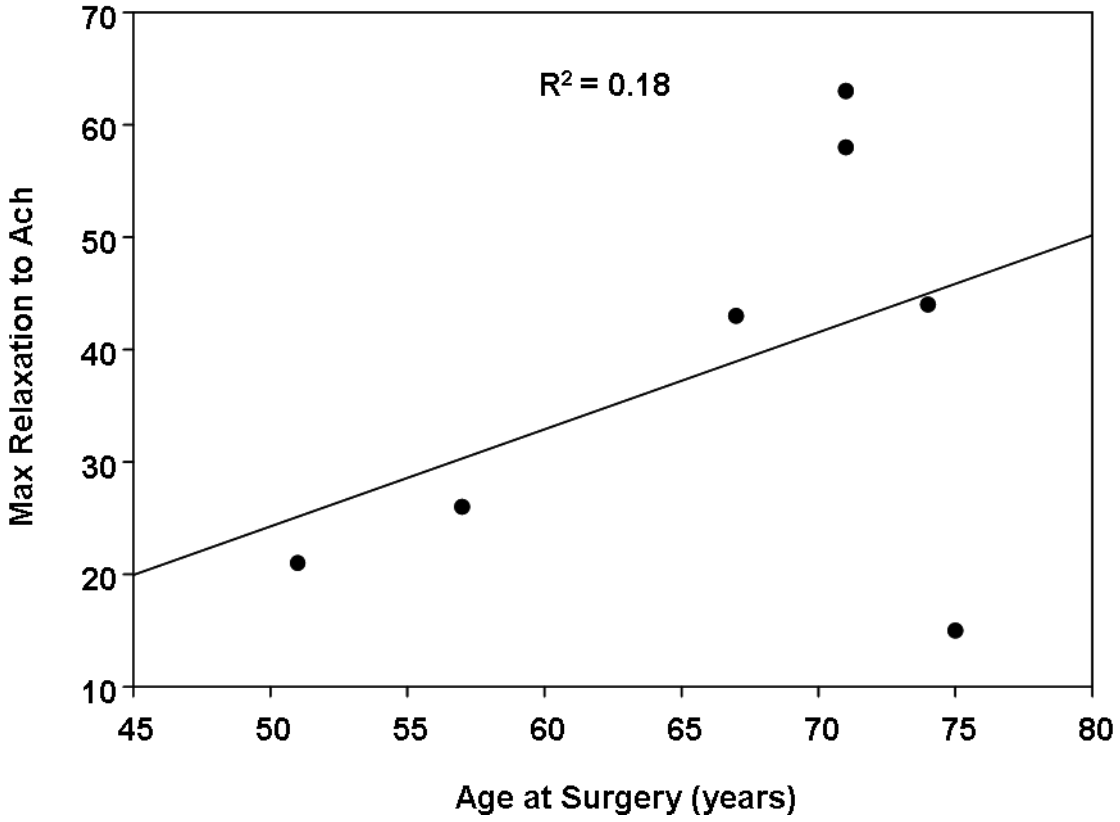

Figure 3-14. Effects of age on endothelial relaxation. Endothelial dependent relaxation was measured in Internal Mammary Artery of human patients with and without diabetes. All patients were undergoing coronary artery bypass grafting between the ages of 18 and 80. Comparing max relaxation vs. age of non-diabetic and diabetic for both female and male patients, rings were constricted with PE (2 µM) and the relaxation response to Ach (500 nM-10 µM) was measured on a wire myograph.

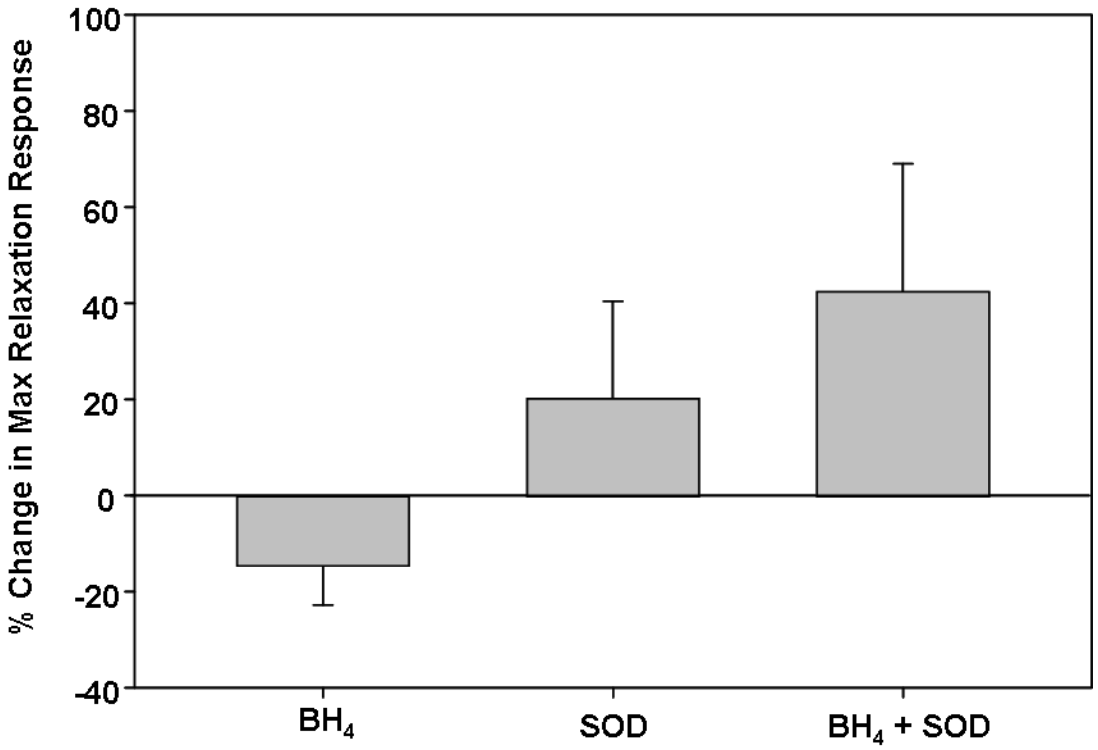

Figure 3-15. Effects of BH_4 and SOD on IMA. Endothelial dependent relaxation was measured in Internal Mammary Artery of human patients with and without diabetes. All patients were undergoing coronary artery bypass grafting between the ages of 18 and 80. Comparing bath supplementation of BH_4, SOD and BH_4+SOD of non-diabetic and diabetic for both female and male patients, rings were constricted with PE (2 µM) and the relaxation response to Ach (500 nM-10 µM) was measured on a wire myograph.

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BIOGRAPHICAL SKETCH

Patrick Kearns graduated from The Ohio State University in 2006 with a Bachelor of Science in biology. Pat moved to Florida, in 2008, where he resumed vascular research work in Dr. Cardounel's lab at the University of Florida. Here, he enrolled in the master's program of medical sciences.