ROLE OF ASYMMETRIC DIMETHYLARGININE (ADMA) IN THE REGULATION OF ENDOTHELIAL DERIVED NITRIC OXIDE

By

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To Mom, Dad, Damecko, and Dannae thank you all for your love and support
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ROLE OF ADMA IN THE REGULATION OF ENDOTHELIAL DERIVED NITRIC OXIDE

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The endogenous NOS inhibitor Asymmetric Dimethylarginine (ADMA) has been demonstrated to be an independent cardiovascular disease risk factor. However, the mechanisms regarding how ADMA levels are modulated and what role they play in disease progression are not clearly understood. Dimethylarginine dimethylaminohydrolase (DDAH) is the enzyme responsible for ADMA metabolism however, how it is regulate in the disease state is unclear. Therefore, we hypothesize that decreased DDAH expression/activity may be involved in the vascular pathophysiology observed in a variety of cardiovascular disease.

Here we present findings that each isoform of the DDAH enzyme regulates endothelial NO production. Over-expression of either DDAH-1 or DDAH-2 was found to increase endothelial NO production. Gene silencing of either isoform attenuated endothelial DDAH activity. Interestingly, dual silencing of the enzymes did not result in an additive effect on DDAH activity suggesting the existence of an alternative pathway of methylarginine metabolism. Furthermore, gene silencing of either isoform results in decreased endothelial NO production.

Subsequent studies aimed at investigating mechanisms of DDAH regulation in a disease state demonstrated that cells exposed to 4-HNE exhibit decreased endothelial NO production and these effects were mediated through increased ADMA levels and decreased DDAH activity. In
addition to methylarginine regulation of NO, it has been hypothesized the ADMA may be involved in the phenomenon of eNOS uncoupling wherein the enzyme switches form an NO producing enzyme to an superoxide producing enzyme. Investigations into this pathway revealed that methylarginines caused a dose dependent increase in eNOS derived superoxide. Interestingly, L-arginine also increased eNOS derived superoxide in a dose dependent manner.

In addition to ADMA accumulation, oxidative stress has also been associated with endothelial dysfunction. The presence of reactive oxygen and nitrogen species decreased the activity of the salvage pathway enzyme, dihydrofolate reductase (DHFR) which regulates the conversion of H₂B to H₄B Physiological levels of OONO- increases enzyme activity. Furthermore, using the diabetic db/db mouse model of diabetes it was observed that DHFR activity was decreased and that these mice had impaired vascular function.

These findings demonstrate that the DDAH-ADMA pathway and oxidative stress plays a critical role in the development of endothelial dysfunction.
CHAPTER 1
INTRODUCTION

In the United States it is estimated that 80,000,000 or 1 in 3 Americans have cardiovascular disease [1]. Data from the Framingham Heart Study demonstrates that 2 out of 3 men and 1 in 2 women will have cardiovascular disease in their lifetime [1]. Of those who have cardiovascular disease, 73,600,000 have high blood pressure, which is defined as having a systolic pressure $\geq 140$ mm Hg or a diastolic pressure $\geq 90$ mm Hg. In 2005, cardiovascular disease was the underlying cause for 35.3% of all deaths in the United States [1]. The number of deaths due to cardiovascular disease surpasses the total number of deaths due to cancer, diabetes, and accidents combined. Of those who die as a result of cardiovascular disease, 52% died as a result of coronary heart disease [1]. However, recent studies have shown that from the years 1980-2000, there was a substantial decrease in the number of deaths due to cardiovascular disease. Furthermore, almost half of the reduction in deaths can be attributed to advances in the treatment of cardiovascular disease, while the other half is due to maintaining a healthy lifestyle [1].

Despite the drop in deaths due to heart disease, it is still the number one cause of death in the United States, and providing care to these patients results in an enormous cost to the health care system. In 2005, 1 out of every 6 hospital stays was related to coronary heart disease and the total cost of hospital care was 71.2 billion dollars. The projected indirect and direct cost for the treatment of cardiovascular disease is expected to rise to 475.3 billion dollars in 2009 [1].

The most prevalent form of heart disease is coronary artery disease (CAD). CAD is caused by the build up of plaque in the coronary artery, which leads to lumen narrowing and a decreased supply of oxygen rich blood to the heart. This pathological process of arterial narrowing and impaired blood flow is termed atherosclerosis and is the most common cause of
coronary heart disease. Coronary heart disease, if untreated, can eventually lead to a heart attack and subsequently heart failure [1]. CAD is the result of various risk factors, including genetics, high blood pressure, smoking and diabetes.

Familial hypercholesterolemia (FH) is an inherited disorder that is caused by a deficiency in the clearance of the Low Density Lipoprotein (LDL). Hypercholesterolemia is defined as having a total serum cholesterol level ≥ 240 mg/dl. Initially, it was believed that familial hypercholesterolemia was caused by the increased production of cholesterol. However, this proved not to be the cause with the discovery of the LDL receptor (LDLR) by Brown and Goldstein in 1973 [2, 3]. Their studies revealed that patients who suffered from FH had dysfunctional LDLRs, therefore, leading to the increased accumulation of cholesterol [2, 3]. The risk of cardiac events in this population has been greatly reduced with the development of statins.

Additional major risk factors taken into account to determine risk for CAD included diabetes, smoking, and high blood pressure. The Framingham Heart Study defines individuals having a blood pressure of <120/80 mm Hg, total serum cholesterol levels <180 mg/dL, non diabetics, and non smokers as those who are least likely to develop CAD [4]. High risk individuals are those who have total serum cholesterol levels that are ≥ 240 mg/dl, hypertension, diabetes and smokers. The risk factors for developing CAD increases with age, however if a healthy lifestyle is maintained the risk remains low. At 50 years of age men have a 5.2% chance and women have a 8.2% chance of developing CAD if they maintain a healthy lifestyle [4]. However, having two or more of the associated risk factors (i.e. hypertension, diabetes) increases the risk of developing CAD to 68.9% for men and to 50% for women [4]. Although increased serum cholesterol levels and the associated risk factors outlined by the Framingham
Heart Study are known to increase the risk of coronary artery disease, it is not just a disease of high cholesterol.

Atherogenesis, once considered mainly a disease of cholesterol storage, is now understood as a complex disease of many interacting risk factors which include cells of the artery wall, the blood and the molecular messengers exchanged between the two. It is now becoming clear that inflammation plays a critical role in atherogenesis [5-9]. It also plays a key role in the local, myocardial and systemic complications associated with atherosclerosis [6, 8, 10].

Dyslipidemia, vasoconstrictive hormones associated with hypertension, and proinflammatory cytokines derived from excess adipose tissue, enhance the expression of adhesion molecules that promote the sticking of blood leukocytes to the inner surface of the vascular wall [6, 8, 10]. Once inside the intima, blood leukocytes activate the smooth muscle cells (SMCs) resulting in their migration to the intima. The SMCs continue to proliferate leading to the creation of a complex extracellular matrix [7, 11-15]. Proteoglycans of the extracellular matrix bind to lipoproteins extending their stay within the intima therefore increasing their chances of becoming oxidized. LDLs undergo oxidative alteration leading to the formation of oxLDL in the arterial wall. Other cellular lipids also undergo redox modifications, which result in the formation of lipid hydroperoxides. These oxidatively modified lipids have been demonstrated to play an important role in the pathogenesis of atherosclerosis [16-21]. Among the mechanisms proposed, Nitric Oxide Synthase (NOS) dysregulation and decreased Nitric Oxide (NO) bioavailability have been implicated as a central mechanism in vascular endothelial dysfunction associated with atherosclerosis.

NO is a potent vasodilator and critical effector molecule that helps the endothelium maintain vascular homeostasis through its anti-proliferative and anti-thrombotic effects. NO is
derived from the oxidation of L-Arginine (L-Arg) and catalyzed by the constitutively expressed enzyme endothelial nitric oxide synthase (eNOS). NO freely diffuses across the vascular endothelium to the vascular smooth muscle cell layer where it activates guanylate cyclase leading to smooth muscle cell relaxation [22, 23]. In addition to its effects on vascular tone, NO also helps to maintain the anti-atherogenic properties of the vascular wall. NO, in association with other cell signaling molecules promotes smooth muscle cell quiescence counteracting pro-proliferative molecules specifically those involved with athero-proliferative disorders [24-29]. Therefore, loss of NO bioavailability is an early symptom of endothelial dysfunction and is implicated as the pathogenic trigger leading to atherosclerosis.

Among the proposed mechanisms that lead to decrease NO bioavailability, is the accumulation of the endogenous NOS inhibitors asymmetric dimethylarginine (ADMA) and N<sup>G</sup>-monomethyl-L-arginine (L-NMMA) [30-34]. ADMA and L-NMMA are both competitive inhibitors of eNOS. ADMA and L-NMMA are derived from the proteolysis of methylated arginine residues on various proteins. Methylation is carried out by a group of enzymes referred to as protein-arginine methyl transferase’s (PRMT’s). Upon proteolysis of methylated proteins, free methylarginines are released where they can then inhibit eNOS activity. The free methylarginines are subsequently hydrolyzed by Dimethylarginine Dimethylaminohydrolase (DDAH) to citrulline, and mono and dimethylarginine [35-37]. Recent studies from our lab and others have shown that the methylarginines ADMA and L-NMMA play a critical role in vascular function and that the dysregulation of the enzymes responsible for metabolizing the methylarginines play an essential role in endothelial dysfunction [38].

In support of this hypothesis several studies from both human and animal models of atherosclerosis have demonstrated that L-Arg enhances the anti-atherogenic properties of the
endothelium by increasing NO bioavailability. Oral L-Arg supplementation has been demonstrated to restore endothelium dependent vasorelaxation in both hyperlipidemic animals and humans [30, 34, 35, 39, 40]. Additionally, oral L-Arg has also been shown to prevent the development of atherosclerosis in LDL receptor knockout mice (LDLR) [30]. The beneficial effects observed following L-Arg supplementation could be explained by the fact there is increased substrate for the NOS enzyme. However, intracellular levels of L-Arg are 50 times above the $K_m$ value for the enzyme therefore, increased NO generation would not be expected as a result of L-Arg supplementation [41]. It has been hypothesized that basal levels of methylarginines can inhibit NOS activity and L-Arg supplementation is able to improve vascular function simply by overcoming the inhibitory effects of the methylarginines [42].

Another potential mechanism for reduced NO bioavailability is through direct scavenging of NO by reactive oxygen species (ROS) [43]. Growing evidence has demonstrated that oxidative stress is associated with the pathogenesis of diseases including hypercholesterolemia, diabetes and hypertension [44-47]. In healthy tissues, superoxide anion ($O_2^-$) is dismutated into hydrogen peroxide ($H_2O_2$) and oxygen by the enzyme superoxide dismutase (SOD). The enzyme, Catalase further reduces $H_2O_2$ to water and oxygen [48]. Increases in oxidative stress seen in the pathological states overwhelm the antioxidant defense systems resulting in an oxidative environment. Failure of the antioxidant system can also lead to the generation of peroxynitrite (OONO$^-$), which is a potent oxidant known to cause damage to proteins and tissues [43]. In this regard, a human variant of ecSOD has been observed in 5% of the population. This ecSOD variant is associated with decreased SOD activity, increased oxidative stress and increased inactivation of NO [49].
Alternatively it has been proposed that increased oxidative stress results in the uncoupling of the NOS enzyme turning it into a superoxide generating enzyme. *In vitro* studies have demonstrated that eNOS depleted of its essential cofactor, tetrahydrobiopterin (H₄B), readily makes superoxide [50, 51]. Furthermore, it is also known that H₄B is highly redox sensitive and can be readily oxidized to its inactive form dihydrobiopterin (H₂B). H₄B is produced via two pathways in the endothelial cell, the de novo synthesis pathway and the salavage pathway. De novo biosynthesis of H₄B is a magnesium, zinc and NADPH dependent pathway. The first step requires the conversion of GTP to 7,8-dyhydronopterin triphosphate. This reaction is catalyzed by the enzyme GTP cyclohydrolase I (GTPCH), and it is the rate limiting step in H4B biosynthesis [52]. Following the GTPCH enzyme reaction pyruvoyl tetrahydroppterin synthase (PTPS) converts 7,8 dihydronopterin triphosphate into 6-pyruvoyl-5,6,7,8-tetrahydroppterin. Alternatively, the salvage pathway enzyme Dihydrofolate reductase (DHFR) is a NADPH dependent enzyme that catalyzes the conversion of H₂B to H₄B.

NOS uncoupling has also been demonstrated to occur in both animal and human models of diseases associated with oxidative stress. In this regard, oral supplementation of H₄B was demonstrated to improve endothelial dependent vascular function in the apoE KO mouse model of hypercholesterolemia. In addition to improved vascular function, a reduction in vascular superoxide production was also observed following oral H₄B supplementation [53]. Moreover, endothelial function has been shown to improve in patients who are chronic smokers, type II diabetics and those with CAD following H₄B supplementation [54, 55].

**Rationale for Study**

It is clear that the mechanisms that lead to vascular endothelial dysfunction are quite complicated. Though the evidence laid out in the introduction points to two possibilities. First, increasing levels of methylarginines have been demonstrated to be an independent risk factor in
the development of cardiovascular disease. However, how methylarginines are modulated and what role they play in disease progression is poorly understood. Additionally, how DDAH is regulated and what role it plays in endothelial dysfunction needs to be explored further. Because NO possess both anti-proliferative and anti-atherogenic properties, methylarginine accumulation in response to decreased DDAH expression and activity has been proposed to be involved in the vascular pathophysiology observed in a variety of cardiovascular disease.

In addition to the accumulation of ADMA, the altered redox status of the endothelium has also been implicated as a central mechanism in endothelial dysfunction associated with cardiovascular disease. Previous studies have demonstrated that in diseases such as diabetes there is an accumulation of H\(_2\)B, the inactive oxidized form of the NOS cofactor H\(_4\)B. This increase has also been associated with increased superoxide production and vascular endothelial dysfunction. However, it is unclear as to what leads to this accumulation, because DHFR should reduce H\(_2\)B back to H\(_4\)B. Therefore, I hypothesized that DHFR activity may decrease in oxidative stress situations. While it may appear that ADMA and oxidative stress are unrelated, it has been suggested that ADMA also plays a role in NOS uncoupling.

Therefore to better establish the role of ADMA in the regulation of endothelial derived NO and vascular endothelial dysfunction, the following aims will be carried out:

Aim 1: To determine the role of DDAH in the regulation endothelial derived NO

Aim 2: To determine the effects of the methylarginines on eNOS derived superoxide.

Aim 3: To determine the effects of oxidative stress on DHFR activity in vitro and in vivo.
CHAPTER 2
REVIEW OF LITERATURE

Endothelial cells were once considered to be a population of elongated cells that were homogenous in nature and that their main function was to serve as a barrier between the vascular space and interstitium. Florey demonstrated in the late 60’s that the endothelium was a permeability barrier and served a much bigger role than previous thought [56]. Subsequently, intense research began to determine the role endothelial cells and their effects on vascular function. Furchgott and Zawadzi were the first to describe that the endothelial layer was necessary for acetylcholine (Ach) mediated vascular relaxation in rabbit aortic rings. Their studies demonstrated that when the endothelial layer was removed, the vessel lost its ability to relax in response to Ach and in fact it resulted in overt vasoconstriction [57]. Moncada, and Ignarro, independently established that the effector previously described by Furchgott and Zawadzi as endothelial derived relaxing factor (EDRF), was in fact NO [58, 59]. A year later, L-Arg was discovered to be the substrate from which NO was synthesized [60]. Since then it has been established that NO is one of the most important regulators of vascular homeostasis and that decreased bioavailability of NO is involved in the endothelial dysfunction observed in cardiovascular disease.

Nitric Oxide

Endothelial derived Nitric Oxide is synthesized from the oxidation of the guanidino carbon of the amino acid L-Arg to NO and L-Cit by the enzyme eNOS [60]. The half life of NO is in the range of 3-5 seconds in the presence of hemoglobin and can undergo rapid oxidation by oxyhemoproteins to nitrate (NO₃⁻) and nitrite (NO₂⁻) [61]. One of the primary functions of NO in the vasculature is to cause vascular smooth muscle cell (VSMC) relaxation. NO does this by freely diffusing from the endothelium into the VSMC layer where it binds to the heme group of
the enzyme guanylate cyclase. Guanylate cyclase then catalyzes the reaction of guanosine triphosphate (GTP) to cyclic guanosine 3’5’-monophosphate (cGMP) and inorganic phosphate [22, 59]. cGMP then activates protein kinaseG (PKG) resulting in the phosphorylation of myosin light chain phosphatase. Myosin light chain phosphatase then dephosphorylates myosin light chain, resulting in vascular smooth muscle cell relaxation. NO in concert with various cell signaling molecules, has been demonstrated to maintain smooth muscle cell quiescence and as such, counteracts pro-proliferative agents, specifically those involved in the propagation of athero-proliferative disorders [25].

**Nitric Oxide Synthase Enzyme**

There are three isoforms of the NOS enzyme, neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS). The cofactors required for the full enzymatic activity of all NOS enzymes are the flavin (FAD, FMN) [28], heme, calmodulin (CaM) and tetrahydrobiopterin (H4B). The enzyme has three domains which are required for catalytic activity, the reductase domain, CaM binding domain and oxygenase domain [62-64]. The cofactors FAD and FMN are located within the reductase domain and in concert with NADPH shuttle electrons to the heme binding site in the oxygenase domain [62-64]. The oxygenase domain contains the heme, H4B, and arginine binding sites [65, 66]. eNOS and nNOS are activated by calcium-calmodulin binding to the CaM binding domain of the enzyme. The binding of calcium-calmodulin to NOS activates the transfer of electrons from the flavin to heme, where oxidation of L-Arg to NO and L-Cit occurs [67, 68].

eNOS, when inactive, is located within invaginations of the plasma membrane called caveolae [69]. Specifically, it has been demonstrated that eNOS binds to caveolin-1 (CAV-1) and that this interaction is inhibitory to enzyme activity [69]. Dissociation of the CAV/eNOS complex occurs when excess amounts of calcium enter the cell and binds to CaM. The resulting
Ca-CaM complex facilitates the dissociation of eNOS from Cav-1 resulting in eNOS activation and NO production.

**Mediators of NO Release**

The release of NO from the vascular endothelium can be activated through both Ca\(^{2+}\) dependent and independent mechanisms. The binding of substances such as acetylcholine (Ach), and bradykinin to their respective receptors activate NOS through a Ca\(^{2+}\) dependent mechanism [70-72]. All of these substances mediate their effects on eNOS activity through phospholipase C (PLC). Activation of PLC results in increased intracellular Ca\(^{2+}\), which subsequently leads to the activation of eNOS [70-73].

Alternatively, laminar shear stress generated by blood flowing over the endothelial cell, which is the main physiological way in which eNOS is activated, and vascular endothelial growth factor (VEGF) can also stimulate the release of NO in a Ca\(^{2+}\) independent manner. Shear stress and VEGF activate the phosphatidylinositol-3-kinase (PI3K) pathway leading to the activation of AKT consequently resulting in eNOS phosphorylation and activation [74].

**Actions of Nitric Oxide**

In addition to modulating vascular tone through VSMC relaxation, NO is also important for maintaining vascular homeostasis through its anti-thrombotic, anti-proliferative and anti-atherogenic effects. NO and prostaglandin (PGI) act in a synergistic manner through a cGMP dependent mechanism to prevent platelet aggregation in the endothelium [75]. It has been demonstrated in both human and animal models that NO is key in preventing platelet aggregation. In this regard, it was observed in a rat model of common carotid artery thrombosis, platelet aggregation increased at the site of the thrombosis following administration of the NOS inhibitor Nitro-L-Arg methyl ester (L-NAME) [76]. Furthermore, studies involving healthy
human volunteers have demonstrated that when the NOS inhibitor L-NMMA is given intravenously bleeding times are decreased [77].

In addition to its anit-thrombotic effects, NO is known for its anti-atherogenic properties. Endothelial cell activation is a process that involves the up regulation of transcription of a number of pro-inflammatory genes, and adhesion molecules such as E and P selectin, VCAM-1 and ICAM-1. Additionally, chemokines such as MCP-1 and IL-1 also increase during endothelial cell activation. Taken together these adhesion molecules and chemokines, increase leukocyte rolling and adhesion to the endothelium. NO through its inhibitory effects on the NF kappa B signaling pathway prevents leukocyte adhesion to the endothelial cell monolayer. Thus, resulting in the inhibition of the pro-atherogenic adhesion molecules P-selectin, E-selectin, and VCAM-1 [78, 79].

The anti-proliferative properties of the endothelium are maintained through a NO mediated mechanism in concert with various other signaling molecules. Balloon angioplasty is a standard treatment for coronary artery stenosis caused by CAD. An unfortunate side effect to this treatment is restenosis, which is caused by VSMC proliferation in response to vascular injury. Several studies have demonstrated that in both human and animal models of restenosis, increasing NO reduces neointimal hyperplasia [80-82]. Though it has been known for quite some time that NO prevents VSMC proliferation, the molecular mechanism of how this occurs was largely unknown. Recently it has been demonstrated that NO inhibits cell cycle progression of VSCMs in the S phase by inducing down-regulation of cyclin-dependent kinase 2 (cdk2) activity and cyclin A gene transcription [83].

In addition to its effects on the endothelium, NO has also emerged as a protein post translational modifier. Several studies have demonstrated that endogenous and exogenous NO
and its oxidative products NO₃ and NO₂ can S-nitrosylate proteins at active cysteine residues altering their function. The exact function of protein S-nitrosylation (SNO) is not clearly defined, however, it has been suggested that it may be involved in storage and transportation of the NO molecule [84]. In support of this hypothesis it has been demonstrated that glutathione and NO interact to form S-nitrosoglutathione (GSNO). GSNO is the most abundant SNO and in-vitro its decomposition has been shown to generate NO [85].

The formation of SNO is prevented during high antioxidant activity. However, when the antioxidant defense system is overwhelmed during times of oxidative stress, SNO formation could be a key in preventing further oxidative damage [86]. SNO has also been shown to partially mediate the antioxidant effects of statins in the endothelial cell by activating the antioxidant enzyme thioredoxin [87].

SNO can also modulate the activity of enzymes important for regulating vascular homeostasis. SNO formation has been demonstrated to occur in the catalytic triad of the DDAH enzyme at cysteine 249 rendering the enzyme inactive [88]. Argininosuccinate synthetase, the enzyme responsible for converting citrulline to argininosuccinate can also undergo SNO formation also inhibiting its activity [89]. In-vitro studies using NO donors demonstrate that formation of SNO on eNOS targets two cysteine residues at 96 and 101 rendering the enzyme inactive [90]. Furthermore, it has been demonstrated in Bovine Aortic Endothelial Cells (BAECs) that SNO formation on eNOS also occurs, but can be rapidly denitrosylated in the presence of VEGF [91]. Though eNOS can be self regulated through SNO formation, it is also regulated by posttranslational modifications, substrate availability and protein-protein interactions.
Regulation of NOS

The role of eNOS in the regulation of cardiovascular function has been the focus of extensive research efforts. Results have demonstrated that eNOS enzymatic activity is regulated by a variety of factors including substrate/inhibitor bioavailability, protein-protein interactions and post-translation modifications. In addition to being a substrate for NOS, L-Arg is also metabolized through various pathways in the cell. Arginine is predominantly metabolized by the enzyme arginase and its activity could play a key role in regulating eNOS. eNOS’s interactions with other proteins and cofactors have been well documented as ways in which eNOS can be regulated. Hsp90, CaM, H4B all promote increased enzyme activity and NO production. On the other hand, eNOS’s interaction with Cav-1 results in the inhibition of enzyme activity. eNOS is also regulated by post-translational modifications. Among them, phosphorylation of eNOS is most extensively studied and has been demonstrated to result in both site specific activation and inactivation of the enzyme. Finally, the last known post translational modification of eNOS that occurs is myristoylation and palmitoylation. Myristoylation and palmitoylation of the enzyme causes it to be targeted to the plasma membrane where it will interact with CAV-1 inhibiting enzymatic activity.

Arginine

The discovery in 1987 that Endothelial Dervied Relaxing Factor (EDRF) was NO was an important milestone in understanding how vascular tone was regulated. However, it was not until a year later that the substrate for eNOS was discovered to be the amino acid arginine [86]. Arginine is available from three main sources; dietary intake, endogenous biosynthesis, and protein turnover. 40% of the arginine that we ingest through our diet is catabolized in the intestine before reaching the whole body [92]. During fasting states, 85% of our circulating arginine is derived from protein turn-over and the rest comes from endogenous biosynthesis [93].
The endogenous biosynthesis of arginine in healthy adult humans is enough that it is not an essential amino acid in the diet. However, in infants, growing children, and adults with kidney or intestinal dysfunction, endogenous arginine synthesis is not enough. Therefore, it is classified as a conditionally essential dietary amino acid [94].

Whole body arginine synthesis occurs primarily between the interaction of the small intestine and the kidney and it is referred to as the gut-kidney axis. Citrulline is produced from glutamine and proline in the small intestine. The kidney then takes up citrulline where it is converted to arginine. A large amount of arginine synthesis also takes place in the liver, however, it is not a significant source as arginine is quickly hydrolyzed to urea and ornithine therefore not contributing a lot to the whole body [95].

Although the primary means of arginine synthesis occurs in the kidney renal tubules, the majority of cell types have the ability to synthesize arginine. Arginine synthesis from citrulline occurs via the synergistic action of argininosuccinate synthase and argininosuccinate lyase (ASL). ASL is the rate-limiting step in the conversion of citrulline to arginine, and it requires aspartate, citrulline and ATP as cofactors for full activity [95]. The citrulline-NO cycle, much like the urea cycle, is recognized as an alternative means to produce arginine in the cell. However, only a fraction of the citrulline produced by eNOS oxidation of arginine is recycled via the citrulline-NO cycle [93].

**Arginine Transportation**

Transportation of the cationic amino acid arginine from the plasma into the cell occurs though the sodium (Na⁺) independent transport system y⁺. The y⁺ transport system family consists of 3 cationic amino acid transporters (CAT) CAT-1, CAT-2 and CAT-3, each having distinct tissue distribution. CAT-1 is ubiquitously expressed, CAT-2A expression is found in the liver, skin and skeletal muscle, and CAT-3 expression is exclusively expressed to the brain. The
amino acids lysine, ornithine and the methylarginines compete with arginine for transport through the CAT transport system. Although intracellular arginine appears to be the most important source of eNOS derived NO, there is evidence to support a role for CAT in the regulation of endothelial NOS [95].

The $y^+$ $K_m$ for arginine is within the physiological range of plasma arginine levels and therefore arginine transportation into the cell maybe an important regulator of NOS. Kinetic studies have demonstrated that the $K_m$ of eNOS for arginine is 2-3 $\mu$M. L-Arg intracellular levels are in the range of 100 $\mu$M. Therefore, substrate availability should not be a limiting factor in NO synthesis [96]. However, studies have clearly demonstrated in both animal and human models that arginine supplementation leads to increases in NO generation. This phenomenon has been termed the “L-Arg paradox” and has been hypothesized that perhaps increased uptake through the $y^+$ transport system may play a role in this paradox [93, 95].

**Arginine Metabolism**

**Arginase**

Arginase is the key urea cycle enzyme involved in arginine metabolism and is responsible for the hydrolysis reaction of arginine to urea, and ornithine. There are two isoforms of arginase that are expressed in the body. The type I isoform is located in the liver and is responsible for the majority of arginase activity. The type II isoform is predominantly expressed as a mitochondrial protein and is expressed in a variety of tissues with the highest expression localized to the kidney, and the lowest in the liver [93].

Recently, several studies have demonstrated that arginase is present in the vasculature and may serve a regulatory role in vasomotor tone. VSMC only express type I, while endothelial cells express both isoforms. The aortic smooth muscle cells of rats were observed to have high arginase activity. Additionall, transforming growth factor-beta (TGF-β) up-regulates the
expression and activity of arginase I in these cells [97]. Furthermore, it has been demonstrated that both isoforms are expressed in the aorta, carotid artery and pulmonary artery [98].

Considering that arginine is also a substrate for arginase, it has been suggested that arginase may compete with NOS for substrate binding. The $K_m$ for arginine for eNOS and arginase are 2 µM and 1-5 mM respectively. Although, arginine has a higher affinity for NOS, the activity of the arginase enzyme is 1000 fold greater therefore suggesting that at physiological levels arginase can compete with NOS for substrate binding [93, 99]. In support of this hypothesis, it was demonstrated in macrophages that L-Arg supplementation resulted in greater urea production, than NO generation [100]. It has also been demonstrated in endothelial cells that over-expression of either arginase isoforms resulted in decreased eNOS derived NO. In microvascular endothelial cells isolated from Dahl salt sensitive rats, the increase in arginase activity counteracts NO mediated relaxation, thus suggestive of a vasoconstrictive role [101]. In contrast, inhibition of arginase activity has been demonstrated to increase endothelial NO production in cultured endothelial cells [102]. Although arginase is the main pathway in which arginine is metabolized; there are other pathways in which its metabolism can also occur.

Arginine:Glycine Amindotransferase and Arginine Decarboxylase

The arginine:glycine amidotransferase [103] enzyme catalyzes the first step and it is also the rate limiting step in creatine formation. In the first step of creatine synthesis arginine donates an amidino group to glycine to form guanidinoacetate and ornithine. The guanidionacetate is then methylated to form S-Adenosylhomocysteine and creatine. Creatine negatively feedbacks to inhibit enzyme activity [104]. Ornithine made from this pathway can be used by ornithine decarboxlyase (ODC) to make polyamines.

Arginine Decarboxylase (ADC) catalyzes the reaction of arginine to carbon dioxide and agmatine. Agmatine is further metabolized into putrescine and urea. Putrescine is used in the
synthesis of polyamines, which are important for cell division [105]. Although AGAT, ADC, and ODC do not appear to compete with NOS for substrate binding, they can play a role in vascular remolding as polyamines are important for cell division and proliferation [74]

NOS Cofactor and Protein-Protein Interactions.

Tetrahydrobipterin (H₄B)

First described as an essential cofactor for the aromatic amino acid hydroxylases, tetrahydrobipterin (H₄B) is also an essential cofactor for all three NOS isoforms [106-108]. The role that H₄B plays in NOS regulation has only recently become more defined. Located within each domain of eNOS is a binding site for a H₄B molecule. In vitro studies demonstrate that H₄B stabilizes and donates electrons to the ferrous-dioxygen complex in the oxygenase domain to help initiate the oxidation of L-Arg [109-111]. Loss of H₄B leads to the phenomenon of “NOS uncoupling” which has been documented in a variety of cardiovascular related diseases [53, 112, 113]. H₄B 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 [50, 51].

As previously stated, H₄B is an essential cofactor for the aromatic amino acid hydroxylases and NOS. The synthesis of H₄B 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 H₄B, tetrhydrobiopterin-4alpha-carbinolamine, is recycled back to H₄B in a two step enzymatic process. First Pterin-4alpha-carbinolamine dehydratase (PCD) reduces tetrhydrobiopterin-4 alpha-carbinolamine to a quinonoid dihydrobiopterin intermediate which is then further reduced by dihydropteridine reductase (DHRP) to H₄B [114, 115]. The recycling pathway has not been shown to represent a critical pathway for production of H₄B in the endothelial cell, nor does it have an effect on eNOS activity [110, 116].
De novo biosynthesis of H₄B is a magnesium, zinc and NADPH dependent pathway. The first step requires the conversion of GTP to 7,8-dihydroneopterin triphosphate. This reaction is catalyzed by the enzyme GTP cyclohydrolase I (GTPCH), and it is the rate limiting step in H₄B biosynthesis [52]. GTPCH can be regulated at both the gene and protein level. Cytokines such as Tumor Necrosis Factor Alpha (TNF-α) and Interferon (IFN-γ) increase GTPCH activity resulting in increased H₄B levels in human endothelial cells [117-119]. Platelet-derived growth factor and angiotensin II (Ang II) have both been demonstrated to increase GTPCH activity by phosphorylation in rat mesangial cells via a phosphokinase C (PKC) dependent pathway. However, this mechanism has not been observed in endothelial cells [120]. Over-expression of GTPCH has been demonstrated to increase the levels of H₄B by ten fold in human endothelial cells [121]. Laminar shear stress also leads to increased GTPCH activity and H₄B production in the vascular endothelium [122]. Additionally, endothelial specific GTPCH transgenic mice have been observed to have a two fold increase in NO synthesis compared to wild type litter mates [123].

GTPCH activity is also regulated by its physical interaction with the GTPCH feedback regulator protein (GFRP). H₄B exerts its inhibitory effects on GTPCH by binding to GFRP[124]. Following exposure to H₂O₂ GFRP mRNA levels have been observed to decrease resulting in increased GTPCH activity and H₄B levels. However, the decrease in GFRP mRNA expression has no effect on NO production [125]. What role if any GFRP plays in regulating eNOS is unknown, however, in a yeast 2-hybrid studies the activator of heat shock protein 90 (Aha1) was recently shown to be a binding partner in the N-terminal region of the GFRP protein [126]. HSP 90 is a known cofactor of the eNOS enzyme, and it’s binding to eNOS results in enhanced enzyme activity (149). Because GFRP binds in a region that is not required for HSP 90
activation it has been proposed that GFRP binding to Aha1 functions to help support local changes in eNOS derived NO generation [126].

Following the GTPCH enzyme reaction, pyruvoyl tetrahydropterin synthanse (PTPS) converts 7,8 dihydroneopterin triphosphate into 6-pyruvoyl-5,6,7,8-tetrahydropterin. In macrophages, induction by cytokines leads to increased GTPCH activity however, the activity of PTPS remains unchanged [127, 128]. Under these conditions PTPS becomes the rate limiting enzyme for H4B 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 [129]. 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, H4B [130]. A mouse SPR KO model has been generated and this model shows impaired synthesis of H4B. To date however, no studies have been done to gather what effect this may have on the vascular endothelial function of these mice [131]. The salvage pathway is another in which H4B can be synthesized. One way in which the salvage pathway works is through the conversion of exogenous sepiapterin. Sepiapterin is metabolized to H2B by sepiapterin reductase and subsequently to H4B by the enzyme dihydrofolate redutase (DHFR). Alternatively, when H4B is oxidized to H2B, DHFR reduces it back to H4B [132]. Recently the role of endothelial DHFR in BAECs as it relates to H4B and NO bioavailability was investigated. As a result of DHFR gene silencing, endothelial NO production and H4B levels in endothelial cell decreased [133]. Additionally, DHFR expression was observed to decrease in BAECs following exposure to H2O2. Following Ang II mediated stimulation of NADPH, increases in eNOS derived O2− were observed. DHFR gene over-expression was able to restore H4B and NO
bioavailability. It also resulted in decreased eNOS derived $\text{O}_2^{-}$ in ANG II treated cells. Overall, this study demonstrates the importance of DHFR in maintaining endothelial $\text{H}_4\text{B}$ and NO bioavailability. Moreover, under conditions of oxidative stress the salvage pathway maybe critical in maintaining endothelial $\text{H}_4\text{B}$ and NO production [133].

**Hsp90**

Hsp 90 is a chaperone protein that is among the most abundant proteins in eukaryotic cells accounting for 1-2 percent of total cytosolic protein [134]. It exists in two isoforms, Hsp90 alpha and HSP90 beta and it is mostly localized to the cytoplasm with a marginal amount found in the nucleus [134]. The role of Hsp90 in the cell is to promote protein folding by preventing protein aggregation of unfolded protein [135, 136]. In addition to promoting protein folding, there is evidence to suggest that Hsp 90 is important for signal transduction in all cell types. In support of this, a variety of signaling proteins including v-Src, Raf-1 and MEK have been shown to interact with Hsp 90 [137-139]

**eNOS-Hsp90**

eNOS was initially shown to interact with a 90 kDa tyrosine phosphorylated protein following bradykinin stimulation in BAECs and this promoted translocation of eNOS to the cytoskeleton [140, 141]. It was later shown that this protein termed endothelial nitric oxide synthase associated protein (ENAP-1) was in fact HSP90 [140, 141]. Hsp90 is recruited for binding to eNOS following VEGF, histamine and fluid shear stress stimulation and enhances the activity of the enzyme [141]. Geldanamycin (GA) is a ansamycin antibiotic that binds to the ATP binding site of Hsp90 preventing the ATP/ADP cycle that is required for protein-protein interactions [142]. In support of Hsp 90 being critical to eNOS activity, it has been shown that GA treatment in isolated mesenteric arteries and rat aortas decreases NO generation [141, 143]. Furthermore, Hsp 90 inhibition by GA has been shown to increase eNOS derived superoxide
production [144]. Recent studies have demonstrated that there is an Hsp90 binding domain present on eNOS. Site directed mutagenesis of this site yields an eNOS mutant that has a weak binding affinity to HSP90 and increased generation of $O_2^-$ [145]. These observations suggest that Hsp90 binding is not only important for enhancing enzyme activity, but that it can also be important in modulating the balance between NO and $O_2$-generation from eNOS.

Calmodulin

In addition to HSP 90, calmodulin has been known to have a positive regulatory effect on eNOS activity [67] and was the first protein known to be involved in eNOS regulation. CaM binding to its binding motif on eNOS displaces the auto-inhibitory loop on eNOS allowing the electrons to flow from the reductase domain to the oxygenase domain [146].

Caveolae

Caveolae are small (50-100 nm) cholesterol rich invaginations located on the surface of the cell membrane. They are found in practically every cell type and are found in copious amounts in VSMC, endothelial cells and adipocytes. The major structural protein of caveolae is caveolin. The caveolin family consists of three protein isoforms, Caveolin-1 (Cav-1), Caveolin-2 (Cav-2) and Caveolin-3 (Cav-3). Cav-1 is expressed in most cell types including adipocytes, endothelial cells and VSMC [147]. Cav-2 is found in the same cell types as Cav-1. In fact Cav-1, and Cav-2 co-localization is required in order for Cav-2 to make caveolae [147, 148]. Furthermore, without Cav-1, Cav-2 is localized to the Golgi complex where it is degraded [148]. Cav-3 expression is limited to muscle tissue and it is found in skeletal, cardiac and smooth muscle cells [149].

Caveolin-1 and eNOS

As previously stated, abundant amounts of Cav-1 are present in the endothelial cell. Numerous studies have demonstrated in vivo and in vitro that Cav-1 is a negative regulator of eNOS activity [150, 151]. eNOS contains a consensus binding sequence for Cav-1 at amino
acids 350-358. In this regard, studies done using a scaffolding peptide corresponding to the consensus sequence have been shown to cause inhibition of enzyme activity [151]. Additionally, it has been demonstrated in cellular studies that over-expression of Cav-1 results in reduced eNOS activity [150]. In further support of its inhibitory effects, in vivo studies using the Cav-1 scaffolding peptide was demonstrated to inhibit endothelial dependent vasorelaxation following Ach stimulation [152]. In contrast, site directed mutagenesis of the Cav-1 consensus sequence inhibits Cav-1 binding and suppression of eNOS activity [151]. Moreover, Cav-1 KO mice exhibit enhanced endothelial dependent vasodilatation in response to acetylcholine stimulation [153, 154].

The inhibitory effects of Cav-1 on eNOS activity can be overcome exogenously with the addition of calmodulin, suggesting a reciprocal relationship between the two proteins [150, 155-157]. In support of this, co-immunoprecipitation experiments demonstrate that in the absence of calcium eNOS remains abound to Cav-1. However, stimulation of cells with calcium ionophore results in reduced formation of the eNOS-Cav-1 complex [150].

**eNOS Posttranslational Modifications**

**Myristoylation and Palmitoylation**

Myristoylation is important for the subcellular targeting of proteins to membranes. eNOS is the only NOS isoform to posses an N-myristoylation consensus sequence [158-162]. Glycine 2 (Gly-2) and serine 6 (ser-6) are the preferred substrate binding sites for N-myristoyltransferase [163]. In this regard, site directed mutagenesis studies have demonstrated that mutation of Gly-2 converts the eNOS membrane bound protein to the cytosolic form [164-166]. However, inhibiting N-myristoylation of eNOS does not effect enzyme activity [166]. eNOS palmitoylation unlike myristoylation is a reversible process. In order for eNOS to be targeted to the plasma membrane myristoylation must precede palmitoylation [167]. Palmitoylation occurs
at the cysteine residues 15 and 26. Mutations at these sites do not affect eNOS activity or protein trafficking to the plasma membrane [167]. The role of palmitoylation is to specifically target eNOS to caveolae. In support of this experiments done using wild type-eNOS and a palmitoylation mutant form of the enzyme, showed that the wild type enzyme colocalized with caveolin while the mutant form did not [69, 168]. Therefore, it appears that the first step in eNOS protein localization to the plasma membrane requires myristoylation, which is then followed by palmitoylation, which stabilizes the enzyme and targets it to the caveolae.

**eNOS Phosphorylation**

Phosphorylation of eNOS typically occurs at serine (Ser) residues, and less frequently at tyrosine (Tyr) and threonine (Thr) residues. Currently five sites on eNOS have been identified as targets for protein phosphorylation, Ser 1177 (human)/Ser 1179 (bovine), Ser 633 (H)/Ser 635 (B), Ser 615(H)/Ser 617 (B), Thr 495 (H)/Thr 497 (B), and Ser 114 (H)/Ser 116 (B).

**Ser 1177/1179**

The phosphatidylinositol 3-kinase (PI3K) pathway was first shown to be involved in eNOS phosphorylation when it was demonstrated that VEGF or insulin stimulated release of NO was attenuated by the pharmacological inhibitors of PI3K wortmannin and LY298004 [169, 170]. The protein kinase Akt is known to be activated by PI3K and to target phosphorylation sites with the particular consensus sequence of RXRXXXS/T which have been identified on eNOS [171]. Two groups independently demonstrated that Akt could directly phosphorylate eNOS at Ser1179 resulting in its activation [172, 173]. Various stimuli including shear stress, bradykinin, VEGF and insulin activate Ser 1179 phosphorylation. It has been hypothesized that the activation of eNOS by Ser 1179 phosphorylation causes a conformational change in the enzyme similar to the effects caused by calmodulin binding [174]. Because of the wide variety of stimulators that can activate Ser 1179, it appears that it is the most important site in the regulation of eNOS activity.
In support of these observations, it has been demonstrated that mutating the Ser 1179 site to an alanine thus preventing phosphorylation, leads to a reduction in basal and stimulated release of NO [175]. Additionally, mutating the Ser 1179 site to aspartate to mimic the negative charge of phosphorylation, results in increased eNOS activity when stimulated with low levels of calcium [172]. Furthermore, it has been demonstrated that adenoviral mediated over-expression of Akt in rabbit femoral arteries resulted in increased resting diameter of the artery [176]. Moreover, the Hmg CoA reductase inhibitor simvastatin has been shown to activate Akt leading to increased eNOS phosphorylation at Ser 1179 [177].

**Thr 495/497**

Phosphorylation of eNOS does not only result in activation of the enzyme, as evident by the inhibitory effects of phosphorylation of Thr 495. The phosphorylation of Thr 495 is mediated through the PKC pathway [178-181]. The site of Thr 495 phosphorylation is located within the Ca^{2+}/CaM binding domain, and it appears that this interferes with the binding of Ca^{2+}/CaM to eNOS [179, 180]. The basal level of Thr 495 phosphorylation is high in cultured endothelial cells [179-181]. Various agonists of eNOS such as bradykinin, VEGF and calcium ionophore have been shown to cause dephosphorylation of Thr 495 [180, 182, 183]. Also, it has been suggested that in order for eNOS activation to occur Thr 495 dephosphorylation must precede Ser 1179 phosphorylation [180-184].

**Ser 633/635**

Phosphorylation at the Ser 633 site also enhances the activity of eNOS. The phosphorylation site is located within the CaM autoinhibitory sequence of eNOS contained within the FMN binding domain [181]. There have been several studies to suggest that Protein Kinase A [74] phosphorylates Ser 633 [181, 182, 185, 186]. The same agonists that lead to the activation of NO via Ser 1177 phosphorylation also stimulate Ser 633 phosphorylation. The rate
of phosphorylation of Ser 633 is much slower than that of Ser 1177 after agonist stimulation. Additionally, phosphorylation of Ser 633 by PKA in endothelial cells can increase NO production without requiring increased intracellular Ca\(^{2+}\) levels [186].

**Ser 615/617**

Ser 617 phosphorylation also occurs in the CaM autoinhibitory sequence of the CaM binding domain, however there is controversy over its function [181]. Various eNOS agonists similar to the ones that trigger enzyme Ser 633 and Ser 1179 phosphorylation also increase phosphorylation at the Ser 617 site [175, 181, 182]. One study showed that mimicking the phosphorylation of Ser 617 with a serine to aspartate mutation increases Ca\(^{2+}\)/CaM sensitivity of eNOS, but not the overall activity of the enzyme [181]. In contrast, another study demonstrated that phosphorylation at Ser 615 does increase eNOS activity. However, it was observed in the same study that the serine to alanine mutation mimicking dephosphorylation also increased enzyme activity [175]. Moreover, the dephosphorylation of Ser 615, led to increased recruitment of Hsp 90 and Akt both which are known to activate eNOS [175]. These observations suggest that the role of Ser 615 phosphorylation is to facilitate eNOS interaction with other proteins and regulate phosphorylation at other sites.

**Ser 114/116**

The final identified site of eNOS phosphorylation occurs at Ser 116 and it is the only known phosphorylation site in the oxygenase domain of eNOS. Currently, its role in eNOS activity much like Ser 615 phosphorylation is controversial. eNOS activation due to VEGF is associated with Ser 116 dephosphorylation [187]. Laminar shear stress and HDL exposure on the other hand have been reported to cause phosphorylation of Ser 116 leading to eNOS activation [188, 189]. Reports on Ser 114 to alanine mutations mimicking dephosphorylation also conflict, with one study showing increased activity and the other reporting no change in
activity, but increased NO release [175, 187]. These conflicting results suggest that further studies need to be done to elucidate the role of Ser114/116 phosphorylation.

Overall the regulation of eNOS has developed into this complex story that involves protein-protein interactions, substrate ability and protein posttranslational modifications. This tight regulation is necessary to maintain vascular homeostasis. However, loss of this regulation has been implicated in the pathology of many diseases that eventually lead to vascular endothelial dysfunction, CAD, myocardial infarctions, heart failure and even death.

**Pathophysiology**

As previously stated, eNOS and its product NO are important for maintaining vascular homeostasis. Given its significance it is important that a healthy environment is maintained within the endothelium. However, it is known that in a variety of conditions such as diabetes, chronic smoking, hypertension, and hypercholesterolemia the endothelium environment loses its anti-atherogenic, anti-proliferative, and anti-thrombotic properties. Vascular endothelial dysfunction is the common link seen in the pathology of all of these diseases and it is the underlying cause to the more serious vascular disease, atherosclerosis. The exact mechanism as to how endothelial dysfunction is caused is not known. However, there is growing evidence that oxidative stress which subsequently leads to the loss of NO plays a significant role. Although oxidative stress can be caused by a variety of ROS generating enzymes, studies have implicated NAPDH oxidase as the main source of ROS in vascular diseases. The increase in ROS generated from NADPH oxidase has also been implicated in playing a role in NOS uncoupling by causing the oxidation of the essential NOS cofactor H₄B. The depletion of H₄B results in decreased NO bioavailability and increased eNOS derived superoxide. This loss of NO bioavailability and the increase in superoxide production results in an endothelium that is no longer able to maintain homeostasis. Moreover, this change in vascular homeostasis results in
impaired vascular relaxation and increased vascular damage eventually leading to vascular remolding, which are all characteristic signs of endothelial dysfunction.

**Pathways Leading to Oxidative Stress Generation**

**NADPH Oxidase**

The NADPH Oxidase (NOX) isoforms NOX-2 and NOX-4 are both found to be highly expressed in the endothelial cell. NOX-2 requires the translocation of many regulatory subunits to the cytosol to become active, those subunits include p22phox, p47phox, Rac, p67phox and p40phox [190]. Upon assembly of the complex, electrons from NADPH are transferred to molecular oxygen to form $\text{O}_2^-$. NOX-4 expression on the other hand is greater than NOX-2 in the endothelial cell. It also appears that NOX-4 is a constitutively active enzyme [191]. Furthermore, it does not require any of the cytosolic subunits that are required for NOX-2 activation [192]. Though ROS can be generated from other superoxide generating enzymes, NOX has emerged as the main culprit, because its activity can be stimulated by many of the substrates involved in vascular endothelial dysfunction such as oxLDL, ANG II, and TNF alpha. In the endothelial cell a key event leading to NOX-2 activation is the phosphorylation of p47phox [193]. The phosphorylation of this subunit has been shown to occur in the response to ANG II, TNF alpha and VEGF [193-195].

In arteries of atherosclerotic patients NOX-2 and NOX-4 expression is increased particularly in the shoulder region of the plaque. The increased expression of these isoforms may also contribute to plaque erosion [196]. Furthermore, there is evidence to support that there is a local increase in the renin angiotensin system in the tissue periphery associated with hypercholesterolemia, as increased concentrations of Ang II are also observed the shoulder region of plaques [197, 198]. Moreover, the expression of the angiotensin type I receptor is increased in the platelets of hypercholesterolemic patients [199].
In addition to being a producer of O$_2^-$, NOX derived O$_2^-$ can also quench eNOS derived NO, resulting in the formation of OONO$^-$. OONO$^-$ can subsequently oxidize lipoproteins in the vasculature, which become trapped in the endothelium, leading to endothelial activation. This activation causes an increase in expression of atherogenic proteins such as VCAM-1, P and E selectin, and chemoattactants thus continuing the cycle of vascular injury and repair resulting in atherosclerosis [200]. Additionally OONO$^-$ has been demonstrated to cause eNOS uncoupling by directly oxidizing the NOS cofactor H$_4$B [201].

**eNOS Uncoupling**

eNOS uncoupling was first shown to occur by two independent groups using purified eNOS. Both groups demonstrated that eNOS depleted of H$_4$B could catalyze O$_2^-$ formation primarily from the oxygenase domain [50, 51]. Furthermore, the first evidence of eNOS uncoupling in-vivo was generated with the desoxycorticosterone acetate (DOCA) salt induced model of hypertension demonstrating that vascular superoxide production was increased, which could be attenuated by the NOS inhibitor L-NAME [202]. Besides hypertension there is evidence to support a role for eNOS uncoupling to occur in the pathology of diabetes, and hypercholesterolemia.

Studies carried out in endothelial cells derived from diabetic mice provided early evidence for altered H$_4$B metabolism and NO production in diabetes. Despite having normal eNOS protein levels, cells derived from the diabetic mice had decreased NO production and H$_4$B levels. Moreover, supplementation with the H$_4$B precursor was able to reverse these effects [203]. Additional studies carried out in human aortic endothelial cells (HAECs) demonstrated that following 48 hours of exposure to high glucose media, eNOS expression was increased while H$_4$B levels were decreased and eNOS derived O$_2^-$ was increased. Adenoviral mediated over-expression of GTPCH I was able restore NO and H$_4$B levels and suppress O$_2^-$ generation [121].
In vivo studies have also provided further evidence that diabetes can result in altered H₄B levels and increased O₂⁻ generation. In this regard, endothelium specific GTPCH transgenic mice have been generated. These mice have been observed to have increased H₄B levels in vascular tissue [49]. To evaluate the effect of H₄B bioavailability, diabetes was induced using the streptozotocin experimental model. The vasculature of both control and diabetic mice exhibited increased oxidative stress. While H₄B levels were undetectable in control diabetic mice, the GTPCH tg diabetic mice maintained modest H₄B levels. Moreover, these mice exhibited decreased eNOS dependent O₂⁻ generation in the vascular endothelium and increased endothelium dependent vasorelaxtion in response to acetylcholine (Ach) [204]. Finally in patients with Type II diabetes, H₄B infusion has been shown to reverse vascular endothelial dysfunction via a NO-dependent mechanism [205]. In addition to being observed in both in-vivo and in-vitro models of diabetes, several studies have also demonstrated that eNOS uncoupling occurs in atherosclerosis.

The pathology of hypercholesterolemia is associated with impaired vascular function, atherosclerosis, decreased H₄B bioavailability and increased O₂⁻ generation. In this regard, the hypercholesterolaemic ApoE KO mice exhibit impaired vascular relaxation and increased vascular superoxide production. Both of which can be attenuated by oral H₄B supplementation [53]. Furthermore, when ApoE KO mice are crossed with GTPCHtg mice, these mice were observed to have a improved vascular relaxation response to Ach. Additionally these mice had increased H₄B levels and decreased O₂⁻ generation in the vascular endothelium [206]. Moreover, when eNOS tg mice are crossed with ApoE KO mice, the progression of atherosclerosis is accelerated and these mice also have increased O₂⁻ generation, which is improved following H₄B supplementation [207]. In addition to the animal studies,
administration of H4B in patients with hypercholesterolemia has been shown to improve vascular endothelial dysfunction [103].

The ratio between H4B and H2B is another important trigger for eNOS uncoupling. Recently, it has been demonstrated that H4B and H2B can bind eNOS with equal affinity. Additionally, intracellular levels of H2B increased 40% after 48 hours of high glucose treatment and this was associated with reduced NO activation and increased eNOS dependent O2\textsuperscript{-} production. [208]. Over all these studies suggest that it is not only important to maintain the levels of H4B, but the ratio of H4B/ H2B may also be important in maintaing NO production.

Over all the studies presented in the section demostrate that oxidative stress plays a significant role in vascular endothelial dysfunction. However, increasing evidence also supports the role for the endogenous NOS inhibitors the methylarginines ADMA and L-NMMA in the pathophysiology of endothelial dysfunction.

**DDAH ADMA Pathway**

The endogenous NOS inhibitor AMDA has been demonstrated to be an independent cardiovascular disease risk factor. However, the mechanisms regarding how ADMA levels are modulated and what role they play in disease progression are not clearly understood. Therefore, ADMA accumulation in response to decreased DDAH expression/activity has been proposed to be involved in the vascular pathophysiology observed in a variety of cardiovascular disease. The following section will describe the production and function of the methylarginines. Futhermore, the significance of the methylarginine metabolizing enzyme DDAH will be described. Finally, this section will end with studies describing the pathophysiology associated with increased levels of methylarginines and decreased DDAH activity.
**PRMT**

The methylation of protein arginine residues is carried out by a group of enzymes referred to as protein-arginine methyl transferase’s (PRMT’s). To date, nine different isoforms of the enzyme have been identified with each subtype exhibiting various levels of activity, substrate specificity and tissue distribution. During PRMT catalysis S-adenosylmethionine serves as its substrate (SAM) and is then subsequently converted to S-adenosylhomocysteine (SAH), which is then enzymatically converted to homocysteine, which is either further metabolized, or remethylated [5]. PRMT’s are separated into two classes depending on what type of methylarginine they generate. In mammalian cells, these enzymes have been classified into type I (PRMT1, 3, 4, 6, and 8) and type II (PRMT5, 7, and FBXO11) enzymes, depending on their specific catalytic activity. Both types of PRMT, however, catalyze the formation of monomethylarginine (MMA) from L-Arg. In a second step, type I PRMT’s produce asymmetric dimethylarginine (ADMA), while type II PRMT catalyzes symmetric dimethylarginine (SDMA) [209]. Arginine methylation by both type of PRMT’s enzyme occurs mostly in the arginine-glycine rich sequences of proteins [210, 211]. PRMT 1 is a member of the type I class of PRMT’s and it specifically catalyzes the formation of L-NMMA and ADMA [212]. The PRMT 1 enzyme is mostly expressed in the heart and testis [213]. Intracellularly, PRMT 1 is expressed predominantly in the nucleus with partial expression in the cytoplasm [214]. During development the expression of PRMT1 is essential, as PRMT1 KO mice have been observed to be embryonically lethal [215]. Until recently protein arginine methylation was thought to be irreversible. Recently, the Jumonji domain-containing protein 6 (JMJD6) has been identified as a histone arginine demethylase, whether or not this has implications for intracellular protein arginine methylation is unknown [216]. The relationship between PRMT1 activity, expression and ADMA synthesis has been demonstrated in several studies. Specifically, it has been
observed in HAEC’s following 24 hour incubation with either LDL or OxLDL within the pathological range of 200-300 mg/dl that PRMT 1 mRNA expression increases 1.5-2.5 fold. Furthermore, ADMA released into the media increased 2 fold following the 24 hour incubation period. The increase in ADMA could be attenuated in the presence of the PRMT inhibitor SAH [217]. In human umbilical vein endothelial cells (HUVECs) exposure to shear stress has been demonstrated to increase gene expression of PRMT-1. Furthermore, low levels of shear stress (5-15 dynes/cm²) increases ADMA release from HUVEC cells after 3-6 hours of exposure. In contrast, high shear stress (25 dynes/cm²) does not result in increased release of ADMA. [218].

**Methylarginine Biochemistry**

The free methylarginines ADMA, L-NMMA, and SDMA are all transported through the y⁺ CAT transport system. However, only ADMA and L-NMMA competitively compete with L-Arg for binding to eNOS, resulting in its inhibition. Inhibition of eNOS activity by the methylarginines is reversible, but only under conditions in which excess L-Arg is added. In support of the role of methylarginines in eNOS inhibition, several studies have reported that L-Arg supplementation enhances endothelium dependent relaxation through increased NO generation. However, considering that the intracellular concentrations of L-Arg is 50 times higher than the Kₘ for eNOS, increased NO generation would not be expected with L-Arg supplementation; this phenomenon has been termed the “L-Arg paradox” [38]. Therefore, it is hypothesized that L-Arg supplementation overcomes the endogenous inhibitory actions of cellular methylarginines ADMA and NMMA [42]. However, whether or not these endogenous methylarginines are present at concentrations sufficient to regulate eNOS is unclear. In this regard, it has been reported that plasma levels of ADMA and L-NMMA are in the range of 0.5-1µM in healthy individuals [219]. We have demonstrated in studies from our lab that the basal endothelial cells level of ADMA and L-NMMA were 3.6 µM and 2.9 µM respectively.
Furthermore, our kinetic studies using purified eNOS demonstrated that the $K_i$ for ADMA and L-NMMA were 0.9µM and 1.1µM respectively [38]. Therefore, it is expected that under normal physiological conditions that methylarginines would not have a significant affect on endothelial NO production. In support of this, it has been demonstrated that at low concentrations of methylarginines modest inhibition of NO production is observed. In isolated human blood vessels, 1 µM of L-NMMA leads to inhibition of bradykinin induced vasodilatation by 20% [220]. Similar reports from a study using plasma from end stage renal patients have shown that plasma levels of ADMA of 2µM, can have a significant inhibitory effect on endothelial NO production [221]. Additionally, in the circulation of the guinea-pig 10 µM ADMA was observed to increase blood pressure by 15% [222]. Although, studies have demonstrated modest inhibition of eNOS at physiological concentrations of methylarginines, there have been several reports of increased methylarginine levels in various disease states including hypercholesterolemia, diabetes and end stage renal disease. 

In the disease state plasma methylarginine levels have been reported to increase 3 to 9 fold [38]. It remains unclear whether or not increases in methylarginine levels will result in significant inhibition of endothelial NO production. Recently, we have addressed this question in cellular studies in an effort to determine the dose dependent effects of the methylarginines on endothelial NO production. Previous studies suggest that compartmentalization of eNOS or L-Arg may occur in the endothelial cell, limiting the ability of L-Arg to overcome the inhibition of methylarginines on eNOS activity. Therefore, cellular studies were carried out in order to determine the effective concentration of cellular methylarginines necessary to cause eNOS inhibition in BAECs. Our results demonstrated that ADMA dose dependently inhibited eNOS derived NO generation as 5 µM and 100 µM ADMA elicited a 38% and 74% inhibition,
respectively. Similar results were obtained with L-NMMA, as 42% and 81% inhibition was seen with 5 µM and 100 µM L-NMMA respectively. In the presence of L-Arg these effects were less prominent. ADMA dose dependently inhibited eNOS derived NO 24% at 10 µM ADMA and 52% at 100 µM. Similar results were obtained for L-NMMA with 17% inhibition observed at 10 µM L-NMMA and 63% at 100 µM. These results were surprising to us because based on kinetic studies we did not expect to see such robust inhibition of endothelial NO production. This led us to speculate that endothelial cells are able to concentrate methylarginines. Therefore, cellular uptake studies were performed. Our results demonstrated that in the absence of physiological levels of L-Arg, 10 µM of exogenous ADMA resulted in intracellular ADMA concentration of 68.4 µM. When this same experiment was repeated in the presence of L-Arg (100 µM), 10 µM ADMA resulted in a markedly lower intracellular ADMA concentration of 23.5 µM [38]. Additional studies were also performed with L-NMMA. Intracellular concentrations of L-NMMA sometimes reach as much as 7 times higher than outside the cell. Moreover, L-NMMA (10 µM) uptake was only inhibited by 65% in the presence of L-Arg (100 µM). As previously mentioned the methylarginines along with L-Arg are all transported through the y+ transporter. Therefore our results would indicate that even in the presence of L-Arg, elevated plasma levels of methylarginines would result in increased uptake through the y+ transporter resulting in even higher intracellular levels. Moreover, this increased uptake through the y+ transporter represents a novel mechanism by which methylarginines can modulate eNOS activity and endothelial NO production.

Overall our studies suggest that under pathological conditions such as hypercholesterolemia, and diabetes where methylarginine levels are increased, methylarginines can modulate eNOS activity. Because NO is known to possess anti-proliferative and anti-
atherogenic properties, methylarginine accumulation could play a significant role in development of atherosclerosis.

**Metabolism of Methylarginines**

Initially it was believed that after proteolysis, free methylated arginine residues were released and excreted through the kidney [224]. On the contrary, subsequent studies into methylarginine metabolism in rabbits demonstrated that the urinary excretion of SDMA was 30 times greater than ADMA and L-NMMA excretion. This led to the assumption that ADMA and L-NMMA were being metabolized through alternate pathways [224]. These early studies led to further investigations into the metabolic fate of C¹⁴ labeled ADMA and SDMA. Sasaoka et al. demonstrated that while both dimethylarginines could be metabolized by the Dimethylarginine: pyruvate Aminotransferase pathway, there existed a specific pathway for ADMA metabolism. In support of this they found that the radioactivity that remained in the tissue of rats injected with C¹⁴ ADMA consisted mainly of citrulline, in complete contrast to rats injected with C¹⁴ SDMA [225].

After the identification of this alternative pathway, DDAH was identified as the metabolizing enzyme of ADMA and was purified from the rat kidney [226]. It was demonstrated that DDAH specifically hydrolyzed ADMA and L-NMMA to citrulline, and mono and dimethylamine. Until recently, DDAH enzyme activity studies have only been performed on bacterial sources and tissue homogenates from either rat kidney or porcine brain. Those studies reported that DDAH hydrolyzes ADMA at a faster rate than L-NNMA with reported Km values of 0.18 and 0.36 mM respectively and that it is responsible for >90% of ADMA metabolism [224, 225, 227]. We have recently purified the human isoform of DDAH-1 (hDDAH-1) and in contrast to previous studies we observed that hDDAH-1 hydrolyzes ADMA and L-NNMA at similar rates 68.7 µM and 53.6 µM respectively [228]. Furthermore, we observed that hDDAH-
1 is maximally active at pH 8.5, contrasting earlier reports that enzyme maximum activity at pH 5.2 to 6.5 [225, 229]. DDAH-1 contains a Zinc (II) binding site, with endogenous bound Zinc(II) inhibiting its catalytic activity [230]. Birdsey et al. and Murray-Rust et al. were the first to demonstrate that ADMA and not SDMA could be metabolized intracellularly [231, 232]. Additional studies by Murray-Rust et al., demonstrated that steric hindrance caused by the methyl groups on both nitrogens of SDMA prevents its binding to the active site of DDAH, therefore it is unable to hydrolyze it [232].

In observance that DDAH expression did not correlate to activity, Leiper et al. discovered a second isoform of DDAH, DDAH-2. DDAH-2 has a 63% homology to hDDAH-1. Currently there are no studies on the enzymatic activity of human DDAH-2, as the only study that has been done uses recombinant bacterial lysates that express DDAH-2. Enzyme activity from bacterial lysates demonstrated that DDAH-2 hydrolyzed L-NMMA at the comparable rates to reported DDAH-1 in bacterial lysates [36].

DDAH-1 and 2 are predominantly located in the cytoplasm, DDAH-1 has also been found in membrane fractions of endothelial cell lysates [231]. DDAH-1 is predominately expressed in the liver and kidney which are major sites of ADMA metabolism [233, 234]. It is also expressed strongly in the aorta and equally in adult and fetal tissues [235, 236]. DDAH-2 expression is predominant in fetal tissues. However, expression decreases and becomes more tissue specific in adults with DDAH-2 expression predominately in the vascular endothelium, kidney, heart, and placenta [36]. DDAH-1 while it is also expressed in the endothelium, studies of mesenteric resistance arteries demonstrate that DDAH-2 mRNA expression is 5.1 fold greater than that of DDAH-1 suggesting an important role for DDAH-2 in the resistance vessels [237].
**In Vivo and In Vitro Significance of DDAH**

The first functional studies of DDAH-1 were done using the inhibitor S-2-amino-4 (3-methylguanidino) butanoic acid (4124W). Treatment with 4124W in cultured human endothelial cells led to accumulation of ADMA in the supernatant thus demonstrating that the role of DDAH-1 was to prevent the accumulation of ADMA. Ex-vivo studies using rat aortic rings demonstrated that inhibition of DDAH-1 by 4124W caused vasoconstriction. However, this effect was reversed in the presence of L-Arg. Additional studies done on human saphenous veins demonstrated that inhibition of DDAH-1, led to the loss of the bradykinin mediated relaxation response [238]. These studies were among the first to demonstrate that DDAH could be important in the regulation of endothelial NOS activity and vascular function. Recently, the in-vivo significance of DDAH-1 has been described by two independent groups using both transgenic and knockout mice [239, 240].

Dayoub et al. described the effects of DDAH-1 over-expression in-vitro and in-vivo, with the creation of DDAH-1 transgenic mouse model. Cellular studies performed in human microvascular endothelial cells and murine endothelial cells demonstrated that over-expression of DDAH-1 yields a 2-fold increase in NO activity and Nitrogen Oxides (NOx) released into the culture media. The in-vivo studies demonstrate that DDAH-1 tg mice have increased NOS activity in the heart and skeletal muscle however, no change was seen in the aorta. DDAH-1 tg mice also have decreased mean arterial blood pressure (MAP). The systemic vascular resistance (SVR) and cardiac contractility are also decreased in response to an increase in NO production. Furthermore, it was observed in DDAH-1 tg mice, that urinary excretion of NOx was increased 2 fold, and this corresponded to a 2-fold drop in plasma ADMA levels [239]. Additional studies done by Jacobi et al. demonstrated that DDAH-1 tg mice exhibit enhanced angioadapatation in response to hind limb ischemia [241]. Subsequent studies by Tanaka and Sydow et al.,

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demonstrated in a cardiac transplantation model that DDAH-1 tg mice exhibit suppressed immune responses as result of increased cardiac NO generation and decreased superoxide production. Also, these mice exhibited less graft coronary artery disease, and improved function of the allograft [242].

In more recent studies, Lieper et al have demonstrated the in-vivo effects of DDAH-1 gene deletion in mice. The first significant finding of this study was that homozygous deletion of the DDAH-1 gene was embryonically lethal. Demonstrating that DDAH-1 is essential to normal embryonic development. Therefore, subsequent studies were performed with DDAH 1+/- mice. The DDAH-1 +/- mice exhibit increased plasma levels of ADMA, indicative of DDAH’s role in regulating ADMA levels. DDAH-2 expression was not altered by the drop in DDAH-1 expression. Tissue DDAH activity in the kidney, lung, and liver was decreased by approximately 50% suggesting that DDAH-2 is not the principle methylarginine metabolizing enzyme in these tissues. Additionally, it was observed that these mice exhibited impaired vascular relaxation in response to Ach treatment. Moreover, hemodynamic studies reveal that mean arterial blood pressure (MAP), systemic vascular resistance (SVR) and right ventricular pressure are all increased in the DDAH 1 +/- mice [240].

Hasegawa et al recently created a transgenic mouse over-expressing the DDAH-2 gene. They have reported that DDAH-2 tg mice have reduced plasma ADMA levels and an elevation in cardiac NO levels. However, in contrast to DDAH-1 mice, there was no change in systemic blood pressure. The difference seen in two models is likely to be due to the fact that plasma ADMA levels are vastly different in these two mice. In the DDAH-1tg ADMA plasma levels decreased by 60%, whereas DDAH-2 tg mice plasma ADMA levels were only reduced by 26%. This further provides evidence that DDAH-1 is the principle methylarginine metabolizing
enzyme. The expression of DDAH-2 was significantly increased in the heart, skeletal muscle and brown adipose tissue. They also reported that DDAH-2 over-expression did not alter the expression of DDAH-1. Furthermore, ADMA induced vascular lesions were attenuated in the DDAH-2tg mice, which they attributed to decrease in angiotensin converting enzyme expression [243]. ANG II infusion over a two week period induced increased medial thickening and perivascular fibrosis in coronary microvessels of WT mice, however this response was attenuated in DDAH-2 tg mice [244].

Studies done by Wang et al. reported that in-vivo DDAH-2 gene silencing in rat mesenteric arteries caused almost complete inhibition of the NO response to Ach in vascular reactivity studies. They also reported DDAH-1 gene silencing increased ADMA, however it had no effect on vascular relaxation in response to Ach [237]. As demonstrated in the previous study by Hasegawa et al., this study provides more evidence that DDAH-1 is the predominate metabolizing enzyme of ADMA. In addition, this study provides some evidence that DDAH-2 regulates endothelial NO production independent of ADMA, because ADMA levels did not rise following DDAH-2 gene silencing.

**ADMA Independent Mechanisms of DDAH**

Wang et al. reported that DDAH-2 gene silencing in mesenteric resistance vessels lead to a significant down-regulation of eNOS mRNA and protein expression [237]. Smith et al. observed that DDAH-2 over-expression in HUVEC cells lead to a 2-fold increase in VEGF mRNA expression [245]. Later it was reported by Hasegawa et al. that DDAH-2 over-expression in BAECs increased transcriptional activation of VEGF, without increasing NO generation. In this study they observed that DDAH-2 mediated its effects by directly binding PKA leading to the phosphorylation of the transcription factor specificity protein 1 (Sp1). Sp1 translocates to the nucleus and binds the promoter region of VEGF activating its transcription. They also
demonstrated that the DDAH-2 effect on VEGF transcription is blocked by gene silencing of Sp1 [246]. Tokuo et al. reported that DDAH-1 in a similar fashion, binds to neurofibromin 1 (NF-1) in a region coinciding specific sites of PKA phosphorylation. DDAH-1 binding to NF-1 increases NF-1 phosphorylation by PKA. Overall these studies demonstrate that DDAH can mediate its effects independent of ADMA [247].

**Regulation of DDAH Activity**

Given the importance that DDAH plays in maintaining NO levels in the vascular endothelium, extensive research efforts have been undertaken to study its regulation. Leiper et al. were the first to report that NO could inactivate DDAH-1 by SNO of a cysteine (Cys) residue. Cys 249 is located within the active site of the DDAH-1 enzyme. It was observed in this study using recombinant bacterial protein expressing DDAH-1 that SNO occurs at the Cys 249 residue, rendering the enzyme inactive [88]. Additional studies done using mouse endothelial cells over-expressing DDAH-2 demonstrated that cytokine mediated induction of iNOS resulted in the SNO of DDAH-2 [88]. Thus, under conditions of enhanced immune response which leads to iNOS induction, inhibiting DDAH activity would be beneficial; because of the accumulation of ADMA which would be expected to inhibit iNOS derived NO. Knipp et al. observed that DDAH-1 in its native form, Zn (II) bound, is resistant to SNO and it is the zinc depleted form that is susceptible to [248]. It has also been suggested that DDAH activity maybe sensitive to oxidative stress. However, studies from our lab and others have shown that DDAH is largely resistance to oxidative species at pathophysiological levels [228, 249].

Studies by Scalera et al. demonstrated that the anti-hypertensive drug, Telmisartan, can positively regulate DDAH. Although Telmisartan is known to function as an ANG II type 1 receptor blocker, it has also been found to activate PPAR γ. PPAR γ signaling is associated with increased NO formation. In this study they observed that in the presence of Telmisartan, DDAH
activity increased and DDAH-2 expression also increased. However, PPARγ inactivation either pharmacologically or by gene silencing mitigated the effects on DDAH activity and expression [250]. Yin et al. reported that pravastatin a cholesterol lowering drug, restores DDAH activity and endothelium relaxation in the rat aorta following exposure to glycated bovine serum albumin (AGE-BSA) [251]. Achan et al. reported that all-trans-retinoic acid could transcriptionally regulate DDAH II, increasing its mRNA expression in HUVECs [252]. Additional studies by Jones et al. demonstrated that there are six single nucleotide polymorphisms (SNP) in the promoter region of the DDAH-2 gene [253]. Furthermore, they observed that the 6G/7G insertion/deletion SNP at position -871 in the promoter region of the DDAH-2 gene, resulted in enhanced promoter activity [253]. Valkonen et al. observed in the Kuopio Ischemic Risk Factor Study that 13 male patients were carriers of a of DDAH-1 gene varient that put them at 50 times greater risk for cardiovascular disease [254].

Overall these studies suggest that ADMA and DDAH may play a role in endothelial dysfunction. The next section will help to provide further evidence as to the exact role of ADMA and DDAH in the disease state.

**Pathophysiology**

Clinical syndromes involving defective NO productions underscore the importance of eNOS and NO in the maintenance of normal vascular function. Although it is well established that NO is a critical effector molecule in the maintenance of vascular tone, NO also maintains the non-atherogenic character of the normal vessel wall. Several studies have linked ADMA and L-NMMA as key players in endothelial dysfunction.

Epidemiological studies have demonstrated a strong correlation between plasma ADMA and incidence of cardiovascular disease. Initial studies by Boger et al. demonstrated that the plasma ADMA levels of young hypercholesterolemic individuals were double that of
normocholesterolemic patients [255]. The increase in ADMA in the hypercholesterolemic patients resulted in impaired endothelium dependent response and reduced nitrate urinary excretion. The effects on nitrate urinary excretion and vascular function, improved following L-Arg supplementation [255]. Subsequent studies by Zoccali et al. were the first to establish ADMA as an independent risk factor for cardiovascular disease in patients with chronic kidney disease [256]. Lu et al. observed in patients following angioplasty that ADMA was the sole predictor of future cardiovascular events [257]. Studies by Valkonen et al. demonstrated that in healthy non smoking men, those in the highest quartile of ADMA plasma levels, had a 3.9 fold increase in risk of acute coronary events [254]. Studies by Abbasi et al. reported that type II diabetic patients, have increased ADMA plasma levels in comparison to healthy individuals. Additional studies of obese woman found that women who are insulin resistant and obese have higher plasma ADMA levels and that ADMA levels decreased following weight loss [258]. In support of these epidemiological studies, in vitro and in vivo data has shown similar effects of ADMA on endothelial function in several disease states.

Chan et al. demonstrated that blood monocytes from hypercholesterolemic individuals adhered to human endothelial cells in culture greater than normocholesterolemic [259]. This increase in adhesion of monocytes is one of the initial steps in the progression of endothelial dysfunction and atherosclerosis. Furthermore, they observed that the adhesion was related to the L-Arg/ADMA ratio. In support of this, monocytoid cells were co-cultured with BAECs exposed to the corresponding L-Arg/ADMA ratios of the hypercholesterolemic patients. It was observed that the adhesion of monocytoid cells increased in a dose dependent manner. Following the initial adhesion studies patients were placed on 12-weeks of L-Arg supplementation which resulted in the normalization of monocyte adhesion [259]. Studies by Azuma et al. demonstrated
that ADMA and L-NMMA levels were increased in regenerated endothelial cells following balloon angioplasty of the rabbit carotid artery. Furthermore, L-Arg levels were significantly depleted in regenerated endothelial cells which resulted in increased neointima formation. The accumulation of ADMA and L-NMMA in these regenerated cells led to decreased endothelium dependent relaxation, which was attenuated with L-Arg supplementation [260]. Overall these studies suggest that the L-Arg/ADMA ratio is an important predictor of endothelial dysfunction.

To add physiological relevance to our biochemistry studies discussed earlier, we wanted to examine whether or not methylarginines inhibition on eNOS could modulate a physiological response. Therefore changes in vascular reactivity in rat carotid rings under varying concentrations of ADMA (1-500 µM) were observed. ADMA dose dependently inhibited the Ach mediated relaxation response with a 52% reduction seen at 5 µM ADMA and a 95% reduction at 500 µM, in the absence of L-Arg. In the presence of L-Arg 10 µM ADMA inhibited the Ach mediated relaxation response by 7%, and an 84% reduction was seen at 500 µM. Because our studies in vitro and ex vivo demonstrated that ADMA could inhibit eNOS, it was unclear if this could occur in-vivo. Using the balloon model of carotid injury we observed that intracellular methylarginine levels increased 4 fold and resulted in a 50% loss of vasculature relaxation response. Overall these results demonstrated that intracellular methylarginine levels are elevated in pathological conditions and that the levels reach high enough to inhibit endothelial NOS activity and vascular function. In addition to increased plasma ADMA levels, dysfunction of the DDAH enzyme and its ADMA independent effects on NO have become a potential mechanism by which endothelial dysfunction can occur.

Ito et al. demonstrate in HUVEC cells that following 48 hours of exposure to either oxLDL or TNFalpha the activity of DDAH decreased but expression remained unchanged [261].
Furthermore, they observed in-vivo that New Zealand White rabbits fed on high-cholesterol diet had significantly reduced aortic, renal, and hepatic DDAH activity [261]. These studies were the first to demonstrate that DDAH activity could be modulated under pathological conditions.

In transplant patients there is an increased incidence of transplant atherosclerosis as a result of the cytomegalovirus (CMV), which is known to promote atherogenesis [262]. Weis et al. demonstrated that human microvascular cells infected with CMV, resulted in increased ADMA and decreased cellular DDAH activity [263]. Therefore, these studies demonstrate that CMV infection contributes to endothelial dysfunction and transplant atherosclerosis that is observed in heart transplant patients by modulating the DDAH-ADMA pathway.

Although previous studies using purified human DDAH enzyme have demonstrated that DDAH was largely resistant to reactive oxygen and nitrogen species, it may be the oxidatively modified products of these reactions that influence DDAH activity [249]. 4-hyrdoxy-2-nonenal (4-HNE) is a lipid hydroperoxide that is biologically active and known to accumulate in membranes at concentrations 10 µM to 5 mM. Mounting evidence suggests that reactive aldehydes such as 4-HNE play a role in the progression of atherosclerosis. We have demonstrated using purified hDDAH-1 that 4-HNE inhibits DDAH activity by binding a histidine residue in the catalytic triad of the enzyme [228]. Therefore, this may represent a novel mechanism by which 4-HNE causes impairment of endothelial NO production, by directly inhibiting DDAH activity. In contrast to our studies, Tain et al reported that DDAH activity was significantly inhibited in the presence of NO and O₂⁻. The differences could be attributed using either using purified recombinant enzyme, or species differences as their studies were done using kidneys from rats [264].
Recent evidence suggests that the DDAH-ADMA pathway may also play a significant role in endothelial dysfunction associated with diabetes. Lin et al. demonstrated that in VSMC and HUVECs cellular ADMA levels increased and DDAH activity decreased following 48 hours of exposure to high glucose media. Furthermore, they observed in vivo that rats placed on a high fat diet and injected with streptozotcin to induce type II diabetes, had elevated plasma ADMA levels. Moreover, these diabetic rats had decreased tissue DDAH activity [265]. Sorrenti et al. demonstrated that DDAH-2 expression and DDAH activity were decreased in human iliac artery cells following five days of exposure to high glucose condition media [266].

Overall, these studies suggest that methylarginines are key in regulating eNOS in a disease state. Based on cellular kinetic studies from our group, a 3-4 fold increase in cellular methylarginines would be expected to inhibit NOS activity by greater than 50%.

It is evident that that NO bioavailability is key to maintaining the anti-atherogenic state of the vascular wall. Furthermore, DDAH and ADMA have emerged as critical factors in diseases associated with increased cardiovascular risk. It is also evident that oxidative stress also plays a significant role in endothelial dysfunction observed in such diseases as hypertension and diabetes.

The observations provided in this dissertation could be of potential clinical importance as CAD is the number cause of all deaths in the United States. Therefore, elucidating the mechanism(s) of how eNOS is modulated by both methylarginines and oxidative stress may provide knowledge for potential therapeutic targets in the treatment of athero-proliferative disorders such as atherosclerosis.
CHAPTER 3
ROLE OF DDAH-1 AND DDAH-2 IN THE REGULATION OF ENDOTHELIAL NO PRODUCTION

Introduction

Endothelium-derived Nitric Oxide (NO) is a potent vasodilator that plays a critical role in maintaining vascular homeostasis through its anti-atherogenic and anti-proliferative effects on the vascular wall. Altered NO biosynthesis has been implicated in the pathogenesis of cardiovascular disease and evidence from animal models and clinical studies suggest that accumulation of the endogenous nitric oxide synthase (NOS) inhibitors, asymmetric dimethylarginine (ADMA) and N⁴-monomethyl arginine (L-NMMA) contribute to the reduced NO generation and disease pathogenesis. ADMA and L-NMMA are derived from the proteolysis of methylated arginine residues on various proteins. The methylation is carried out by a group of enzymes referred to as protein-arginine methyl transferase’s (PRMT’s) [35]. Protein arginine methylation has been identified as an important post-translational modification involved in the regulation of DNA transcription, protein function and cell signaling. Upon proteolysis of methylated proteins, free methylarginines are released which can then metabolized to citrulline through the activity of Dimethylarginine Dimethylamino Hydrolase (DDAH). Currently there are two known isoforms of DDAH each having different tissue specificity. DDAH-1 is thought to be associated with tissues that express high levels of Neuronal Nitric Oxide (nNOS), while DDAH-2 is thought be associated with tissues that express eNOS. Decreased DDAH expression/activity is evident in disease states associated with endothelial dysfunction and is believed to be the mechanism responsible for increased methylarginines and subsequent ADMA mediated eNOS impairment. However, the contribution of each enzyme to the regulation of endothelial NO production has yet to be elucidated.
The strongest evidence for DDAH involvement in endothelial dysfunction has come from studies using DDAH gene silencing techniques and DDAH transgenic mice. Specifically, Cooke et.al. has demonstrated that DDAH-1 transgenic mice are protected against cardiac transplant vasculopathy [241, 242]. Using in-vivo siRNA techniques, Wang et.al. demonstrated that DDAH-1 gene silencing increased plasma levels of ADMA by 50%, but this increase had no effect on endothelial dependent relaxation. Conversely, in-vivo DDAH2 gene silencing had no effect on plasma ADMA, but reduced endothelial dependent relaxation by 40% [237]. These latter findings are particularly intriguing and demonstrate that elevated plasma ADMA is not associated with impaired endothelial dependent relaxation while loss of DDAH-2 activity is associated with impaired endothelial dependent relaxation, despite the fact the plasma ADMA levels are not increased [237]. Given the obvious inconsistencies in the literature regarding the individual roles of DDAH-1 and DDAH-2, the current study establishes the specific role of each DDAH isoform in the regulation of endothelial NO production and its potential role in disease pathogenesis.

**Materials and Methods**

**Cell Culture**

Bovine aortic endothelial cells (BAECs) were purchased from Cell-Systems and cultured in MEM (Sigma, St Louis, MO) containing 10% FBS, 1% NEAA, 0.2% Endothelial Cell Growth Factor Supplement (ECGS) and 1% Antibiotic-Antimyotic (Gibco, Carsbad, CA) and incubated at 37° 5% CO₂ -95% O₂.

**EPR Spectroscopy and Spin Trapping**

Spin-trapping measurements of NO were performed using a Bruker Esca spectrometer with FE-MGD as the spin trap (22,38). 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 x in KREBS and incubated at 37° C 5% CO₂ in 0.2 ml of KRBES buffer containing the spin trap complex FE-MGD (0.5 mM Fe^{2+}, 5.0 mM) was added and the cells stimulated with calcium ionophore (1 uM). 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; 20 mW, modulation amplitude 3.00 G and modulation frequency; 86 kHz.

**HPLC**

BAEC’s were collected from confluent 75 mm culture flask and sonicated in PBS followed by extraction using a cation exchange column. Samples were derivatized with OPA and separated on a Supelco LC-DABS column (4.6 mm x 25 cm i.d., 5 µm particle size) and methylarginines were separated and detected using an ESA (Chelmsford, MA) HPLC system with electrochemical detection at 400mV. Homoarginine was added to the homogenate as an internal standard to correct for the efficiency of extraction. The mobile phase consisted of buffer A (50 mM KH₂PO₄ pH 7.0) and buffer B (ACN/MeOH 70:30) run at room temperature with a flow rate of 1.3 mL/min. The following gradient method was used 0-10 min 90% A 10-40 min a linear gradient from 90% A to 30% A (22,39).

**DDAH-1 and 2 Gene Silencing**

21-bp siRNA nucleotide sequences targeting the coding sequences for DDAH-1 (accession no. NM_001102201) and DDAH-2 (accession no. NM_001034704) were purchased from Ambion. Control cells received GAPDH siRNA also purchased from Invitrogen. 400 μl of nuclease free water was added to the dried oligonucleotides to obtain a final concentration of 100
Transfections were done using the lipid mediated transfection reagent RNAiMax (Invitrogen). The procedure was as follows, 240 nM or 5 ul of siRNA per well of a six well plate was diluted into 250 μl of Opti MEM (Invitrogen) and 5 ul of RNAiMax was diluted in 250 μl of Opti MEM. The siRNA and RNAiMax were then combined into one Eppendorff tube and then incubated at room temperature for 20 minutes. Following the 20 minute incubation period, the RNAi MAX-siRNA complexes were added to each well of a six well plate. The mixture was rocked back and forth to allow for coating of the entire well. BAECs were trypsinized and spun down at 1000 x g for 4 minutes and then resuspended in 1.5 mls of Opti MEM + 10% MEM medium containing 10% FBS, 1% NEAA, 0.2% Endothelial Cell Growth Factor. The cells were then added on top of the RNAiMAX-siRNA complexes and incubated at 37° 5% CO2 -95% O2 for 6 hours. After the 6 hour incubation period, 1ml of MEM medium containing 10% FBS, 1% NEAA, 0.2% Endothelial Cell Growth Factor was added. 24 hours later 1ml of MEM medium containing 10% FBS, 1% NEAA, 0.2% Endothelial Cell Growth Factor was added. At 48 hours 2 ml of medium was removed and replaced with fresh MEM medium containing 10% FBS, 1% NEAA, 0.2% Endothelial Cell Growth Factor and the transfection was continued for another 24 hours.

DDAH Activity

DDAH activity was measured from the conversion of L-[3H]L-NMMA to L-[3H]citrulline. A T-75 flask was used for each measurement, BAECs were trypsinized, pelleted and resuspended in 150 μL of 50mM Tris (pH 7.4). The cells were then sonicated 3 x 2 seconds and 150 μL of reaction buffer (50 mM Tris, 20 μM L-[3 H]L-NMMA, 180 μM L-NMMA, pH 7.4) was added. The samples were then incubated in a water bath at 37°C for 90 minutes. Following the 90 minute incubation, the reaction was stopped with 1 ml of ice-cold stop buffer using 20
mM N-2 Hydroxyethylpiperazine-N’-2 ethanesulfonic acid (HEPES) with 2 mM EDTA, pH 5.5

(15) Separation of L-[\textsuperscript{14}C]citrulline from L-[\textsuperscript{3}H]L-NMMA was performed using the cation exchange resin Dowex AG50WX-8 (0.5 ml, Na\textsuperscript{+} form, Pharmacia). The L-[\textsuperscript{14}C]citrulline in the eluent was then quantitated using a liquid scintillation counter.

**DDAH Over-Expression**

Following the 48 hours of adDDAH-1 or adDDAH-2 transduction, cells were trypsinized and spun down at 1000 x g for 4 minutes. The cell pellet was then washed 1x with PBS and then centrifuged at 1000 x g for an additional 4 minutes. The cell pellet was then homogenized using RIPA buffer containing sodium orthovanadate (2 mM), phenylmethylsulphonyl fluoride (1 mM), and protease inhibitor cocktail (Santa Cruz biotechnology). Following homogenization the cell pellet was briefly sonicated 2 x 2 sec. Protein concentration was quantified using the Bradford assay. 1x sample buffer containing DTT was added to 40 μg of protein and boiled at 95° for 3 minutes and then spun down briefly and cooled for 2 minutes. The samples were then loaded on to a SDS Tris -Glycine gradient gel 4-12% (Invitrogen) and run at 130V for 2 hours. The gel was then removed and the protein was transferred on to a nitrocellulose membrane using the semi dry transfer blot system (BioRad). Following the transfer, the nitrocellulose membrane was blocked for 1 hour in Tris Buffer Saline and 0.05% Tween (TBST) with 5% milk powder. After the blocking period was over the membrane was washed 3x for 5 minutes with TBST and then the respective primary antibody was added and incubated over night at 4°C. DDAH was detected by anti-DDAH-1 and DDAH-2 rabbit IgG obtained from Dr. Renke Mass (Hamburg, Germany) and diluted 1:1000. Following the overnight incubation with the primary antibody the membrane was washed for 15 minutes 3x with TBST and the secondary goat-anti rabbit hpr tag antibody diluted 1:2000 was added. After 1 hour of incubation at room temperature, detection
was preformed using an enhanced chemiluminescence kit purchased from Amersham Biosciences.

**Assessment of mRNA Levels Following DDAH Gene Silencing**

Following the 72 hour siRNA transduction period, BAECs were trypsinized and spun down at 1000 x g for 4 minutes. The cell pellet was then washed 1 x with PBS and centrifuged at 1000 x g for an additional 4 minutes. The cell pellet was then homogenized in lysis buffer from the Qiagen (Valencia,CA) RNAeasy Mini Kit. Following lysis, RNA was extracted using a Qiagen (Valencia,CA) RNAeasy Mini Kit. cDNA was then isolated using the Invitrogen (Carsbad, CA) One Step RT-PCR kit. Semiquantitive PCR was preformed in order to detect changes in mRNA expression following DDAH-1 or DDAH-2 gene silencing. Bovine Primers for DDAH-1 Forward (GAGGAAGGAGGCTGACATGA), DDAH-1 Reverse (TTCAAGTGCAAAAGCATCCAC), and DDAH-2 Forward (CTAGCCAAAGCTCAGAGGGACAT), DDAH-2 Reverse (TCAGTCAACACTGCCATTGCCCT) were purchased from Invitrogen (Carsbad, CA).

**eNOS Activity**

eNOS activity was measured from the conversion of L-[^14]C arginine to L-[^14]C citrulline. A T-75 flask was used for each measurement, BAECs were trypsinized, pelleted and resuspended in 132 µL of 50 mM Tris (pH 7.4). The cells were then sonicated 3 x 2 seconds and 28 µL of reaction buffer (50 mM Tris containing 5 µM L-[^14]C arginine, 50 µM L-arginine 500 µM NADPH/50 µM CaCl2/50 µM H4B pH 7.4) The samples were then incubated in a water bath at 37°C for 30 minutes. Following the 30 minute incubation the reaction was stopped with 1ml of ice-cold stop buffer using 20 mM N 2-Hydroxyethylpiperazine-N’-2 ethansulfonic acid (HEPES) with 2mM EDTA, pH 5.5. Separation of L-[^14]C arginine from to L-[^14]C citrulline
was performed using the cation exchange resin Dowex AG50WX-8 (0.5ml Na\(^+\) form, Pharmacia). The L-\(^{14}\text{C}\) citrulline in the eluent was then quantitated using a liquid scintillation counter.

**Results**

**Effects of DDAH 1 and 2 Over-Expression on Endothelial NO Production**

Previous studies have demonstrated that both DDAH-1 and DDAH-2 are expressed in the vasculature. However, it is presently unknown which of the DDAH isoforms is responsible for the regulation of endothelial NO. Therefore, studies were carried out using adenoviral mediated over-expression of both DDAH-1 and DDAH-2 in order to determine which isoform is responsible for endothelial methylarginine metabolism and NO regulation. Endothelial cells were grown to 90% confluency and then transduced with either ad-DDAH-1 (50 MOI) or ad-DDAH-2 (50 MOI) for 48 hours. Western blot analysis demonstrated robust increases in endothelial expression of both DDAH-1 and DDAH-2 following respective adenoviral treatment (Figure 3-1) At the end of the 48 hour period NO production was measured by EPR as previously described. Results demonstrated that following 48 hours of transduction, adDDAH-1 mediated over-expression resulted in a 24% increase in NO production over basal NO levels (Figure 3-2). It was anticipated that if DDAH over-expression is increasing NO through the metabolism of basal methylarginines, then L-arg supplementation should prevent the increase. Endothelial cells were transduced with adDDAH-1 for 48 hours as previously described. After the 48 hour exposure the media was removed and BAECs were incubated with L-Arg (100 \(\mu\)m) for 30 minutes in KREBS-HEPES buffer. Results demonstrated that L-Arg supplementation alone resulted in a 30% increase in basal NO production in control cells (Figure 3-2). Moreover, in the presence of DDAH-1 over-expression, L-arginine supplementation resulted in an additive effect with a 13% increase in NO compared to L-arg supplementation alone.
Similar to adDDAH-1, DDAH-2 over-expression resulted in an 18% increase in endothelial cell NO production (Figure 3-3). Similar results were obtained with L-arginine supplementation of DDAH-2 over-expressing cells in which we observed a 45% increase NO production following the addition of L-arg compared to a 28% increase with L-arg supplementation alone (Figure 3-3). The observation that the effects of L-arg supplementation on NO production are not attenuated in the presence of DDAH-1 or DDAH-2 over-expression possibly demonstrates that ADMA is not responsible for the “arginine paradox” as has been proposed.

**Effects of DDAH-1 and DDAH-2 Over-Expression on ADMA Inhibition**

The previous studies assessed the effects of DDAH over-expression on NO production in the presence of normal physiological levels of methylarginine. Given that normal intracellular methylarginines are in the low micromolar range it would not be expected that physiological levels of these competitive NOS inhibitors would elicit pathological eNOS inhibition. Therefore, additional studies were performed in the presence of exogenously added ADMA to assess whether DDAH over-expression can overcome ADMA accumulation at levels observed with cardiovascular disease states [96]. Results demonstrated that exogenously added ADMA (10 µM) resulted in 40% inhibition of endothelial cell NO production from BAECs and that over-expression of either DDAH-1 or DDAH-2 was able to restore 50% of the loss in endothelial NO production (Figure 3-4). These results indicate that both DDAH-1 and DDAH-2 may serve as potential therapeutic targets for the treatment of diseases associated with elevated ADMA.

**Effects of DDAH 1 and 2 Silencing on Endothelial NO Production**

Previous studies have suggested that a decrease in DDAH activity, as has been observed in vascular disease, contributes to endothelial dysfunction through a mechanism involving increased cellular ADMA levels. In support, ADMA levels are an independent risk factor for
cardiovascular disease and results from numerous clinical and basic science studies have revealed increased ADMA levels in a variety of diseases including diabetes, pulmonary hypertension, coronary artery disease and atherosclerosis [240, 241, 249, 265, 267-269]. However, whether loss of DDAH activity is directly responsible for the impaired NO production and which specific isoform is responsible for NO regulation in the endothelium are unknown. Therefore, in order to determine the role of each DDAH isoform in the regulation of endothelial NO, cellular studies were performed using BAECs to assess the effects of DDAH-1 and 2 gene silencing on NO production. Bovine aortic endothelial cells were cultured in 6 well plates and using the reverse transfection protocol described in the methods, DDAH-1 and DDAH-2 genes were silenced with specific siRNA’s. The degree of gene knock-down was evaluated using semi-quantitative PCR analysis of DDAH-1 and DDAH-2 mRNA expression. This approach was used in lieu of protein analysis because basal levels of DDAH-2 are undetectable by western blot. Results demonstrated that DDAH-1 and DDAH-2 silencing resulted in greater than 70% reduction in mRNA expression for the respective gene (Figure 3-5). In addition to DDAH mRNA expression, the effects of siRNA mediated DDAH gene silencing on endothelial DDAH activity was measured. Following 72 hours of DDAH-1, DDAH-2 or dual silencing, BAECs were assessed for DDAH activity by measuring the conversion of L-[3H]NMMA to L-[3H]Citrulline. Results demonstrated that DDAH-1 gene silencing resulted in a 64% decrease in totally DDAH activity. DDAH-2 gene silencing resulted in a 48% decrease in total DDAH activity (Figure 3-6). Interestingly, silencing of both DDAH-1 and DDAH-2 resulted in only a 50% drop in total DDAH activity suggesting that other methylarginine metabolic pathways may be invoked as a consequence of loss of DDAH activity (Figure 3-6).
The functional effects of DDAH gene silencing were assessed using EPR spin trapping to measure endothelial derived NO production. Results demonstrated that DDAH-1 silencing reduced endothelial NO production by 27% (Figure 3-7). In order to determine whether the effects of DDAH gene silencing on NO production resulted from increased intracellular levels of ADMA, L-arg supplementation experiments were carried out to assess the ability of L-arg to overcome ADMA mediated eNOS inhibition. Specifically, DDAH gene silencing studies were carried out in the presence of L-arg (100 µM). Results demonstrated that L-arginine (100 µM) supplementation restored 50% of the siDDAH-1 mediated loss of endothelial NO production (Figure 3-7). DDAH-2 gene silencing resulted in a 57% reduction in endothelial NO production. L-arginine supplementation did not increase endothelial NO production in DDAH-2 silenced BAEC’s (Figure 3-8). These results suggest that the effects of DDAH-2 silencing on endothelial NO production are independent of ADMA-mediated eNOS inhibition. Additional studies were performed in which both genes were silenced. Silencing of both the DDAH-1 and DDAH-2 genes resulted in 55% inhibition which was not increased with L-arg supplementation (Figure 3-9).

These results are surprising given that L-arg would be expected to overcome the accumulation of methlyarginines. Therefore, to confirm that L-arg supplementation can in fact ameliorate ADMA mediated inhibition, additional studies were carried out with cells treated with exogenous ADMA and the ability of L-arg supplementation to overcome eNOS inhibition was measured. Cellular studies were carried out using BAEC’s stimulated by calcium ionophore A23187 (1 µM). EPR-based NO measurements were preformed in modified KREBS buffer (0.5 mM Fe²⁺ and 5 mM MGD) in the presence or absence of L-arg (100 µM). The dose-dependent effects of ADMA (0-10 µM) were then measured. Results demonstrated that ADMA dose-
dependently inhibited eNOS-derived NO production with 5µM ADMA eliciting 46% inhibition and 10 µM ADMA, exhibiting 58% inhibition in the absence of L-arg. In the presence of physiologically relevant L-arg levels (100 µM), ADMA treatment resulted in a dose-dependent inhibition of endothelial NO with < 20 % inhibition seen at ADMA concentrations of 5µM. Overall these results demonstrate that L-arg supplementation can only partially restore the loss in NO production occurring after ADMA administration. Although the addition of exogenous L-arg would be expected to fully restore ADMA mediated eNOS inhibition, these results are consistent with previous studies demonstrating partial restoration of endothelial NO with L-arg following exposure to exogenous ADMA [96] (Figure 3-10).

Effects on DDAH Gene Silencing on Methylarginine Metabolism

As demonstrated earlier, when both DDAH-1 and DDAH-2 were silenced, total DDAH activity was only inhibited by 50%, suggesting the endothelium may possess alternate metabolic pathways for methylarginine metabolism. These are unexpected results given that DDAH is considered to be the principle metabolic pathway for ADMA metabolism and was previously demonstrated to mediate >80% of cellular methylarginine metabolism [267]. Therefore, studies were carried out using HPLC techniques with radiolabeled NMMA to assess the metabolites of methylarginine metabolism. Results demonstrated that in BAECs that were not silenced three radiolabeled peaks were identified, arginine, citrulline and L-NMMA. However, in BAECs that were silenced an additional unidentified radiolabeled peak was observed suggestive of induction of an alternate metabolic pathway. Furthermore, following dual gene silencing the concentration of the unknown metabolite increased 2-fold (Table 3-1). These results would suggest that BAECs have an alternate inducible pathway for methylarginine metabolism in response to loss of DDAH activity or methylarginine accumulation.

Effects of DDAH 1 and 2 Gene Silencing on eNOS Activity
The studies on DDAH silencing demonstrate that loss of DDAH-2 expression/activity may elicit ADMA independent effects given that L-arg supplementation was not able to enhance endothelial NO production from DDAH-2 silenced cells. Therefore, studies were carried out in order to determine whether gene silencing has any direct effects on eNOS activity independent of ADMA. Studies were performed measuring the conversion of L-[14C] Arginine to L-[14C] Citrulline from BAEC homogenates following DDAH gene silencing. Results demonstrated that DDAH-1, DDAH-2 and dual silencing resulted in no change in total eNOS activity based on L-NAME inhibitable counts (Figure 3-11).

**Discussion**

ADMA plasma levels have been shown to be elevated in diseases related to endothelial dysfunction including hypertension, hyperlipidemia, diabetes mellitus, and others [267, 268, 270-272]. Moreover, it has been shown that ADMA predicts cardiovascular mortality in patients who have coronary heart disease (CHD). Recent evidence published from the multicenter Coronary Artery Risk Determination investigating the Influence of ADMA Concentration (CARDIAC) study has indicated that ADMA is indeed an independent risk factor for CAD [273]. There is a growing body of evidence implicating ADMA as a key player in endothelial dysfunction and a independent risk factor involved in the pathophysiology of a variety of cardiovascular diseases including hypertension, and atherosclerosis (21-26). Recently several groups have demonstrated that modulating DDAH activity can have a profound effect on endothelial NO production [237, 240]. In this regard, our group and others have shown that over-expression of DDAH-1 results in increased NO production [242, 274]. Furthermore, OxLDL and TNFα have been shown to decrease DDAH activity leading to decreased endothelial NO production [275]. It has also been demonstrated that 4-hyrdoxynonenal (4-HNE), the
highly reactive oxidant product of lipid peroxidation, inhibits DDAH activity and leads to impaired NO generation through the formation of Michael addition products in the catalytic triad of DDAH [274]. Thus, evidence suggests that DDAH-1 activity is under redox control and loss of enzyme function impairs endothelial NO generation.

Whether the increased risk associated with elevated ADMA is a direct result of NOS impairment is an area of controversy. Significant debate about the contribution of ADMA to the regulation of NOS-dependent NO production has been initiated. In pathological conditions such as pulmonary hypertension, coronary artery disease, diabetes and hypertension, plasma ADMA levels have been shown to increase from an average of ~0.4 µM to ~0.8 µM [269, 272, 273, 276-278]. Given that these values are at least 2 orders of magnitude lower than the plasma L-arg levels it is unlikely that elevated plasma ADMA can significantly regulate eNOS activity. It is more likely that elevated plasma ADMA levels reflect increased endothelial concentrations of ADMA. In support of this hypothesis, we and others have demonstrated that endothelial ADMA levels increase 3-4 fold in restenotic lesions and in the ischemia reperfused myocardium [96, 279]. Based on the kinetics of cellular inhibition, these concentrations of ADMA would be expected to elicit a 30-40% inhibition in NOS activity [96]. These studies however involve lesion specific increases in ADMA and are not associated with increased plasma levels of ADMA and would not be expected to contribute to systemic cardiovascular pathology. In this regard, there is little direct evidence that elevated plasma ADMA levels are associated with increased endothelial ADMA nor is it clear whether ADMA directly contributes to the NOS inhibition observed in chronic cardiovascular diseases. The current hypothesis in the field suggests that decreased DDAH activity, as has been observed in cardiovascular disease, results in impaired endothelial methylarginine metabolism with subsequent elevation in ADMA leading
to NOS inhibition. However, identification of the endothelial DDAH isoform responsible for NOS regulation and direct evidence for its role in modulating endothelial NO production has not been demonstrated. Therefore, the current study was undertaken to evaluate the roles of both DDAH-1 and DDAH-2 in the regulation of methylarginine metabolism and endothelial NO production.

Initial studies were carried out to determine how cellular endothelial NO production is regulated by the DDAH isoforms. DDAH-1 and DDAH-2 over-expression was induced using an adenoviral construct carrying either the human DDAH-1 gene (adDDAH-1) or DDAH-2 gene (adDDAH2). Results demonstrated that adenoviral mediated overexpression of both DDAH-1 and DDAH-2 increased cellular endothelial NO production. These initial studies were done in the presence of basal methylarginine levels and demonstrate that normal endogenous levels of these NOS inhibitors are present at concentration sufficient to regulate eNOS activity. It had previously been proposed that ADMA may be responsible for the “arginine paradox” and these studies would appear to support the hypothesis. However, subsequent studies using L-arg supplementation with DDAH over-expression demonstrated an additive effect which suggests that ADMA is not involved in the “arginine paradox”.

It has been estimated that more than 80% of ADMA is metabolized by DDAH [267], however, it is unclear which DDAH isoform represents the principal methylarginine metabolizing enzyme. PCR and western blot analysis revealed that the endothelium contains mRNA and protein for both DDAH-1 and DDAH-2. However, in order to assess the relative contribution of each isoform a detailed analysis of the enzyme kinetics of each isoform is necessary. Unfortunately, detailed biochemical studies have only been published for DDAH-1 [228, 249]. Using purified recombinant hDDAH-1 we and others have demonstrated the precise
enzyme kinetics of this isoform and results demonstrated $K_m$ values of 68.7 and 53.6 $\mu$M and $V_{\text{max}}$ values of 356 and 154 nmols/mg/min for ADMA and L-NMMA, respectively [228, 249]. In regards to DDAH-2, previous attempts at purifying the protein have been unsuccessful primarily due to solubility issues with recombinant enzyme. Therefore, to investigate the role of the DDAH isoforms in the regulation of endothelial NO production, studies were performed using siRNA to silence both the DDAH-1 and DDAH-2 genes in BAECs. It was anticipated that silencing of DDAH would lead to increased cellular methylarginines and decreased endothelial NO production. Results supported this prediction and demonstrated that DDAH-1 silencing reduced endothelial NO production by 27% while DDAH-2 silencing reduced it by 57%. These studies were then repeated with L-arg supplementation in order to establish the ADMA dependence of the DDAH effects. The addition of L-arg (100 $\mu$M) was able to restore ~ 50% of the loss of endothelial NO generation observed with DDAH-1 silencing. Although it may be predicted that L-arg supplementation should completely restore NO production given that ADMA is a competitive inhibitor of NOS, these result are consistent with previously published studies and suggest that DDAH-1 silencing may lead to ADMA accumulation in sites that are not freely exchangeable with L-arg. In support of this hypothesis it has been demonstrated by Simon et al. that within the endothelial cell exists two pools of arginine both of which eNOS has access to. Pool I is largely made up of extracellular cationic amino acids transported through the CAT transport system, however Pool II does not freely exchange with extracellular cationic amino acids. Furthermore they also demonstrated that Pool II is separated into two components. Pool II A participates in the recycling of citrulline to arginine, while Pool II B is occupied by protein derived by-products. It is within this Pool II B where the methylarginines are likely to accumulate, thus rendering its inhibitory effects on eNOS [280]. Alternatively, ADMA and/or
DDAH may elicit effects that are independent of NOS, this appears to be the most plausible explanation with regards to DDAH-2 wherein loss of activity reduced endothelial NO production by greater than 50% and the loss was unaffected by L-arg supplementation. This is strong evidence that DDAH may elicit effects that are independent of ADMA. Although this may represent an overall paradigm shift with regards to the role of DDAH in the endothelium, it is not without support. The most convincing evidence that DDAH may regulate cellular function through mechanisms independent of ADMA mediated NOS inhibition come from data on the DDAH-1 knockout mouse. Homozygous null mice for DDAH-1 are embryonic lethal while the NOS triple knockout mice are viable [240]. This further supports are hypothesis that DDAH effects are not limited to ADMA dependent regulation of eNOS.

It has been widely reported that DDAH-2 is the predominant DDAH isoform in the vascular endothelium; however these studies have widely relied on assessing the expression of the DDAH isoforms in various cell and tissue types [237, 240, 281, 282]. Consequently, studies were carried out in BAECs to determine which isoform is responsible for the majority of the DDAH activity in the endothelial cell. DDAH-1 and DDAH-2 gene silencing decreased total DDAH activity by 64% and 48%, respectively. Additional studies demonstrated that dual gene silencing only resulted in a 50% loss total DDAH activity in BAECs thus suggesting that other methylarginine metabolic pathways may be invoked as a consequence of loss of DDAH activity. To investigate the possibility that loss of DDAH activity may lead to the induction of other methylarginine metabolic enzymes we used HPLC techniques to measure the metabolic products of 14C-L-NMMA. In control cells we observed 3 peaks with radioactive counts and they were identified as L-NMMA, L-arginine and L-citruline. The formation of radiolabelled L-citruline is likely from the metabolism of L-NMMA by DDAH while radioactive L-arg is generated from
citrulline recycling through ASS and ASL. In contrast, results from DDAH-1 and DDAH-2 silenced cells indicated the presence of 4 radioactive peaks including L-NMMA, L-arginine, L-citruline and a yet unidentified peak. The concentration of this unidentified peak increased 2 fold in the dual silencing group as compared to the levels in either the DDAH-1 or DDAH-2 silencing groups alone. Initial mass spec analysis has been unsuccessful in identifying the unknown species and is currently an area of active investigation in our lab. Regardless, the results clearly indicate that the endothelium possesses an alternate inducible pathways for metabolizing methylarginines. Together, these results demonstrate that both DDAH-1 and DDAH-2 are involved in the regulation of endothelial NO production. However, while DDAH-1 effects are largely ADMA-dependent, DDAH-2 effects appear to be ADMA-independent.

To determine whether the ADMA-independent effects of DDAH silencing on endothelial NO production involved changes in eNOS protein, we measured eNOS activity from BAEC homogenates following DDAH-1, DDAH-2 and dual silencing. Analysis of eNOS activity demonstrated that DDAH gene silencing had no effect on the enzyme. These experiments were carried out in the presence of saturating concentrations of substrate and cofactors and can rule out DDAH effects on endothelial substrate/cofactor bioavailability.

Overall these results demonstrate that loss of DDAH activity, as has been demonstrated in a number of cardiovascular diseases, leads to significant inhibition of endothelial NO production. Moreover, the effect of DDAH-1 and DDAH-2 on endothelial NO appear to manifest through very different mechanisms. DDAH-1 appears to be largely and ADMA dependent effect, while DDAH-2 appears mostly to mediate its effects independent of ADMA. Moreover, we have demonstrated for the first time an alternative pathway through which methlyarginines can be metabolized.
<table>
<thead>
<tr>
<th>Multiplicity of Infection (MOI)</th>
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![Image showing DDAH-1 and DDAH-2 expression](image.png)

Figure 3-1. DDAH Over-expression. DDAH-1 and DDAH-2 expression was measured by western blot techniques from BAECs transduced for 48-hours with adDDAH-1(10, 25, 50 MOI) and adDDAH-2(10, 25, 50 MOI)
Figure 3-2. Effects of adDDAH-1 over expression on endothelial cell NO production. NO generation from calcium ionophore A23187 (1 µM) stimulated BAECs (1x10⁶) was measured by EPR spin trapping with the Fe²⁺-MGD complex. The left side of the panel represents the amplitude of the NO triplicate EPR spectra of a 30 consecutive 20 second scans following a 30 minute incubation period. B) The right panel represents the characteristic triplicate NO spectra and the effects of adDDAH-1 over-expression on NO production. Results are means ± SD. * Significance at p<0.05 as compared to the control. n=9
Figure 3-3. Effects of adDDAH-2 over expression on endothelial cell NO production. NO generation from calcium ionophore A23187 (1 µM) stimulated BAECs (1x10⁶) was measured by EPR spin trapping with the Fe²⁺-MGD complex. The left side of the panel represents the amplitude of the NO triplicate EPR spectra of a 30 consecutive 20 second scans following a 30 minute incubation period. The right panel represents the characteristic triplicate NO spectra and the effects of adDDAH-2 over-expression on NO production. Results are means ± SD as compared to the control. * Significance at p<0.05 as compared to the control. n=9
Figure 3-4. Effects of DDAH-1 and DDAH-2 over-expression on ADMA mediated inhibition of endothelial NO production. NO generation from calcium ionophore A23187 (1 µM) stimulated BAECs (1x10^6) was measured by EPR spin trapping with Fe^{2+}-MGD complex. Experimental groups consisted of adGFP (control) adDDAH-1 and adDDAH-2. These experiments were performed in the absence and presence of ADMA (5 µM). Results are ±SD *Significance at p<0.05 as compared to the respective control.
Figure 3-5. Effects of DDAH gene silencing on DDAH mRNA expression. DDAH mRNA expression was measured by semi quantitative PCR, and ran on an agarose gel to check for differences in DDAH expression following siRNA treatment. Experimental groups consist of 60 nM siRNA (DDAH-1, DDAH-2) and 240 nM siRNA (DDAH-1 and DDAH).
Figure 3-6. Effects of DDAH gene silencing on endothelial cell DDAH activity. DDAH activity was measured from BAEC homogenates following 72 hours of DDAH gene silencing. Experimental groups consisted of si-DDAH-1, si-DDAH-2, si-DDAH1/2. Results are means ± SD. * Significance at p<0.05 as compared to the control. n=3
Figure 3-7. Effects of DDAH-1 gene silencing on endothelial cell NO production. NO generation from calcium ionophore A23187 (1 μM) stimulated BAECs (1x10^6) was measured by EPR spin trapping with the Fe2+-MGD complex. Experimental groups consisted of scrambled siRNA (control), and si-DDAH-1. These experiments were performed both in the presence and absence of L-arginine (100μM). Results are means ± SD. * Significance at p<0.05 as compared to the respective control. n=9
Figure 3-8: Effects of DDAH-2 gene silencing on endothelial cell NO production. NO generation from calcium ionophore A23187 (1 µM)-stimulated BAECs (1x10^6) was measured by EPR spin trapping with the Fe^{2+}-MGD complex. Experimental groups consisted of scrambled siRNA (control) and siDDAH-2. These experiments were performed both in the presence and absence of L-arginine (100 µM). Results are means ± SD. * Significance at P<0.05 as compared to the respective control.
Figure 3-9. Effects of DDAH-1 and DDAH-2 gene silencing on endothelial cell NO production. NO generation from calcium ionophore A23187 (1 µM) stimulated BAECs (1x10^6) was measured by EPR spin trapping with the Fe^{2+}-MGD complex. Experimental groups consisted of scrambled siRNA (control) and siDDAH-2. These experiments were performed both in the presence and absence of L-arginine (100 µM). Results are means ± SD. * Significance at p<0.05 as compared to the respective control. n=9
Figure 3-10. Effects of ADMA on endothelial cell NO production. NO generation from calcium ionophore A23187 (1 µM) stimulated BAECs (1x10^6) was measured by EPR spin trapping with the Fe^{2+}-MGD complex. Experimental groups consisted of control (0 µM), 5 µM and 10 µM ADMA. These experiments were preformed both in the presence and absence of L-arginine (100 µM). Results are means ± SD. * Significance at p<0.05 as compared to the respective control. n=9
Figure 3-11. Effects of DDAH gene silencing on endothelial cell eNOS activity. eNOS activity was measured from BAEC homogenates following 72 hours of DDAH gene silencing. Experimental groups consisted of si-DDAH-1, si-DDAH-2, si-DDAH1/2. Results are means ± SD. n=3
### Table 3-1. L-NMMA Metabolism

<table>
<thead>
<tr>
<th></th>
<th>Arginine (µM)</th>
<th>Citrulline (µM)</th>
<th>Unknown (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.5</td>
<td>4.07</td>
<td>0</td>
</tr>
<tr>
<td>siDDAH-1</td>
<td>5</td>
<td>1.5</td>
<td>2.2</td>
</tr>
<tr>
<td>siDDAH-2</td>
<td>4.25</td>
<td>2.05</td>
<td>2.8</td>
</tr>
<tr>
<td>siDDAH-1/2</td>
<td>5.1</td>
<td>1.27</td>
<td>4.4</td>
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</table>

BAECs were cultured in 6 well plates and transfected with siRNA. Following the 72 hour transfection period, cellular amino acid content was measured and quantified using HPLC techniques with ESA peak integration software.
CHAPTER 4
ROLE OF DDAH-1 IN THE 4-HYDROXY-2-NONENAL MEDIATED INHIBITION OF ENDOThELIAL NITRIC OXIDE GENERATION

Introduction

Endothelium-derived nitric oxide (NO) is a potent vasodilator that plays a critical role in maintaining vascular homeostasis through its anti-atherogenic and anti-thrombotic effects on the vascular wall [283-285]. In this regard, impaired endothelial derived NO production has been implicated in the pathogenesis of atheroproliferative disorders [286]. Among the proposed mechanisms for the impaired NOS activity observed in these conditions are the elevated levels of oxidatively modified lipids [287, 288]. Polyunsaturated fats in cholesterol esters, phospholipids and triglycerides are subjected to free radical initiated oxidation. These polyunsaturated fatty acid peroxides can yield a variety of highly reactive smaller molecules such as the aldehyde 4-hydroxy-2-nonenal (4-HNE) upon further oxidative degradation [289]. 4-HNE is a major biologically active aldehyde formed during lipid peroxidation of w6 polyunsaturated fatty acids which has been shown to accumulate in membranes at concentrations from 10 μm to 5 mM [290]. There is a body of evidence which suggests that reactive aldehydes such as 4-HNE play a role in the progression of atherosclerosis. Plasma concentrations of these reactive aldehydes are known to increase relative to the progression of aortic atherosclerosis, and during the oxidation of LDL high concentrations of the reactive aldehydes are generated [291, 292]. It has been suggested that the elevations in these highly reactive lipid hydroperoxide degradation products result in impaired endothelial function and atherosusceptibility, secondary to NOS impairment [288, 293, 294]. In support of the importance of the reactive aldehyde involvement in endothelial dysfunction, here we demonstrate that exposure of aortic endothelial cells to 4-HNE dose-dependently inhibits NO bioavailability. We hypothesize that the decrease in NO
bioavailability is a result of increased levels of the NOS inhibitors, asymmetric dimethy arginine (ADMA) and $N^G$-monomethyl arginine (L-NMMA).

ADMA has been shown to be increased in conditions associated with increased risk of atherosclerosis and independently predicts total and cardiovascular mortality in individuals with angiographic coronary artery disease [31, 34, 273, 295-297]. However, little is known with regards to the pathways leading to the methylarginine accumulation observed in cardiovascular diseases. These endogenous inhibitors of NOS are derived from the proteolysis of methylated arginine residues in various proteins. The methylation is carried out by a group of enzymes referred to as protein-arginine methyl transferase’s (PRMT’s). Subsequent proteolysis of proteins containing methylarginine groups leads to the release of free methylarginine into the cytoplasm where NO production from NOS is inhibited [35, 298, 299]. These methylarginines are subsequently degraded by the enzyme DDAH which hydrolyzes the conversion of ADMA to L-citrulline and dimethylamine [36, 300]. The activity of DDAH has been shown to be decreased by oxidized LDL and tumor necrosis factor (TNF-α), yielding increased methylarginine levels with subsequent impairment of NOS-derived NO generation [239, 261, 301, 302]. Because NO is known to possess anti-proliferative and anti-atherogenic properties, methylarginine accumulation in response to the decreased DDAH expression/activity has been proposed to be involved in the vascular pathophysiology observed in a variety of cardiovascular diseases [35, 96, 303-305]. However, the mechanisms as to how methylarginines are modulated and what role they play in disease progression are not understood. Therefore, the current studies were performed in order to establish the effects of the lipid peroxidation degradation product 4-HNE on NO production and determine if methylarginines are involved in the lipid peroxidation mediated pathogenesis of endothelial dysfunction.
Materials and Methods

Materials

4-HNE was purchased from Biomol (Plymouth Meeting, PA). BAECs were purchased from Cell-Systems (Kirkland, WA). All other reagents were purchased from Sigma (St.Louis, MO).

Cell Culture

Bovine aortic endothelial cells (BAECs) were purchased from Cell-Systems and cultured in DMEM containing 10% FBS, 1% NEAA, 0.2% endothelial cell growth factor supplement and 1% antibiotic-antimyotic and incubated at 37°C under a humidified environment containing 5% CO₂ – 95% O₂. For experiments involving exposure to 4-HNE, 4-HNE was prepared as a stock solution in ethanol at a concentration of 50 mM. The 4-HNE was then added to the media of BAECs and incubated for 24 hrs. Dilutions of 4-HNE were performed in order to maintain the final ethanol concentration below 0.2%.

Epr Spectroscopy and Spin Trapping

Spin-trapping measurements of NO were performed using a Bruker ESP 300E spectrometer with Fe-MGD as the spin trap [96, 306]. 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 (1 x 10⁶ cells/ well). In these studies, cells attached to the substratum were utilized since scraping or enzymatic removal leads to injury and membrane damage with impaired NO generation. The medium from each well was removed and the cells were washed 3 x with PBS (w/o CaCl₂ or MgCl₂). Next, 0.3 ml of PBS containing glucose (1 g/L), CaCl₂, MgCl₂, the NO spin trap FE-MGD (0.5 mM Fe²⁺, 5.0 mM MGD), and calcium ionophore (1 μM) was added to each well and the plates were incubated at 37°C under a humidified environment containing 5% CO₂ – 95% O₂ for 30 min. Following incubation, the
medium from each well was removed and the trapped NO in the supernatants was quantified using EPR. Spectra recorded from these cellular preparations were obtained using the following parameters: microwave power; 20 mW, modulation amplitude; 3.16 G and modulation frequency; 100 kHZ.

**Measurement of Endothelial Cell ADMA and L-Arg Levels**

BAEC’s were collected from confluent 75 cm² culture flask by gentle scraping followed by sonication in PBS followed by extraction using a cation-exchange column. Samples were derivatized with OPA and separated on a Supelco LC-DABS column (4.6 mm x 25 cm i.d., 5 µm particle size) and L-Arg and methylarginines separated and detected using an ESA (Chelmsford, MA) HPLC system with electrochemical detection at 400 mV [306]. Intracellular levels of L-Arg and methylarginines were determined from values derived from standard curves of each analyte using the ESA peak integration software assuming the endothelial cell intracellular water content of 2 pL.

**DDAH-1 and eNOS Expression**

DDAH-1 was detected by anti-DDAH-1 goat IG purchased from IMGENEX and diluted 1:2000 (San Diego, CA). eNOS was detected using an anti-eNOS antibody purchased from Calbiochem (San Diego, CA). The secondary antibodies were donkey anti-goat IgG-HRP and goat anti-rabbit IgG-HRP, respectively, and purchased from Santa Cruz (Santa Cruz, CA). The secondary antibodies were diluted 1:2000. Western blot detection was performed using an enhanced chemiluminescence kit purchased from Amersham Biosciences (Piscataway, NJ).

**DDAH Activity**

DDAH activity was measured from the conversion of L-[¹⁴C]L-NMMA to L-[¹⁴C]citrulline. BAECs grown to confluence in T-75 flasks were trypsinized, pelleted and resuspended in 150 µL of 50 mM Tris (pH 7.4). The cells were then sonicated 4 x 2 seconds and
150 μL of the reaction buffer (50 mM Tris, 20 μM L-[\textsuperscript{14}C]L-NMMA, 180 μM L-NMMA, pH 7.4) was added to each sample. The samples were then incubated in a water bath at 37°C for 90 minutes. Following the incubation, the reaction was stopped with 1 ml of ice-cold stop buffer using 20 mM N-2 Hydroxyethylpiperazine-N’-2 ethanesulfonic acid (HEPES) with 2 mM EDTA, pH 5.5. Separation of L-[\textsuperscript{14}C]citrulline from L-[\textsuperscript{14}C]L-NMMA was performed using the cation exchange resin Dowex AG50WX-8 (0.5 ml, Na\textsuperscript{+} form, Pharmacia). The L-[\textsuperscript{14}C] citrulline in the eluent was then determined using a liquid scintillation counter.

**Results**

**Effects of 4-HNE on Endothelial Cell NO Production**

Previous studies have demonstrated that lipid hydroperoxide levels are elevated in atherosclerotic lesions and the presence of these oxidized lipid congeners may contribute to the endothelial dysfunction observed in CAD [291, 292]. Therefore, in order to determine the effects of lipid hydroperoxides on endothelial function, cellular studies were carried out using BAECs stimulated with calcium ionophore to assess the effects of 4-HNE on NO production from endothelial cells. Endothelial cells were cultured in 6 well plates and upon reaching confluence were exposed to 4-HNE (10-100 μM) for 24 hours. 4-HNE was dissolved in ethanol and added directly to the media (0.1 % EtOH). This compound is highly lipophilic and readily crosses cellular membranes, as such no carrier is needed to deliver this agent into the cell [307]. At the end of the incubation period, EPR spin trapping measurements were performed to measure endothelial-derived NO production. Results demonstrated that 4-HNE dose dependently inhibited NO generation from BAECs, with 10 μM 4-HNE inhibiting NO generation by 14%, 50 μM inhibited NO generation by 45% and at 100 μM a 72% inhibition was observed (Figure 4-1). Results from these studies demonstrated that 4-HNE, at pathologically
relevant levels, dose-dependently inhibited eNOS-derived NO generation. Measurements of cell viability demonstrated no increase in cell death with 4-HNE doses up to 50 µM, however, at 100 µM 4-HNE cell viability decreased by 18 % following 24-hours of 4-HNE exposure. Thus all subsequent studies were performed using 4-HNE concentrations \( \leq 50 \mu M \). Control studies using the non-oxidized carbonyl hexanol demonstrated no significant inhibition in cellular NO production with concentrations up to 50 µM (Figure 4-2). The concentration of 4-HNE (50 µM) used for subsequent studies represents pathologically relevant levels of this reactive lipid oxidation product as previous studies have shown concentrations exceeding 50 µM 4-HNE in the plasma of dogs following reperfusion injury [308, 309].

**Effect of 4-HNE on eNOS Expression**

In order to determine whether the inhibitory effects of 4-HNE on cellular NO production were due to alterations in eNOS expression, western blot analysis was performed and measurements of eNOS expression were carried out (Figure 4-3). NOS phosphorylation status was measured using the western blotting techniques with anti-pSer1179 and pThr-497 antibodies (Figure 4-3). In addition, because calcium ionophore stimulation is known to alter Ser1179 phosphorylation, western blot experiments were also performed on A23187 stimulated BAECs following 4-HNE treatment (Figure 4-4). Results demonstrated that the loss of NOS activity was independent of both eNOS protein expression and phosphorylation status as both of these outcomes were unchanged following exposure to 4-HNE.

**Restoring NO Generation from Cells**

Because the observed NO inhibition did not result from changes in protein expression or phosphorylation state, we carried out additional studies aimed at assessing whether substrate or cofactor depletion was involved in the observed decrease in NO bioavailability. In this regard, oxidant stress, which has been shown to occur following exposure to lipid oxidation products,
has been shown to reduce the bioavailability of the critical NOS cofactor H4B [290, 310, 311]. Loss of this cofactor results in NOS uncoupling with the enzyme primarily generating superoxide. Moreover, oxidant injury has also been demonstrated to increase cellular levels of the endogenous methylarginine, ADMA [88]. Therefore, cellular studies were carried out to investigate the effects of adding both an antioxidant to prevent H4B oxidation as well as the eNOS substrate L-Arginine, to overcome endogenous methylarginine mediated NOS inhibition. Results demonstrated that 24 hour exposure of BAECs to 50 µM 4-HNE resulted in a 51% decrease in endothelial NO generation (Figure 4-5). When these experiments were repeated in the presence of GSH (1 mM) or with L-Arg supplementation (1 mM), NO production increased by 26% and 7% respectively. Moreover, when these experiments were repeated in the presence of both GSH and L-Arg, endothelial cell NO production was restored to near normal levels (87% of control) (Figure 4-5). These results suggest that 4-HNE-mediated effects on NO production involve multiple mechanisms which include elevated levels of methylarginines.

**Effects of 4-HNE on Superoxide Production and Nitrotyrosine Formation**

Because our previous results demonstrated that GSH was able to partially restore NO production in BAEC treated with 4-HNE, studies were done in order to determine the effects of 4-HNE on superoxide production (Figure 4-12). Following 24 hours of 4-HNE (50µM) treatment, resulted in increased superoxide production in our BAEC cells. The superoxide signal was largely quenched by the SOD mimetic. Furthermore, because it is known that oxidative stress can increase eNOS derived by causing the oxidation of the essential NOS cofactor H4B we then repeated these studies in the presence of the NOS inhibitor L-NAME. Results demonstrated that following 24 hours of 4-HNE exposure, L-NAME treatment caused a 20% reduction in superoxide production (Figure 4-12). Finally, because increases in superoxide production are
also know to increase OONO- production which can result in increased protein nitrosylation, western blot analysis was done to measure nitrotyrosine formation. Results demonstrated that there were no significant changes in protein nitrotyrosine formation. (Figure 4-11)

**Effects of 4-HNE on Cellular ADMA Levels**

Our observation that 4-HNE treatment impairs cellular NO production and that this inhibitory effect can be reversed with L-Arg administration suggests that intracellular levels of the NOS inhibitor ADMA may be elevated. In order to confirm this hypothesis, cellular levels of ADMA and L-Arg were measured following exposure of BAECs to 50 μM 4-HNE for 24 hours. Results demonstrated that at 24 hours post-exposure to 4-HNE, endothelial cell concentrations of ADMA increased from 3.2 ± 0.5 to 6.5 ± 0.7, while L-Arg levels were not significantly different (Figure 4-6). These results support our conclusion that the inhibitory effects of 4-HNE on endothelial NO production are due, at least in part, to the increased levels of the competitive NOS inhibitor, ADMA.

**Effect of 4-HNE on DDAH Expression and Activity**

Cellular methylarginine levels are regulated by DDAH, the enzyme responsible for the metabolism of both ADMA and L-NMMA. Recent studies have demonstrated that the expression and activity of this methylarginine-regulating enzyme decreases in variety of cardiovascular diseases. Therefore, to determine whether the observed elevations in intracellular ADMA were a result of changes in DDAH, measurements of DDAH expression and activity were performed following exposure of BAECs to 4-HNE. BAECs were treated with 4-HNE (50 μM) followed by western blotting and enzyme activity assays. Results demonstrated that exposure of endothelial cells to 4-HNE did not affect the protein expression (Figure 4-7), but resulted in a 40% decrease in cellular DDAH activity (Figure 4-8). Studies were then performed
with purified recombinant hDDAH-1 to evaluate whether the observed cellular inhibition of DDAH activity was a result of direct 4-HNE effects on the enzyme. Incubation of purified hDDAH-1 with 50 μM 4-HNE resulted in a 41% decrease in activity from the purified enzyme (Figure 4-8). This loss in activity was largely restored by GSH (1 mM) pre-incubation. Together, these results demonstrate that 4-HNE directly inhibits DDAH activity resulting in increased methylarginine levels and thus impaired eNOS-derived NO.

Effects of DDAH Over-Expression on Endothelial NO Production Following Exposure to 4-HNE

Our results have demonstrated that the exposure of BAECs to the lipid peroxidation product, 4-HNE, results in the impaired NO production and accumulation of ADMA, secondary to the loss of DDAH activity. Therefore, studies were carried out in order to determine whether over-expression of DDAH-1 could restore endothelial NO production following 4-HNE challenge. BAECs were grown to 80% confluence and then transduced with adDDAH-1 (25 MOI) which resulted in a 3-fold increase in DDAH 1 expression. After 24 hours of adenoviral transfection, cells were challenged with 4-HNE and allowed to incubate for an additional 24 hours. At the end of the 24 hour challenge, EPR analysis of NO production was carried out as described in the Material and Methods. Results demonstrated that exposure to 4-HNE (50 μM) resulted in a 36% decrease in NO generation in cells transduced with a control vector (Figure 4-9). Cells over-expressing DDAH-1 demonstrated a 22% basal increase in NO generation as compared to the control vector, suggesting that the endogenous levels of methylarginine are sufficient to significantly inhibit cellular NO production (Figure 4-9). Exposure of cells over-expressing DDAH-1 to 4-HNE (50 μM) resulted in a 58% decrease in NO production, thus demonstrating that DDAH alone cannot restore eNOS function. However, when these experiments were repeated in the presence of GSH, DDAH over-expression was able to almost
completely restore NO production following 4-HNE challenge, while GSH alone had only modest effect (Figure 4-9).

In order to confirm that these NO-restoring effects were dependent on increased DDAH activity, studies were performed measuring the conversion of L-[^14C]NMMA to L-[^14C]citrulline in BAECs. We found that exposure of BAECs to 4-HNE (50 μM) resulted in a 38% decrease in DDAH activity, supporting our previous HPLC results (Figure 4-10). Over-expression of DDAH-1 increased DDAH activity by ~50% and this increase in activity was reduced by 25% following exposure of BAECs to 4-HNE. Although DDAH activity was significantly higher in the DDAH over-expressing cells exposed to 4-HNE as compared to the control, this increase in DDAH activity was not accompanied by an increased NO production (Figure 4-9). Treatment of BAECs with the antioxidant GSH had modest effect on the DDAH activity and did not significantly prevent the loss of DDAH activity following 4-HNE challenge, while the combination of DDAH over-expression and GSH increased DDAH activity by ~50% (Figure 4-10) with near complete restoration of NO production (Figure 4-9). These results suggest that 4-HNE causes NOS impairment through multiple mechanisms the first involving methylarginine accumulation. The second one being NOS uncoupling, because of its known oxidative effects on H_4B(Vivar Vasquez). Evidence for NOS uncoupling is supported by our data demonstrating that DDAH over-expression in the presence of 4-HNE actually exacerbates the effects of 4-HNE on NO production (Figure 4-9). In this regard, we have previously reported that methylarginines inhibit nNOS derived superoxide, and as such, over-expression of DDAH would reduce this inhibitory effect resulting in increased NOS derived superoxide and reduced NO bioavailability [312]. Evidence for the multiple mechanisms through which 4-HNE mediates its effects are supported by our results demonstrating that GSH treatment alone or DDAH over-expression
alone has only moderate protection from 4-HNE induced NOS dysfunction. However, in combination, these two treatments largely restored endothelial NO generation (Figure 4-9). Therefore, complete protection of endothelial-derived NO generation from 4-HNE damage can only be achieved by both preventing NOS uncoupling and oxidase generation from other oxidative sources (GSH treatment) and methylarginine accumulation (DDAH over-expression).

Discussion

There is a growing volume of literature implicating ADMA as a key player in endothelial dysfunction and strong correlative data suggesting that ADMA is involved in the pathophysiology of a variety of cardiovascular diseases including hypertension and atherosclerosis [35, 303]. More recently we and others have shown that methylarginines are elevated in response to vascular injury and that this elevation in ADMA and L-NMMA results in impaired endothelial function [96, 313]. In addition to mechanical injury, studies have also demonstrated that exposure of endothelial cells to pro-atherogenic lipoproteins such as LDL, results in increased cellular ADMA levels [261]. Polyunsaturated fats in cholesterol esters, phospholipids and triglycerides are subjected to free radical oxidation. These polyunsaturated fatty acids can yield a variety of lipid hydroperoxides and highly reactive lipid peroxidation products such as the aldehyde 4-hydroxy-2-nonenal (4-HNE). During inflammation and oxidative stress levels of 4-HNE have been shown to accumulate in membranes at concentrations from 10 μm to 5 mM [290]. Moreover, studies have suggested that reactive aldehydes/carbonyls such as 4-HNE may play a critical role in the progression of atherosclerosis [291, 292]. Plasma concentrations of these lipid peroxidation products are known to increase relative to the progression of atherosclerosis, and during the oxidation of LDL high concentrations of these reactive aldehydes/carbonyls are formed. We thus hypothesized that elevations in lipid
peroxidation products may result in impaired endothelial function and atherosusceptibility, secondary to NOS impairment.

Therefore, studies were performed in order to determine the effects of the highly reactive lipid peroxidation product, 4-HNE, on endothelial-derived NO generation. Results demonstrated that the exposure of BAECs to 4-HNE caused a dose-dependent inhibition of cellular NO production. The observed 4-HNE effects were independent of changes in either NOS expression or phosphorylation state, as the Western blotting analysis revealed no changes in either endpoint. These results suggested that the observed NOS impairment involved mechanisms other than those related to protein expression. As such, subsequent experiments were performed in order to determine whether alterations in NOS cofactors or substrate may be involved in the decreased NO bioavailability. In this regard, oxidant stress, which has been shown to occur following exposure to lipid peroxidation products, has been shown to reduce the bioavailability of the critical NOS cofactor, H₄B [290, 311]. Loss of this cofactor results in NOS uncoupling evident by impaired NO synthesis and enhanced superoxide production from the enzyme [310]. Moreover, oxidant injury has also been demonstrated to increase the cellular levels of the endogenous methylarginine, ADMA [35]. Therefore, cellular studies were carried out to investigate the effects of adding both an antioxidant (GSH) to prevent H₄B oxidation as well as the eNOS substrate L-Arginine to overcome endogenous methylarginine-mediated NOS inhibition. Our data demonstrate that the addition of either GSH or L-Arginine alone had only modest NO-enhancing effects, however, co-incubation with both GSH and L-Arg was able to almost completely restore endothelial NO production. These data suggest that the observed NOS impairment involves both oxidant induced NOS inhibition (alleviated by the addition of GSH) as well as methylarginine accumulation (alleviated by the addition of excess substrate).
Direct measurement of ADMA levels and DDAH activity within cells by HPLC demonstrated that following 4-HNE challenge intracellular ADMA levels were increased greater than 2-fold. Based on previously published studies demonstrating the kinetics of ADMA mediated cellular inhibition, a 2 fold increase in methylarginine levels would be expected to inhibit NOS dependent NO generation by 20-30 % [96]. The additional inhibition observed could be due to compartmentalization or NOS uncoupling and increased NOS derived superoxide production in the presence of ADMA. To test this hypothesis, western blotting studies to measure nitrotyrosine formation (Figure 4-11). Although no significant increase in ONOO⁻ formation was observed, this does not rule out NOS uncoupling as superoxide generation from the enzyme is likely below detection limits. In this regard, we have also employed EPR spin-trapping techniques to measure eNOS derived endothelial superoxide production. These studies demonstrated increased levels of oxygen radicals which were inhibited by ~ 20% by L-NAME (Figure 4-12). L-NAME is currently the only known specific inhibitor of NOS derived superoxide production, however, this observation is based primarily on studies from purified enzyme. Because L-NAME is a methyl ester and is subject to modification by cellular esterases, its intracellular kinetics on NOS derived superoxide production are not well characterized. Nevertheless, increased endothelial superoxide production was observed from BAECs exposed to 4-HNE, however not all can be contributed to eNOS derived superoxide.

To determine whether the increased levels of ADMA observed following 4-HNE exposure resulted from changes in the activity of the ADMA metabolizing enzyme DDAH, its activity was measured. Studies of DDAH activity demonstrated a 40% decrease in hydrolytic activity, suggesting that the mechanism for the observed 4-HNE-directed NOS impairment was via an inhibition of DDAH. Additional studies were performed on purified recombinant hDDAH-1 in
order to determine whether 4-HNE effects were through direct interaction with the enzyme. Results demonstrated that incubation of hDDAH-1 with 4-HNE (50 µM) resulted in a > 40% decrease in enzyme activity. These effects were specific to 4-HNE as incubation with the non-oxidized carbonyl hexanol (10-500 µM) had no effect on DDAH activity (Figure 4-13). Similar studies were performed with purified recombinant eNOS and no inhibition was observed following 4-HNE exposure. 4-HNE forms Michael adducts with histidine and cysteine residues on proteins. In this regard, the catalytic triad of DDAH contains both cysteine and histidine residues and mutation of either amino acid has been demonstrated to render the enzyme inactive (Figure 4-14) [314-316].

As further support to the role of DDAH in mediating the inhibitory effects of 4-HNE on endothelial NO production, studies were performed using DDAH over-expressing BAECs. Over-expression of DDAH should lead to a decrease in cellular methylarginines with the concomitant increase in NOS-derived NO. DDAH over-expression was induced using an adenoviral construct carrying the human DDAH-1 gene (adDDAH1) (Figure 4-15). Preliminary studies demonstrated that incubation of BAECs with adDDAH1 at 25 MOI, resulted in a 3-fold increase in protein expression and a > 50% increase in DDAH activity following a 48 hour incubation. DDAH over-expression increased cellular DDAH activity in control cells by 50% and resulted in a 22% increase in cellular NO production (Figures 4-9 and 4-10), demonstrating that the endogenous levels of ADMA and L-NMMA are sufficient to significantly inhibit endothelial NO generation. If one then considers the 2-fold increase in the levels of ADMA observed following the 4-HNE treatment, a ~40 % inhibitory effect would be predicted [96].

Subsequently, a series of studies were performed using this same transduction protocol to examine the effects of DDAH over-expression on 4-HNE mediated endothelial NO inhibition.
Although DDAH over-expression did increase DDAH activity and decrease endogenous methylarginines, the over-expression of the enzyme alone was not sufficient to prevent the 4-HNE-induced decrease in NO production. In fact, our results demonstrated that exposure of DDAH over-expressing cells to 4-HNE resulted in worsened outcome as NO levels were significantly lower than that in the control cells exposed to 4-HNE. Although these results may appear contradictory to our hypothesis, they in fact support it and demonstrate that NOS uncoupling is likely occurring. We have previously demonstrated that ADMA inhibits nNOS-derived superoxide, and as such, DDAH over-expression in the presence of uncoupled NOS would be expected to eliminate ADMA and thus prevent ADMA mediated inhibition of NOS-derived superoxide. The outcome of this would be reduced NO bioavailability through the reaction of available NO with superoxide, a reaction which occurs at diffusion limited rates.

Our hypothesis would predict that treatment of DDAH over-expressing cells with an antioxidant would restore NO to levels similar to those observed with L-Arg and GSH treatment, if in fact methylarginines are contributing to the inhibition in NO generation seen with 4-HNE challenge. Indeed, we have demonstrated almost complete protection of cellular NO production following 4-HNE challenge using a combination of viral over-expression of DDAH and treatment with GSH, when compared to the respective control. These results would indicate that GSH alone reduces NOS uncoupling but not the methylarginine accumulation, while L-Arg supplementation and/or DDAH over-expression overcomes the 4-HNE-induced increase in methylarginines but not the NOS uncoupling.

In conclusion, our results demonstrate for the first time that the lipid peroxidation product 4-HNE can inhibit the endothelial NO production. The doses used in this study represent pathological levels of this highly reactive lipid peroxidation product and suggest that this
bioactive molecule may play a critical role in the endothelial dysfunction observed in a variety of cardiovascular diseases. The inhibitory effects of 4-HNE appear to be mediated through both oxidant stress and elevated levels of the endogenous NOS inhibitors ADMA and L-NMMA, as either L-Arg supplementation or DDAH over-expression in the presence of an anti-oxidant were able to restore NO production. Together, these results represent a major step forward in our understanding of the regulation, impact, and role of methylarginines and lipid peroxidation in cardiovascular disease.
Figure 4-1. Effects of 4-HNE on NO production. NO generation from BAECs stimulated with calcium ionophore A23187 (1 μM) was measured by EPR spin trapping with the Fe-MGD complex. The left panel shows the amplitude of the NO triplicate EPR spectrum over 10 consecutive 1 minute scans after a 30 minute incubation period. The right panel shows the EPR spectra and the dose dependent effects of the 4-HNE treatment on NO production. Results represent the mean ± SD. * indicates significance at p<0.05.
Figure 4-2. Effects of Hexanol on NO production. NO generation from BAECs stimulated with calcium ionophore A23187 (1 μM) was measured by EPR spin trapping with the Fe-MGD complex. The left panel shows the amplitude of the NO triplicate EPR spectrum over 10 consecutive 1 minute scans after a 30 minute incubation period. The right panel shows the EPR spectra and the dose dependent effects of the 4-Hexanol treatment on NO production. Results represent the mean ± SD. * indicates significance at p<0.05.
Figure 4-3. 4-HNE effects on eNOS expression and phosphorylation. Protein was obtained from BAECs treated with varying concentrations (1-50 µM) of 4-HNE for 24 hours. For measurements of eNOS, p-eNOSser1179 and p-eNOSThr 497 expression, 30 µg of protein was loaded into each well, Lane 1 is non treated cells, Lanes 2-5 are cells treated with 4-HNE.

Figure 4-4. Effects of 4-HNE on Ser1179 phosphorylation following calcium ionphore (5 µM, A23187) stimulation. Protein was obtained from BAECs treated with varying concentrations (1-50 µM) of 4-HNE for 24 hours. For measurements of eNOS and, p-eNOSser1179 expression, 30 µg of protein was loaded into each well. Lane 1 is the cells treated with A23187, Lane 2 is non treated cells, Lanes 3-6 are cells treated with 4-HNE
Figure 4-5. Effects of L-arginine and GSH supplementation on NO generation. NO generation from BAECs stimulated with calcium ionophore A23187 (1 μM) was measured by EPR spin trapping with the Fe-MGD complex. Experimental groups consisted of untreated (Control); 1 mM L-arginine supplementation (L-arg); 1 mM GSH supplementation (GSH); and 1 mM L-Arg with 1 mM GSH (L-arg + GSH). These experiments were performed both in the presence and absence of 4-HNE (50μM). Results represent the mean ± SD. * indicates significance at p<0.05.
Figure 4-6. Effects on 4-HNE on the levels of ADMA in BAECs. BAEC’s were cultured in T-75 flasks and exposed to 50 μM 4-HNE for 24 hours. Total cellular ADMA levels were measured using HPLC techniques and concentrations were determined as a factor of cell amount, cell volume and protein amount. Results represent the mean ± SD. * indicates significance at p<0.05.

Figure 4-7. 4-HNE effects on DDAH expression. DDAH-1 expression was measured by western blot techniques from BAECs treated with 50 μM 4-HNE for 24 hours.
Figure 4-8. 4-HNE effects on DDAH activity. DDAH activity was measured from BAEC homogenates following a 24 hour incubation with 4-HNE (50 μM) and DDAH activity was measured from purified recombinant hDDAH-1 (5 μg) following a 60 minute incubation with 50 μM 4-HNE. Experimental groups consisted of 50 μM 4-HNE (4-HNE) and 50 μM 4-HNE + 1 mM GSH (GSH). Results represent the mean ± SD. * indicates significance at p<0.05.
Figure 4-9. Effects of DDAH over-expression on endothelial cell NO production following 4-HNE challenge. NO generation BAECs stimulated with calcium ionophore A23187 (1 μM) was measured by EPR spin trapping with the Fe-MGD complex. Experimental groups consisted control vector (Control), DDAH over-expressing (DDAH), glutathione (1 mM) treated (GSH) and DDAH over-expression with GSH (1 mM) treatment (DDAH + GSH). These experiments were performed both in the presence and absence of 4-HNE (50 μM). Results represent the mean ± SD. * indicates significance at p<0.05.
Figure 4-10. Effects of 4-HNE on endothelial cell DDAH activity. DDAH activity was assessed by measuring the conversion of C14-L-NMMA to C14-Citrulline. Experimental groups consisted control vector (Control); DDAH over-expressing (DDAH); 1 mM glutathione supplementation (GSH); and DDAH over-expression with 1 mM GSH treatment (DDAH + GSH). These experiments were performed both in the presence and absence of 4-HNE (50 μM). Results represent the mean ± SD. * indicates significance at p<0.05.
Figure 4-11. Effects 4-HNE on nitrotyrosine formation in BAECs. BAECs were treated with 50 μM 4-HNE for 24 hours. Following the incubation period, cells were stimulated with calcium ionophore (5 μM A23187) and homogenized for western blot analysis. Results demonstrate no significant increase in protein tyrosine nitration.
Figure 4-12. Effects 4-HNE on ROS formation from BAECs. BAECs were treated with 50 µM 4-HNE for 24 hours. Following the incubation period, cells were stimulated with calcium ionophore (5 µM A23187) and EPR measurements were performed using the spin trap DMPO (50 mM). Results demonstrated an increase in the DMPO-OH adduct following 4-HNE treatment. This adduct was superoxide derived as it was largely quenched by the SOD mimetic M40403 (10 µM). The DMPO-OH adduct was inhibited by ~ 20% with L-NAME. ↓’s indicates the four peaks corresponding to the DMPO-OH adduct. The other 3 peaks are consistent with a carbon center radical.
Figure 4-13. Effects of Hexanol on DDAH-1 activity. DDAH activity was measured from purified recombinant hDDAH-1 (5 μg) following 60 minute incubation with Hexanol (10-500 μM). Results represent the mean ± SD. * indicates significance at p<0.05.
Figure 4-14. MS/MS spectra of a tryptic peptide generating the sequence b/y-ion series from the in-gel digest of the hDDAH-1 reacted with 4-HNE. The peptide observed at m/z 969.9+3 corresponds to the aa sequence 150-175 with H173 modified by HNE with the y3 – y13, y15 and y 20 ions labeled along with the corresponding b4 – b17, b24 – b25 ions labeled.
Figure 4-15. Adenoviral transduction of hDDAH-1 in BAECs. BAECs were treated with adDDAH-1 at various MOI (10-50) to determine optimum viral titer. Results demonstrate a dose dependent increase in DDAH-1 expression at 48 hours post infection.
REGULATION OF ENDOTHELIAL DERIVED SUPEROXIDE BY THE METHYLARGININES

Introduction

The biological significance of guanidino-methylated arginine derivatives has been known since the inhibitory actions of $\text{N}^\text{G}$–monomethyl-L-Arginine (L-NMMA) on macrophage induced cytotoxicity were first demonstrated. This naturally occurring arginine analog together with its structural congener asymmetric dimethylarginine (ADMA), are L-Arginine derivatives that are intrinsically present in tissues and they have the ability to regulate the L-Arginine:NO pathway. These two compounds, along with $\text{N}^\text{G}$–nitro-L-Arginine methyl ester (L-NAME), have been shown to be potent inhibitors of eNOS activity [35, 298, 306, 317].

NO has been demonstrated as a critical effector molecule in the maintenance of vascular function [318-320]. In the vasculature, NO is derived from the oxidation of L-Arginine (L-Arg), catalyzed by the constitutively expressed enzyme, eNOS [158, 162, 321]. This endothelial-derived NO diffuses from the vascular endothelium and exerts its effects on the smooth muscle cell layer where it activates guanylate cyclase leading to smooth muscle cell relaxation [318-320]. In addition to its role in the maintenance of vascular tone, NO helps to maintain the anti-atherogenic character of the normal vascular wall. NO, in concert with various cell signaling molecules, has been demonstrated to maintain smooth muscle cell quiescence and as such, counteracts pro-proliferative agents, specifically those involved in the propagation of atheroproliferative disorders [24-29, 322]. As such, eNOS dysfunction is an early symptom of vascular disease and is manifested through insufficient NO bioavailability. Among the potential mechanisms proposed for this NO deficiency is the uncoupling of NOS and subsequent production of superoxide anion radical ($\text{O}_2^-$)
Our laboratory and others have demonstrated that when cells are depleted of the NOS substrate L-Arginine (L-Arg) or the cofactor tetrahydrobiopterin (H4B), NOS switches from production of NO to O$_2^-$ [310, 323-329]. In the absence of either of these requisite substrates or co-factors, NOS mediated NADPH oxidation is uncoupled from NO synthesis and results in the reduction of O$_2$ to form O$_2^-$[323, 324, 328, 330]. O$_2^-$ exerts cellular effects on signaling and function that are quite different and often opposite to those of NO. Thus, O$_2^-$ is another very important NOS product, and its production may also be regulated by methylarginines. Furthermore, in view of their strong inhibition of NO generation, methylarginines could profoundly modulate the balance of NO and O$_2^-$ generation from the enzyme.

ADMA and L-NMMA are derived from the proteolysis of various proteins containing methylated arginine residues. The methylation is carried out by a group of enzymes referred to as protein-arginine methyl transferases (PRMTs). Subsequent proteolysis of proteins containing methylarginine groups leads to the release of free methylarginine into the cytoplasm where NO production from NOS is inhibited [35, 298, 317]. In addition to inhibition of NO generation, methylarginines may have other important effects on NOS function.

Cytosolic L-Arg concentrations are generally in the range of 100 to 200 μM, and moderate L-Arg depletion has been observed in conditions such as wound healing and aging [306, 331-336]. The redox active cofactor H$_4$B has been shown to be highly susceptible to oxidative stress. Oxidation of H$_4$B has been shown to result in NOS-derived O$_2^-$ generation [326, 327]. We have previously reported on the effects of methylarginines on nNOS-derived O$_2^-$ generation, however, little is known regarding the effects on eNOS [312]. Although L-NAME has been shown to block O$_2^-$ production from eNOS, studies using L-NMMA have suggested that this endogenous methylarginine does not appear to inhibit O$_2^-$ generation [324, 328, 329]. Furthermore, the
effects of ADMA on $O_2^-$ release from eNOS have not been reported. In addition, there have been no studies of the effects of endogenous methylarginines on the $O_2^-$ production that occur in H$_4$B -depleted enzyme. Therefore, critical questions remain regarding the fundamental effects of methylarginine analogues on eNOS function and the process of $O_2^-$ release from the enzyme. Since the levels of the intrinsic methylarginines, L-NMMA and ADMA, have been shown to be sufficient to modulate basal eNOS function in a variety of cardiovascular disease settings, it is critical to understand the concentration-dependent effect of these compounds on $O_2^-$ generation from the enzyme.

Therefore, in the present study, we have applied EPR spectroscopy and spin trapping techniques to measure the dose-dependent effects of ADMA and L-NMMA on the rates of $O_2^-$ production from eNOS under conditions of H$_4$B depletion with normal or depleted levels of L-Arg. We observe that while both of these endogenous methylarginines inhibit NO formation from H$_4$B -replete eNOS, in the presence of uncoupled-eNOS they significantly enhance eNOS-derived $O_2^-$. In addition, we observed that the native NOS substrate, L-Arg, also enhances eNOS-derived $O_2^-$. All of these substrates, result in enhanced NADPH consumption and shift in heme spin state of eNOS resulting in increased eNOS derived $O_2^-$. This observation has important pathological relevance as NOS uncoupling is know to occur in a variety of cardiovascular diseases.

**Materials and Methods**

**Expression and Purification of the Human Full Length eNOS and eNOS Oxygenase Domain (eNOSox)**

Human eNOS and eNOSox were expressed in E. coli similar to that previously described [337] and purified using metal affinity chromatography on a HisTrap FF column (GE Biosciences), followed by size exclusion chromatography using a HiLoad 16/60 Superdex 200
column (GE Biosciences). Full-length human eNOS and eNOSox expressed in bacteria are devoid of biopterin. All eNOS preparations were stored at liquid nitrogen temperature in buffer containing 50 mM HEPES, pH 7.5, 10% glycerol, and 0.15 M NaCl. H₄B (+) eNOS and H₄B (+) eNOSox were prepared by anaerobic incubation of purified proteins with 1 mM H₄B and 1 mM L-Arginine overnight at 4°C. Excess H₄B and L-Arginine were removed by gel filtration through a HiTrap desalting column at 4°C. Protein fractions were pooled, concentrated by Centriprep 30 (Amicon), and stored at liquid nitrogen temperature in the buffer described above. Typical NO generation activity of the final purified eNOS ranged between 80-120 nmol/mg/min, with eNOS concentration based upon heme content as determined by the pyridine hemochromogen assay.

**EPR Spectroscopy and Spin Trapping**

Spin-trapping measurements of NO and oxygen radical generation was performed using a either a Bruker ER 300 or a Bruker EMX spectrometer. The reaction mixture consisted of purified eNOS (50 nM) in 50 mM Tris, pH 7.4, containing 1 mM NADPH, 1 mM Ca²⁺, 30 μM EDTA, 10 μg/ml calmodulin, and 10 μM H₄B. For NO measurements, 25 nM eNOS and 100 μM L-Arg was added to the reaction system with Fe²⁺-MGD (0.5 mM Fe²⁺ and 5.0 mM MGD) used to trap NO, as previously described [338]. The samples were measured at X-band in a TM₁₁₀ cavity. Spectra were obtained using the following parameters: microwave power; 20 mW, modulation amplitude; 3.16 G, modulation frequency; 100 kHz. For the detection of O₂⁻, eNOS (50 nM) was used in a reaction system containing 10 mM DEPMPO as the spin-trap. Spectra were obtained using the following parameters: microwave power; 20 mW, modulation amplitude; 0.5 G, modulation frequency; 100 kHz. Although multiple EPR spectrometers were used for the studies, quantitation of the free radical signals was normalized to each system by comparing the double integral of the observed signal with that of a known concentration of
TEMPO free radical in aqueous solution. To quantify rates of $O_2^-$ generation, adduct signals were corrected for trapping efficiency and decay rate as previously described [339, 340]. Rates of $O_2^-$ formation were determined from the DEPMPO-OOH signal over the first 20 minutes of acquisition.

**NADPH Consumption by eNOS**

NADPH oxidation was followed spectrophotometrically at 340 nm [326]. The reaction systems were the same as described in EPR measurements, and the experiments were run at room temperature. The rate of NADPH oxidation was calculated using an extinction coefficient of 6.22 mM$^{-1}$ cm$^{-1}$.

**UV/Visible Spectroscopy**

Spectra were recorded on H$_4$B-free eNOSox (7.5 µM) in 50 mM sodium phosphate (pH 7.4) from 300 to 800 nm, and then again in the presence of either ADMA (500 uM) or L-NMMA (500 uM) using an Agilent 8453 diode array spectrophotometer.

**Results**

**Effects of Methylarginines on $O_2^-$ Production from H$_4$B Free eNOS**

We have previously reported that in the absence of H$_4$B, NOS generates $O_2^-$. Therefore, to measure NOS-derived $O_2^-$ EPR measurements were carried out as previously described with the nitrene spin-trap DEPMPO, which forms a stable $O_2^-$ adduct with half life of $\sim$16 minutes [340]. Initial studies were performed in the presence of L-Arg in order to determine the ability of H$_4$B-free eNOS to generate $O_2^-$. EPR results demonstrated a significant DEPMPO $O_2^-$ adduct which was inhibited by >80% in the presence of L-NAME (1 mM) and imidazole (5 mM). In addition, in the absence of Ca$^{2+}$, $O_2^-$ generation was almost completely blocked (Figure 5-1). These results demonstrate that the observed $O_2^-$ generation is eNOS-dependent and largely generated from the oxygenase domain, as the signal was quenched with imidazole.
Subsequent studies were performed in order to determine the concentration-dependent effects of ADMA, L-NMMA and L-Arginine on $O_2^-$ production from eNOS. EPR spin-trapping measurements were performed on $H_4B$-free eNOS as described in the methods. Purified eNOS was incubated in L-Arginine free buffer in the presence of NOS cofactors (NADPH, calmodulin, calcium). In the absence of L-Arg, eNOS gave rise to a strong DEPMPO-OOH signal characteristic of trapped $O_2^-$ (Figure 5-2). The effects of ADMA on $O_2^-$ release were then determined by adding varying concentrations of ADMA (1.0 to 100 μM). ADMA dose-dependently increased NOS-derived $O_2^-$ generation, with a 43 % increase at 1.0 μM, a 125 % increase at 10 μM, and a 151 % increase at 100 μM ADMA (Figure 5-2). Experiments were repeated in the presence of L-NMMA (1.0-100 μM). L-NMMA dose-dependently increased NOS-derived $O_2^-$ generation similar to that observed with ADMA, with a 18 % increase at 1.0 μM, a 80 % increase at 10 μM, and a 102 % increase at 100 μM L-NMMA (Figure 5-3). A final set of experiments were carried out to examine previous observations that the native substrate L-Arginine is capable of increasing eNOS-derived $O_2^-$ (21). Results demonstrated that L-Arginine dose-dependently increased eNOS-derived $O_2^-$ with a 26 % increase at 1.0 μM, a 116 % increase at 10 μM, and a 152 % increase at 100 μM L-Arginine (Figure 5-4). These results demonstrate that when eNOS is depleted of the critical cofactor $H_4B$, as has been shown to occur under conditions of oxidative stress, ADMA, L-NMMA and L-Arginine enhance $O_2^-$ generation.

Subsequent studies were then performed using an in-vitro system which could more closely mimic the disease setting wherein eNOS is uncoupled through reduced $H_4B$ bioavailability and cellular methylarginines are elevated in the presence of normal physiological levels of L-Arginine. Using this model, we measured the effects of ADMA and L-NMMA on $H_4B$-free eNOS-derived $O_2^-$ production in the presence of physiological levels of L-Arg (100
μM). As expected exposure to L-Arginine increased the rate of O$_2^-$ production 3 fold, and this increase was only mildly affected by the addition of ADMA (0.1 - 100 μM) (Figure 5-5). In contrast, L-NMMA (0.1 -100 μM) inhibited the formation of the observed O$_2^-$ adduct, with a ~30 % inhibition of the arginine-induced increase at 100 μM L-NMMA (Figure 5-6). Taken together, these results suggest that L-Arginine, ADMA, and L-NMMA, independently increase eNOS-derived O$_2^-$ . However, in the presence of physiological levels of L-Arginine, ADMA has little effect on eNOS-derived O$_2^-$, while L-NMMA inhibits O$_2^-$ . We hypothesized that these effects are mediated through alterations in the heme reduction potential upon ligand binding, leading to a faster transfer of electrons from the reductase domain to the heme. If so, we would then expect to observe an increase in NADPH consumption rate as a consequence of increased electron flow through the heme.

**Effects of methylarginines and L-Arginine on NADPH Consumption from H$_4$B-Free eNOS**

Experiments were performed to determine the effects of ADMA, L-NMMA and L-Arg on NADPH consumption rate from H$_4$B-free eNOS. Results demonstrated that ADMA dose-dependently increased the rate of NADPH consumption from H$_4$B-free eNOS from an initial rate of 55 nmols/mg/min at 0 μM ADMA to 86 nmols/mg/min at 10 μM (Table 5-1). L-NMMA also dose-dependently increased NADPH consumption rate with values of 79 nmols/mg/min observed in the presence of 10 μM L-NMMA (Table 5-1). L-Arginine had the most pronounced effects and like the methylarginines dose-dependently increased the rate of NADPH consumption with an observed rate of 92 nmols/mg/min at 10 μM L-Arginine (Table 5-1). Results from these studies support our previous observations that methylarginines and L-Arginine enhance electron flux through the H$_4$B -free enzyme, thus increasing O$_2^-$ generation.
Effects of Methylarginines on the Heme of eNOSox

L-Arginine and L-NMMA binding to NOS is known to alter the spin-state of the heme iron, and this change in spin-state is accompanied by a blue-shift in the Soret absorbance peak of the NOS heme [341-344]. By using only the oxygenase domain we can remove any spectral contributions due to the flavins. Additionally, expression of eNOSox is much more robust than the full length enzyme. Therefore, studies were performed in order to measure the effects of methylarginine binding on the heme spin-state of the eNOS-oxygenase domain. Results demonstrated that, both ADMA and L-NMMA caused a blue-shift in the Soret absorbance, from a ~412 nm in the resting eNOSox to ~397 nm (Figure 5-7). Thus, just as for arginine and L-NMMA, binding of ADMA produces a shift in the eNOS heme spin state to high-spin.

In summary, these results demonstrate for the first time that the methylarginines as well as the native NOS substrate, L-Arginine, enhance O$_2^-$ generation from H$_4$B -free eNOS. We hypothesize that these effects are mediated through increased electron transfer to the heme via a mechanism involving a change in the heme spin-state and the associated increase in the heme reduction potential that occurs upon inhibitor/substrate binding.

Discussion

It is well known that the endogenous methylarginine derivatives, ADMA and L-NMMA, are capable of regulating NO generation from purified eNOS, and we have previously shown that their intrinsic levels in the endothelium are ~10 μM and are able to basally regulate endothelial NO production [306]. However, their role in controlling O$_2^-$ release from the enzyme was unknown. Therefore, the current studies were carried out in order to characterize and quantify the dose-dependent effects of L-Arginine and the endogenous methylarginines on the O$_2^-$ generation from eNOS.
Over the last several years, studies have shown that in addition to producing NO, NOS is also capable of producing $O_2^-\text{ under conditions of L-Arginine or tetrahydrobiopterin depletion}$ [310, 324-328]. In the endothelium, this $O_2^-$ generation has been shown to be a significant mechanism of cellular injury [306, 328]. Although questions remain regarding the severity of these conditions that arise in normal cells, there is evidence that normal cellular oxidation of H$_4$B can increase $O_2^-$ release [327]. Furthermore, a range of disease conditions favor H$_4$B depletion. These include hypertension, diabetes, ischemia/reperfusion injury, and inflammatory processes [331-333, 345-349].

While prior studies have demonstrated that loss of the critical NOS cofactor, H$_4$B, results in NOS uncoupling and subsequent $O_2^-$ generation from the enzyme, the effects of the native substrate L-Arginine and its methylated NOS inhibitors, ADMA and L-NMMA, on eNOS-derived $O_2^-$ have been previously unknown. Prior studies from our laboratory have characterized the effects of ADMA and L-NMMA on nNOS-derived $O_2^-$. Results from the neuronal isoform demonstrated that that the endogenous methylarginines, ADMA and L-NMMA modulate NO production and that their effects on $O_2^-$ generation are H$_4$B dependent. In the presence of H$_4$B, ADMA selectively inhibited $O_2^-$ generation from the enzyme, while L-NMMA had no effect despite their structural similarities. However, when NOS was depleted of H$_4$B, ADMA no longer had any effect on $O_2^-$ production, while L-NMMA treatment resulted in a marked increase in $O_2^-$ production from the enzyme. Based on these observations, we carried out an extensive set of studies aimed at establishing the role of the methylarginines in regulating eNOS-derived $O_2^-$. 

Initial experiments were carried out in order to determine the ability of eNOS to produce $O_2^-$. Results demonstrated that in the absence of H$_4$B, eNOS gave rise to a strong DEPMPO-
OOH adduct characteristic of $O_2^\cdot$. This signal was calcium-dependent and largely quenched in the presence of L-NAME (1 mM) and imidazole (5 mM). Thus the observed $O_2^\cdot$ generation is eNOS-dependent and largely generated from the heme of the oxygenase domain as the signal was quenched with imidazole.

We observed that both ADMA and L-NMMA dose-dependently enhanced $O_2^\cdot$ generation from eNOS in the absence of H₄B. A significant, (43%) enhancement, of NOS-derived $O_2^\cdot$ was seen with 1 μM ADMA, increasing to a 151% enhancement at 100 μM. Of note, this $O_2^\cdot$ production is blocked by imidazole indicating that the observed increase is due to an increase in heme-derived $O_2^\cdot$. Results obtained using L-NMMA demonstrated that the monomethylarginine also enhanced heme-dependent $O_2^\cdot$ production with an 18% increase observed at 1.0 μM reaching a maximum of 102% at 100 μM.

Furthermore, the native eNOS substrate, L-Arg, which had been previously thought to reduce NOS generated $O_2^\cdot$, also significantly enhanced $O_2^\cdot$ production from H₄B-free eNOS in a dose-dependent manner. At 1 μM, L-Arginine increases NOS-derived $O_2^\cdot$ generation by 26%, 116% at 10 μM and by 152% at 100 μM. In support of our observations, it has recently been reported that the oxygen consumption of H₄B-replete eNOS is also stimulated by the addition of arginine [350, 351]. These results have important pathophysiological relevance, as normal cellular levels of L-Arg exceed 100 μM and would thus be expected to significantly augment NOS-derived $O_2^\cdot$ under conditions of reduced H₄B bioavailability. This raises important questions with regards to the current practice of nutraceutical supplementation with L-Arg in the treatment of cardiovascular diseases such as hypertension and atherosclerosis in which NOS-uncoupling is known to occur through oxidative loss of the cofactor H₄B. In this setting, L-
Arginine supplementation may actually exacerbate the disease resulting in increased NOS-derived $O_2^-$ and further reduced NO bioavailability.

Next we performed studies to determine the effects of the ADMA and L-NMMA on eNOS-derived $O_2^-$ in the presence of physiological levels of L-Arg (100 $\mu$M). Results from these studies demonstrated that the addition of ADMA and L-NMMA did not further increase NOS-derived $O_2^-$ and in fact, L-NMMA decreased $O_2^-$ with a ~30% reduction observed at 100 $\mu$M L-NMMA. Thus, as arginine is replaced by L-NMMA there is decreased enhancement of the eNOS-derived $O_2^-$, because L-NMMA binding produces less stimulation of the eNOS-derived $O_2^-$ compared to the stimulation induced by L-Arginine binding. ADMA competition has very little effect because ADMA and L-Arginine binding produce very similar levels of stimulation of eNOS-derived $O_2^-$. It should be noted that the precise interpretation of the competition data must include differences in binding affinities and binding cooperatively for L-Arg, ADMA, and L-NMMA. Nevertheless, our results suggest that under normal or pathological conditions wherein total methylarginines would not be expected to exceed 20-30 $\mu$M, their major effect on eNOS would be to inhibit NO generation with only modest effect on NOS-derived $O_2^-$. However, if L-Arginine levels are low, the methylarginines would then increase NOS-derived $O_2^-$ from uncoupled eNOS. These results differ significantly from what was previously observed with nNOS, wherein we demonstrated that only L-NMMA was capable of enhancing nNOS-derived $O_2^-$ generation. In this previously published study [312], we demonstrated that L-Arginine and ADMA had no effect on nNOS-derived $O_2^-$ under conditions of $H_2B$/L-Arg depletion, while L-NMMA increased $O_2^-$ production by greater than 2 fold. Moreover, when experiments were carried out using $H_2B$-free nNOS in presence of L-Arg (100 $\mu$M), L-NMMA
effects were maintained and $\mathrm{O}_2^-$ generation dose-dependently increased with L-NMMA concentration, while ADMA had no effect [312].

The mechanism of $\mathrm{O}_2^-$ production from the heme in NOS first requires the transfer of an electron from the reductase domain to the heme, generating the ferrous iron which can bind oxygen. Subsequently, the one electron reduced $\mathrm{O}_2^-$ can dissociate, regenerating the ferric heme. The rate limiting step in this process for eNOS is the initial reduction of the heme [352, 353]. As such, if the reduction of the heme is made more favorable, then the rate of $\mathrm{O}_2^-$ production will be increased. It has been shown that binding of arginine and L-NMMA to the NOS isoforms produces a shift in heme spin-state, which can be monitored spectrophotometrically. This arginine-induced shift in spin-state is accompanied by an increase in the NOS heme redox potential to less negative values [354], theoretically this would produce an increased rate of electron transfer from the reductase domain to the heme. This correlation between spin-state and heme midpoint potential is also found in the related cytochrome P450 family [355, 356]. Furthermore, it is known that the less negative heme redox potential produced by L-Arginine binding to the inducible NOS (iNOS) is accompanied by an increase in NADPH oxidase activity [357]. Thus, we hypothesized that the mechanism for the observed arginine and methylarginine-enhanced $\mathrm{O}_2^-$ production was via an increase in electron flow through eNOS produced by a change in the heme redox potential in response to a ligand-induced change in heme spin-state.

Indeed, we found that just like L-Arginine and L-NMMA, ADMA binding to eNOS produced a shift in the heme to the high-spin state, which in turn will result in a less negative heme redox potential. Results from the NADPH consumption studies supported this hypothesis and demonstrated that ADMA, L-NMMA and L-Arginine dose-dependently increased electron transfer through the heme, consistent with a ligand-induced increase in heme reduction potential.
The rate of NADPH consumption increased in the following order: no-substrate < L-NMMA < ADMA < L-Arg. These data support the hypothesis that the inhibitory actions of the methylarginines on $O_2^-$ generation in the presence of L-Arginine resulted from less enhancement of electron transfer relative to L-Arginine. Thus, taken together our data support the hypothesis that ligand-induced changes in the heme spin-state induced by L-Arginine and the methylarginines are at least partially responsible for the observed increase in eNOS-derived $O_2^-$. However, it is clear that there are other factors to consider.

L-NAME, which also induces the formation of the high-spin eNOS upon binding, very effectively inhibits $O_2^-$ formation from eNOS. This discrepancy has been noted for iNOS, and it was proposed that an electrostatic interaction between an electron rich ligand and the NOS heme inhibits reduction of the NOS heme and thus decreases NADPH oxidation [358]. Conversely, an electrostatic interaction between a positively charged arginine or methylarginine side chain and the heme iron, would theoretically favor the ferrous form of the heme, producing a less negative midpoint potential, and thus increasing the rate of electron transfer to the heme. Additionally, substrate binding is known to stabilize the dimeric form of the enzyme, and since heme reduction is via an inter-monomer electron transfer, this ligand-induced structural stabilization could affect the rate of this transfer.

It has been proposed that the NOS isoforms can produce $H_2O_2$ via a two electron reduction of molecular oxygen [351, 359, 360]. For this to occur, the rate of transfer of a second electron to the ferrous-heme $O_2^-$ complex, either from $H_4B$ or from the reductase domain, must exceed the rate of superoxide release. It has been demonstrated that in the absence of L-Arginine (or other substrate) the sole product of $H_4B$ -free eNOS is $O_2^-$ [351]. It is possible that our proposed substrate-induced increase in reductase-to-heme transfer rate in the $H_4B$-free eNOS could allow
for the direct production of H$_2$O$_2$. However, in preliminary experiments comparing O$_2^-$ consumption to NADPH oxidation of the H$_4$B -free enzyme, the addition of substrates produced similar increases in both O$_2^-$ consumption and NADPH oxidation (unpublished results). Thus, although more definitive work is necessary, we have found no evidence for the substrate/inhibitor-induced direct production of H$_2$O$_2$ from uncoupled eNOS.

In conclusion, the substrate L-Arginine, and the endogenous inhibitors ADMA and L-NMMA, increase the O$_2^-$ generation from uncoupled eNOS by making the transfer of electrons to the heme more favorable via mechanisms involving the modulation of the heme spin-state, altering the electrostatic environment of the heme, and/or by altering the structural stability of the active dimer. These findings have important clinical implications as methylarginine levels have been demonstrated to be elevated in a variety of cardiovascular diseases associated with oxidative stress. In addition, L-Arginine supplementation in these conditions may exacerbate the NOS uncoupling observed in these conditions.
Figure 5-1. Inhibition of NOS-derived O2.- from H4B depleted eNOS. EPR spin-trapping measurements of O2.- production from eNOS (50 nM) were performed in the presence of L-arg (100 μM) as described in Methods. The right panel shows the spectra of the O2.- adduct observed. The left panel shows the total amount of NOS-derived O2.- generation occurring over a 30-minute period. The results show the effects of L-NAME (500 μM), Imidazole (1 mM) and Ca2+-CAM removal on NOS-derived O2.- production. Both inhibitors largely blocked NOS-derived O2.- generation. In the absence of calcium and calmodulin, no signal was observed. Results shown represent the mean ± SEM of 5 experiments.
Figure 5-2. Effects of ADMA on eNOS-derived \( \text{O}_2^- \). EPR spin-trapping measurements of \( \text{O}_2^- \) production from H\(_4\)B-free eNOS (50 nM) were performed with the addition of ADMA (0.01-100 \( \mu \)M) and NOS cofactors as described in Figure 5-1. The right panel shows the spectra observed after 30 min. The DEPMPO-OOH adduct signal was clearly seen. The left panel shows the time-course of NOS-derived \( \text{O}_2^- \) generation determined from the observed EPR spectra recorded over a 40-minute period in a series of experiments. Results graphed are the mean ± SEM. In the absence of H\(_4\)B, NOS gave rise to a prominent DEPMPO-OOH signal characteristic of \( \text{O}_2^- \) and this was dose-dependently increased by ADMA (1.0 \( \mu \)M-100 \( \mu \)M).
Figure 5-3. Effects of L-NMMA on eNOS-derived $\mbox{O}_2^-$ EPR spin-trapping measurements of $\mbox{O}_2^-$ production from H$_4$B-free eNOS (50 nM) were performed with the addition of L-NMMA (0.01-100 μM) and NOS cofactors as described in Figure 5-1. The right panel shows the spectra observed after 30 min. The DEPMPO-OOH adduct signal was clearly seen. The left panel shows the time-course of NOS-derived $\mbox{O}_2^-$ generation determined from the observed EPR spectra recorded over a 40-minute period in a series of experiments. Results graphed are the mean ± SEM. In the absence of H$_4$B, NOS gave rise to a prominent DEPMPO-OOH signal characteristic of $\mbox{O}_2^-$ and this was dose-dependently increased by L-NMMA (1.0 μM-100 μM).
Figure 5-4. Effects of L-arg on eNOS-derived O$_2^-$ EPR spin-trapping measurements of O$_2^-$ production from H$_4$B-free eNOS (50 nM) were performed with the addition of L-arg (0.01-100 μM) and NOS cofactors as described in Figure 5-1. The right panel shows the spectra observed after 30 min. The DEPMPO-OOH adduct signal was clearly seen. The left panel shows the time-course of NOS-derived O$_2^-$ generation determined from the observed EPR spectra recorded over a 40-minute period in a series of experiments. Results graphed are the mean ± SEM. In the absence of H$_4$B, NOS gave rise to a prominent DEPMPO-OOH signal characteristic of O$_2^-$ and this was dose-dependently increased by L-arg (1.0 μM-100 μM).
Figure 5-5. Effects of ADMA on O$_2^-$ production from H$_4$B-depleted NOS in the presence of L-arg. EPR spin-trapping measurements of O$_2^-$ production from eNOS (50 nM) were performed in the presence of 100 μM L-arg, with the addition of ADMA (0.1-100 μM) and NOS cofactors (w/o H$_4$B) as described in Figure 5-1. Results show the time-course of NOS-derived O$_2^-$ generation determined from the observed EPR spectra recorded in a series of experiments. H$_4$B depleted eNOS gave rise to a prominent DEPMPO-OOH signal characteristic of O$_2^-$, which was increased in the presence of L-arg and unaffected by ADMA (0.1-100 μM). Results graphed are the mean ± SEM.
Figure 5-6. Effects of NMMA on NOS-derived O$_2^-$ in the presence of L-arg. EPR spin-trapping measurements of O$_2^-$ production from eNOS (50 nM) were performed in the absence of 100 μM L-arg, with the addition of NMMA (0.1-100 μM) and NOS cofactors as described in Figure 5-1. Results show the time-course of NOS-derived O$_2^-$ generation determined from the observed EPR spectra recorded in a series of experiments. H$_4$B-depleted eNOS gave rise to a prominent DEPMPO-OOH signal characteristic of O$_2^-$, which was increased in the presence of L-arg and unaffected by ADMA (0.1-100 μM). Results graphed are the mean ± SEM.
Figure 5-7. Methylarginines alter the eNOS-bound heme. The UV/Vis spectrum for the eNOS oxygenase domain (7.5 µM) in 50 mM sodium phosphate (pH 7.4) was recorded from 300 to 800 nm, and then again in the presence of either ADMA (500 uM) or NMMA (500 uM).
Table 5-1. Effects of Methylarginines and L-arg on NADPH consumption from H₄B-free eNOS (100 nM)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>0.0 µM</th>
<th>0.1 µM</th>
<th>1.0 µM</th>
<th>10.0 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-arginine</td>
<td>55±2</td>
<td>67±3</td>
<td>79±3</td>
<td>92±4</td>
</tr>
<tr>
<td>ADMA</td>
<td>55±2</td>
<td>62±2</td>
<td>74±3</td>
<td>86±2</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>55±2</td>
<td>61±3</td>
<td>70±4</td>
<td>79±3</td>
</tr>
</tbody>
</table>

The dose-dependent effects of ADMA, L-NMMA and L-arg on NADPH oxidation was followed spectrophotometrically at 340 nm [326]. The reaction systems were the same as described in EPR measurements, and the experiments were run at room temperature for 2 minutes. Results are ± SEM.
CHAPTER 6
REGULATION OF DIHYDROFOLATE REDUCTASE IN THE DIABETIC ENDOTHELIUM

Introduction

Endothelial derived Nitric Oxide (NO) is synthesized from the oxidation of the guanidino carbon of the amino acid L-Arginine to NO and L-citrulline. This reaction is catalyzed by the enzyme nitric oxide synthase (NOS). NO is a potent vasodilator and critical effector molecule involved in the maintainence of vascular homeostasis, through its anti-proliferative and anti-thrombotic effects. NO, in concert with various cell signaling molecules, has been demonstrated to maintain vascular smooth muscle cell quiescence and as such, counteracts pro-proliferative agents specifically those involved in the propagation of athero-proliferative disorders. Diabetes has long been associated with increased oxidative stress and impaired vascular function. NOS dysregulation and decreased NO bioavailability have been implicated as a central mechanism in vascular endothelial dysfunction observed in diabetes. Several studies have demonstrated that while eNOS protein levels are increased in the diabetic state, NO bioavailability decreases and superoxide production increases [361]. Among the proposed mechanisms that lead to decreased NO bioavailability and eNOS uncoupling is oxidation of the essential NOS cofactor Tetrahydrobipetrin (H4B). In vitro studies have demonstrated that H4B stabilizes and donates electrons to the ferrous-dioxygen complex in the oxygenase domain of eNOS to help facilitate the oxidation of the substrate L-Arginine. Furthermore, studies have demonstrated that depletion of H4B causes electrons to be donated to molecular oxygen, turning NOS from a NO generating enzyme to an oxidase. This phenomenon has been termed “NOS uncoupling” and it has been documented in the pathophysiology of various diseases including atherosclerosis and diabetes (56,119,120). H4B is highly redox sensitive and can be readily oxidized to its inactive form dihydrobipterin (H2B). Therefore, it is likely that during increased oxidative stress intracellular
levels of H₄B fall leading to NOS uncoupling and the enzyme primarily being a O₂⁻ generating enzyme.

The synthesis of H₄B occurs via two pathways in the endothelial cell, the de novo and salvage pathways (Figure 6-1). De novo biosynthesis of H₄B is a magnesium, zinc and NADPH dependent pathway. The first step requires the conversion of GTP to 7,8-dihydroneopterin triphosphate. This reaction is catalyzed by the enzyme GTP cyclohydrolase I (GTPCH), and it is the rate limiting step in H₄B biosynthesis [52]. Following the GTPCH enzyme reaction pyruvoyl tetrahydropterin synthase (PTPS) converts 7,8 dihydroleopterin triphosphate into 6-pyruvoyl-5,6,7,8-tetrahydropterin. Alternatively, the salvage pathway enzyme dihydrofolate reductase (DHFR) is a NADPH dependent enzyme that catalyzes the conversion of H₂B to H₄B (140). The functionality of the DHFR enzyme in the endothelial cell was unknown until recently. The strongest evidence for the involvement of DHFR involvement in regulating endothelial NO production has come from DHFR gene silencing studies. Specifically, Chalupsky et al. demonstrated that DHFR gene silencing resulted in a significant reduction in H₄B levels, as well as a 50% reduction in endothelial NO production [133]. Furthermore, DHFR over-expression was able to abolish the production of O₂⁻ in angiotensin II stimulated cells [133]. Because DHFR activity has been demonstrated to be involved in the regulation of NO bioavailability, it is important to understand how the enzyme activity is affected in disease states.

Therefore, studies were carried out to determine the effects of oxidative stress and metabolic dysregulation on DHFR activity. We observed that DHFR is highly resistant to most oxidants at concentrations within the pathophysiological range. We also observed that at low concentrations of OONO- DHFR activity is significantly increased. Additionally, using the diabetic db/db mouse model we observed reduced DHFR activity, impaired vascular function
and increased eNOS derived superoxide in the aorta. These observations have important implications for the role of oxidative stress and its effects on DHFR activity as it relates to the diabetic state.

Materials and Methods.

Materials

DHFR, H$_2$O$_2$, Xanthine and Xanthine Oxidase were purchased from Sigma-Aldrich (St Louis, MO). Peroxynitrate was purchased from Millipore (Lake Placid, NY) Diethylamine NONOate was purchased from Sigma=Aldrich (St.Louis, MO)

DHFR Activity Assay

For kinetic measurements of enzyme activity, human recombinant DHFR (6.0 ug) was incubated for 5 minutes at 25°C in 50 mM Tris buffer (pH 7.5) in the presence of .01-1000 µM substrate with a total reaction volume of 100 µl. Following incubation each sample was added to a 96 well plate and NADPH consumption was measured at 340 nM. The rate of NADPH consumption was calculated using an extinction coefficient of 6.22 mM$^{-1}$ cm$^{-1}$.

Tissue DHFR Activity

For kinetic measurements of tissue DHFR activity, kidneys were removed from age matched control mice and db/db mice on a C57/Blk6 background. The samples were homogenized in diionized water with ascorbic acid (1 mg/ml) to prevent auto-oxidation. Protein concentration was measured by the Bradford assay. 250 µg of protein, 200 µm H$_2$B and 1 mM NADPH were incubated together for 30 minutes at 37°C in a water bath. Following the incubation the samples were then loaded into a Centricon filter with a 3,000 molecular weight cut off and centrifuged at 10,000 x g, 4° C for 60 minutes.
HPLC Techniques

DHFR activity assay was performed by measuring the conversion of H₂B to H₄B using HPLC techniques. 20 µl of the filtrate was then injected into onto the HPLC column using an ESA HPLC with electrochemical gradient detection a 400 mV and 800 mV. The mobile phase consisted of Buffer A (100 mM KH₂PO₄, 25 mM octyl sodium sulfate, 0.6 mM EDTA PH 2.5), Buffer B (2% MeOH) run at room temperature with a flow rate of 1.3 ml/ml.

Vascular Reactivity

Contraction and relaxation of isolated aortic rings were measured in an organ bath containing modified Krebs-Henseleit buffer (118 mM NaCl, 24 mM NaHCO₃, 4.6 mM KCl, 1.2 mM NaH₂PO₄, 1.2 mM CaCl₂, 4.6 mM HEPES and 18 mM glucose) aerated with 95%CO₂/5%O₂, 37°C. Aortic rings were cut into 2-to-3 mm segments and mounted on a wire myograph (Danish Myo, Aarhus, Denmark). Contraction was measured via a force transducer interfaced with Chart software for data analysis. Following a 30-min incubation equilibration period, the rings were stretched to generate a tension of 0.5 g. 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 1 µM phenylephrine. The vascular relaxation response was determined using increasing concentrations of acetylcholine (0.1 nM to 10 µM).

EPR Spin Trapping Studies

Given the millisecond-range half-life of superoxide in situ, electron paramagnetic resonance (EPR) assay involves the approach of using the superoxide spin-trap, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl pyrroldidine HCl (CMH hydrochloride; Axxora) (8, 9, 11, 16, 18) to generate a stable chemical product, 3-methoxycarbonyl- proxyl, by a general method. Stock solution of CMH (10 mM) dissolved in EPR buffer [PBS containing 2µM Diethyldithiocarbamate (Sigma-Aldrich) and 50 µM Desferrioxamine (Sigma-Aldrich)] and
purged with Nitrogen, were prepared daily and kept under nitrogen on ice. Six aortic ring segments (1 mm) were placed in 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). Frozen samples were analyzed with a Benchtop ESR Spectrometer (Bruker Biospin) in a finger Dewar filled with liquid nitrogen with following EPR 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.

Results

Enzyme Kinetics of DHFR

Enzyme kinetic studies were performed to establish the $K_m$ and $V_{max}$ values for H$_2$B. The kinetic activity of human DHFR (hDHFR) was measured by the detection of NADPH consumption as described under the “Material and Methods.” $K_m$ and $V_{max}$ values were derived using the Michaelis-Menten equation and generated values of 48 µM and 6.7 µmols/mg/min, respectively (Figure 6-2).

Effect of Oxidants on DHFR Activity

Previous studies have demonstrated that DHFR expression can be affected following exposure to H$_2$O$_2$. Because H$_4$B is known to be highly sensitive to oxidants leading to its oxidation to H$_2$B, it may also be possible that DHFR is modulated by the redox environment which could result in impaired H$_4$B recycling. Therefore, we carried out a series of studies aimed at determining the dose dependent effects of NO, OONO’, H$_2$O$_2$, and O$_2$’on DHFR activity. NO studies were carried out using the NO donor compound DEANONate (1 µM-1 mM). H$_2$O$_2$ (1 µM-1 mM) and OONO’ (0.01 µM-1 mM) studies were carried out using the authentic oxidant. For O$_2$-, we used a generating system consisting of xanthine and xanthine oxidase (1 µM-1 mM). In order to prevent Fenton type reactions in the H$_2$O$_2$ experiments, the Fe chelator DTPA (100 µM) was added. The exposure of DHFR to oxidants was found to have a
modest effect on enzyme activity. Results demonstrated that following exposure to NO, hDHFR activity was dose dependently reduced with 38 % inhibition observed at 100 µM NO and 53% inhibition observed at 1 mM (Figure 6-3). Results also demonstrated that exposure to H₂O₂ dose dependently decreased activity with 31% inhibition at 100 µM H₂O₂ and 53% inhibition at 1 mM (Figure 6-4). Furthermore, O₂.dose dependently inhibited hDHFR activity as 1 µM and 1 mM elicited a 28% and 70% inhibition respectively (Figure 6-5). Additional studies demonstrated that hDHFR activity was significantly increased 56-58% following exposure to 0.01 µM and 1 µM of OONO⁻ (Figure 6-6). In contrast, at the 10 µM and 100 µM concentrations no significant changes in activity were observed. However, exposure to 1 mM OONO- resulted in a modest inhibition of 24%.

Effects of the Diabetic State on In-Vivo DHFR Activity

Previous studies have suggested that increases in oxidative stress, as have been observed in diabetes, contribute to decreased endothelial NO generation through a mechanism involving the loss of H₄B. In support, it has been observed in several studies that H₄B supplementation restores endothelial NO generation (56,54,209). However, whether DHFR activity is also sensitive to the redox environment is unknown. Therefore, in order to determine the effects of the diabetic disease state on DHFR activity, basal activity in the kidney of db/db mice and age matched controls was measured. Using HPLC techniques, hDHFR activity was measured as described in the “Materials and Methods”. Results demonstrated that db/db mice had significantly more H₂B than the age matched control mice. Furthermore, the control mice produced significantly more H₄B than the db/db mice (Figure 6-7). Overall, these results suggest that the diabetic condition of the db/db mice results in a significant decrease in DHFR activity, which is likely involved in eNOS uncoupling as a result of altered B₂H/B₄H ratios. To confirm
this, we preformed additional studies to determine the effects of decreased DHFR activity on vascular function.

**Effects of the Diabetic State on Vascular Reactivity**

Our previous results demonstrated that tissue DHFR activity was reduced in the diabetic db/db mouse model, which resulted in reduced levels of H4B. Therefore, vascular studies were performed using mouse aortic rings and the vascular relaxation in response to acetylcholine (1 µM) was measured. The percent relaxation to 1 µM Ach was then compared among the control and db/db groups. Results demonstrated that db/db mice had a 35% reduction in vascular relaxation in response to 1 µM Ach, when compared to their aged matched controls (Figure 6-8).

**Effects of the Diabetic State on eNOS Derived O2⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻--; -production in the Aorta

Previous studies have demonstrated that depletion of H4B under oxidative stress conditions increases the production of eNOS derived superoxide in the vasculature (54,211). Although our data demonstrated that db/db mice have a significant reduction in DHFR activity and impaired vascular function, we wanted to examine whether the loss of DHFR activity would also resulted in increased vascular eNOS derived O2⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻--; production in the aorta. Therefore, EPR spin trapping studies were carried out to measure eNOS derived O2⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻--; in the aorta. Results demonstrated that the aorta of db/db mice produced significant O2⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻--;, while in wt type age matched controls it was undetectable. This increase in O2⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻--; production was attenuated following exposure to the NOS inhibitor L-NAME (Figure 6-9). Overall, these studies support the hypothesis that loss of DHFR activity is involved in the endothelial dysfunction associated with diabetes and that loss of DHFR activity increases eNOS derived O2⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻--; in the aorta.

**Discussion**

There is a growing body of evidence indicating that the increased cardiovascular risks associated with diabetes are due to oxidative stress and eNOS dysfunction. In support, several
studies have reported that the pathology of diabetes results in decreased endothelial NO production, impaired vascular function, and increases in eNOS derived O$_2^-$ generation (54,56,211). The primary pathway way for H$_4$B synthesis is through the de novo pathway which involves the rate limiting enzyme GTPCH I. Previous studies have demonstrated that inhibiting GTPCH I leads to impaired vascular relaxation in response to Ach. Over-expression of GTPCH I was found to partially restore vascular function in diabetic mice (208). However, under pathological conditions in which oxidative stress increases and in which H$_4$B can be readily oxidized, DHFR activity maybe critically important in maintaining H$_4$B levels and endothelial NO production. Therefore, studies were carried out in order to investigate the redox regulation of DHFR and its role in diabetic vasculopathy.

Despite its importance of H$_4$B regulation during oxidative stress, few studies have been done regarding the enzymatic activity of DHFR. Furthermore, the few studies which exist have been conducted using rat brain homogenates. In this regard, we have recently measured the kinetic parameters of the human isoform of DHFR. Results from these studies demonstrated the $K_m$ value of 48 µM and $V_{max}$ value of 6.7 µmols/mg/min for H$_2$B. The $K_m$ value obtained correlates well with the previously published report in which the value of 88 µM was reported. However, the maximal enzymatic activity that we report here differs from the previously published using DHFR from rat brain, in which a value of 30 pmole/mg/min was reported, however this was for dihydrofolate metabolism, as DHFR is also known to be an important enzyme in folate metabolism [362].

Cai et al. demonstrated that following exposure to H$_2$O$_2$, DHFR expression decreased in cultured endothelial cells [133]. These studies suggest that DHFR activity may be modulated by the redox environment. Therefore, studies were conducted in order to determine the effect of
various reactive oxygen and nitrogen species on DHFR activity. Following exposure of DHFR to NO, OONO-, H2O2, O2-, we observed that at concentrations between 1 µM -100 µM, NO elicited a 31-38% decrease in DHFR activity. Additionally, at the 1 mM concentration a 53% loss in enzyme activity was observed. In support of our results, previous studies have demonstrated that following exposure to NO, proteins can be s-nitrosylated at active cysteine residues resulting in altered enzyme activity (95,96,97). Similar effects on DHFR activity were observed following exposure to H2O2. We also observed a dose dependent decrease in DHFR activity in the presence of O2- with 1 µM and 1mM eliciting a 28% and 70% decrease in enzyme activity respectively. Additional studies exposing DHFR to OONO- at low pathophysiological relevant concentrations (0.1 µM- 1 µM) demonstrated a significant increase DHFR activity. This result was surprising as OONO- has been demonstrated to increased eNOS derived superoxide production in vascular aortic rings, suggesting it has a role in eNOS uncoupling [363]. In contrast, higher pathophysiological concentrations of OONO'(10-100 µM) resulted in no change to DHFR activity. Only a modest inhibition of 24% was observed following exposure to 1 mM OONO' however, this does not represent physiological or pathophysiological relevant concentrations. Overall these results suggest that the DHFR enzyme is moderately sensitive to ROS mediated inhibition in the pathophysiological/physiological relevant dose ranges.

However, OONO- at pathophysiologically relevant levels induced a significant increase DHFR activity. This is an intriguing finding given that ONOO- has been shown to be the most potent oxidizer of H4B and may represent a novel compensatory mechanism for the cell to maintain adequate H2B / H4B ratios.

In prior studies it has been observed that diabetes is associated with increased oxidative stress, and NOS uncoupling. However, no studies to date have examined the role of diabetes and
its effects on DHFR activity. Therefore, we carried out in vivo studies in order to determine the 
effects of the diabetic state on tissue DHFR activity. We observed that in the kidney of db/db 
mice, DHFR activity was significantly inhibited when compared to wt age matched control. This 
decrease in DHFR activity resulted in increased H2B levels in db/db mice. Our findings are in 
line with previous reports of increased H2B levels in diabetic mice [361, 364]. In addition, 
previous reports have also demonstrated that alterations in the H2B/ H4B ratio is an important 
trigger in NOS uncoupling [208]. Taken together these results represent a potential mechanism 
linking diabetes to vascular endothelial dysfunction.

Next, we carried out studies to determine the effect of the loss of DHFR activity on aortic 
vascular relaxation. Results demonstrated a 35% impairment of the NO mediated vascular 
relaxation in db/db mice when compared to the wt age matched controls. Additional studies 
were carried out to determine the effect of decreased DHFR activity and eNOS derived O2− in 
the aorta. In contrast to the wild type mice, resulted demonstrated that eNOS derived O2− was 
detectable in the isolated aorta of db/db mice. In support of our findings, in the streptozotocin 
induced model of diabetes, mice were observed to have impaired vascular function and increased 
aortic eNOS derived O2− that was attenuated in the presence of H4B [204].

Overall these results provide evidence for our hypothesis that loss of DHFR activity leads 
to endothelial dysfunction in diabetes. Future studies using a gene therapy approach will be 
carried out in order to provide further evidence for the importance of the modulation of DHFR 
activity and its role in vascular endothelial dysfunction. In addition to animal studies, cellular 
studies will examine the effects of the H2B/ H4B ratio in regards to preserving endothelial NO 
production. We hypothesis that adenoviral mediated over expression of the DHFR gene will
result in improved vascular endothelial function and endothelial NO generation in the diabetic condition.

In conclusion, this is the first study to demonstrate that DHFR can be regulated by redox environment in vivo. However, most oxidants have a modest effect on DHFR activity. Also, we have demonstrated for the first time that OONO- increases DHFR activity, which could potentially be protective mechanism in which the cell acts to preserve endothelial NO generation. Furthermore, we have also demonstrated that the loss of DHFR in db/db mice results in impaired vascular relaxation and increased eNOS derived $O_2^-$. Moreover, the loss of DHFR activity may represent a novel mechanism in endothelial dysfunction associated with diabetes.
Figure 6-1. H₄B biosynthesis pathway. H₄B is synthesized in the endothelial cell by either the de novo or salvage pathway.
Figure 6-2. DHFR enzyme kinetics. hDHFR was incubated in the presence of varying concentrations of B$_2$H(1 µM-1 mM). DHFR activity was measured by the rate of NADPH consumption as measured by absorbance at 340 nm. The Km and Vmax were fitted using the Michaelis-Menten equation. The $K_m$ was found to be 48 µM and the $V_{max}$ was found to be 6.7 µmols/mg/min.
Figure 6-3. Effects of Nitric Oxide on hDHFR activity. hDHFR was exposed to varying concentrations (1 µM-1 mM) of Nitric Oxide. hDHFR activity measured by the rate of NADPH consumption as measured by absorbance at 340 nm. Nitric oxide was found to dose dependently reduce hDHFR activity with 38% inhibition observed at 100 µM NO and 53% inhibition observed at 1mM. Results represent the mean ± SD n=3
Figure 6-4. Effects of H$_2$O$_2$ on hDHFR activity. hDHFR was exposed to varying concentrations (1 µM-1 mM) of H$_2$O$_2$. hDHFR activity measured by the rate of NADPH consumption as measured by absorbance at 340 nm. H$_2$O$_2$ dose dependently decreased hDHFR activity with 31% inhibition at 100 µM H$_2$O$_2$ and 53% inhibition at 1 mM. Results represent the mean ± SD n=3
Figure 6-5. Effect of $\text{O}_2^-$ on dDHFR activity. hDHFR was exposed to varying concentrations of (0.01 µM-1 mM) of Xanthine Oxidase and Xanthine (1 unit/mg) to generate $\text{O}_2^-$. hDHFR activity was measured by NADPH consumption as measured by absorbance at 340 nm. $\text{O}_2^-$ was demonstrated to dose dependently inhibit DHFR activity with 1 µM and 1 mM eliciting a 28% and 70% inhibition respectively. Results represent the mean ± SD. * indicates significance at $p<0.05$. n=3
Figure 6-6. Effects of OONO⁻ on hDHFR activity. hDHFR was exposed to varying concentrations of (0.01 µM-1 mM) of OONO⁻. hDHFR activity was measured by NADPH consumption as measured by absorbance at 340 nm. OONO⁻ was observed to increase DHFR activity 56-58% following exposure to 0.01 µM and 1 µM. Exposure to 1 mM OONO⁻ resulted in a modest inhibition of 24%. Results represent the mean ± SD. * indicates significance at p<0.05. n=3
Figure 6-7. Effects of the diabetic condition on in-vivo DHFR activity. HPLC studies were carried out to measure basal DHFR activity in the kidneys of db/db and wild type age matched control mice. DHFR activity was observed to be significantly decreased in the db/db mice population (2) vs. the age matched controls (1). Peak (3) is a H₂B standard.
Figure 6-8. Effects of the diabetic state on vascular reactivity. The effects of the diabetic state on vascular relaxation response to AcH(0.1-1 μM) were determined using mice aortic rings from db/db mice and age matched wild type controls. Following phenylephrine-induced constriction, Ach (0.1 nM to 10 μM) was added to the bath and the relaxation response was measured. Results represent the mean ± SD. * indicates significance at p<0.05. n=4
Figure 6-9. Effects of the diabetic condition on eNOS derived $O_2^-$ in the aorta. EPR spin-trapping measurements of $O_2^-$ production from mouse aortic rings were performed. The panel shows the spectra of the $O_2^-$ adduct observed. The results show the effects of L-NAME (500 µM) on eNOS derived $O_2^-$ production, which blocked eNOS derived $O_2^-$. 
ADMA and L-NMMA are endogenous NOS inhibitors derived from the proteolysis of methylated arginine residues on various proteins. The methylation is carried out by a group of enzymes referred to as protein-arginine methyl transferase’s (PRMT’s) [35]. In mammalian cells, these enzymes have been classified into type I (PRMT1, 3, 4, 6, and 8) and type II (PRMT5, 7, and FBXO11) enzymes, depending on their specific catalytic activity. Both types of PRMT, however, catalyze the formation of mono-methylarginine (MMA) from L-arginine (L-Arg). In a second step, type I PRMT’s produce asymmetric dimethylarginine (ADMA), while type II PRMT catalyzes symmetric dimethylarginine (SDMA) [365, 366]. Subsequent proteolysis of proteins containing methylarginine groups leads to the release of free methylarginine into the cytoplasm where NO production from NOS can be inhibited. Free cytoplasmic MMA and ADMA are degraded to citrulline and mono- or dimethylamines by dimethylarginine dimethylaminohydrolases (DDAH) [367]. While on the other minor clearance of unchanged plasma methylarginines are cleared from the circulation by renal excretion and hepatic metabolism [304, 367]. In addition to the DDAH pathway, ADMA can also be converted to α-keto valeric acid by alanine:glyoxylate aminotransferase [368], although the influence of this pathway on total ADMA metabolism has not been extensively studied thus far. Moreover, the demethylation of methylarginines is believed to be restricted to free methylarginines, as a potential mechanism for possible demethylation of protein-incorporated methylarginines in situ have not yet been identified. It should be noted, however, that the conversion of protein-incorporated L-NMMA to citrulline by peptidylarginine deiminase 4 was recently demonstrated, which prevented histone methylation by PRMT 1 and 4 [369, 370]. This may influence protein methylation directly, as L-NMMA deimination will decrease the amount
of protein-incorporated MMA that is available for dimethylation by PRMT, but the relevance of protein deimination of protein-incorporated MMA by PAD enzymes has been challenged recent [369].

Asymmetric dimethylarginine (ADMA) plasma levels have been shown to be elevated in diseases related to endothelial dysfunction including hypertension, hyperlipidemia, diabetes mellitus, and others [267, 268, 270-272]. Moreover, it has been shown that ADMA predicts cardiovascular mortality in patients who have coronary heart disease (CHD). Recent evidence published from the multicenter Coronary Artery Risk Determination investigating the Influence of ADMA Concentration (CARDIAC) study has indicated that ADMA is indeed an independent risk factor for CAD [273]. However, whether the increased risk associated with elevated ADMA is a direct result of NOS impairment is an area of controversy. Significant debate about the contribution of ADMA to the regulation of NOS-dependent NO production has been initiated.

In pathological conditions such as pulmonary hypertension, coronary artery disease, diabetes and hypertension, plasma ADMA levels have been shown to increase from an average of ~0.4 µM to ~0.8 µM [269, 272, 273, 276-278]. Given that these values are at least 2 orders of magnitude lower than the plasma L-arg levels it is unlikely that elevated plasma ADMA can significantly regulate eNOS activity. It is more likely that elevated plasma ADMA levels reflect increased endothelial concentrations of ADMA. In support of this hypothesis, we and others have demonstrated that endothelial ADMA levels increase 3-4 fold in restenotic lesions and in the ischemia reperfused myocardium [96, 279]. Based on cellular kinetic inhibition studies from our lab in which we observed significant inhibition of NO production at 5µM ADMA, these concentrations of ADMA would be expected to elicit a 30-40% inhibition in NOS activity [96]. These studies however involve lesion specific increases in ADMA and are not associated with
increased plasma levels of ADMA and would not be expected to contribute to systemic cardiovascular pathology. In this regard, there is little direct evidence that elevated plasma ADMA levels are associated with increased endothelial ADMA nor is it clear whether plasma ADMA directly contributes to the NOS inhibition observed in chronic cardiovascular diseases and other disease such as end stage renal disease.

The principal mechanism put forth to explain the pathological role of ADMA in cardiovascular diseases has focused on DDAH. It has been demonstrated that diabetes and hypertension are associated with reduced DDAH activity which is believed to result in ADMA accumulation to levels associated with NOS inhibition. However, a direct cause-effect relationship between DDAH activity and NOS inhibition has not been demonstrated. It has been estimated that more than 80% of ADMA is metabolized by DDAH [267], however, it is unclear which DDAH isoform represents the principal methylarginine metabolizing enzyme. PCR and western blot analysis has revealed that the endothelium contains mRNA and protein for both DDAH-1 and DDAH-2. However, in order to assess the relative contribution of each isoform a detailed analysis of the enzyme kinetics of each isofrom is necessary. Unfortunately, detailed biochemical studies have only been published for DDAH-1. Using purified recombinant hDDAH-1 we and others have demonstrated the precise enzyme kinetics of this isofrom and results demonstrated $K_m$ values of 68.7 and 53.6 μM and $V_{max}$ values of 356 and 154 nmols/mg/min for ADMA and L-NMMA, respectively [228, 249]. In regards to DDAH-2, previous attempts at purifying the protein have been unsuccessful primarily due to solubility issues with recombinant enzyme expressed in e.coli. Recently we have successfully purified recombinant human DDAH-2 from bacterial inclusion bodies using a protein refolding method with L-arginine and cyclodextrin. Initial results demonstrate a $K_m$ value of 16 μM and $V_{max}$
value of 14.8 nmols/min for ADMA (unpublished results). Thus the apparent rate of ADMA metabolism for DDAH-2 is almost 10 times less than that of DDAH-1. Based on these enzyme kinetics, DDAH-1 is likely the principal ADMA metabolizing pathway in the endothelium. Nevertheless, there is significant controversy in the field regarding which DDAH isoform is responsible for endothelial methylarginine metabolism. Along these same lines, it is also unclear whether diseases associated with reduced DDAH activity represent loss of DDAH-1 or DDAH-2 activity. Therefore, the studies described in chapter 3 were carried out in order to address these issues and identify the role of DDAH-1 and DDAH-2 in the regulation of endothelial NO production.

It has been widely reported that DDAH-2 is the predominant DDAH isoform in the vascular endothelium; however these studies have widely relied on assessing the expression of the DDAH isoforms in various cell and tissue types [237, 240, 281, 282]. Consequently, studies were carried out in BAECs to determine which isoform is responsible for the majority of the DDAH activity in the endothelial cell. DDAH-1 and DDAH-2 gene silencing decreased total DDAH activity by 64% and 48%, respectively. There is a possibility that DDAH-2 gene silencing could have an effect on DDAH-1 but further studies need to be done. Additional studies demonstrated that dual gene silencing only resulted in a 50% loss total DDAH activity in BAECs thus suggesting that other methylarginine metabolic pathways may be invoked as a consequence of loss of DDAH activity. To investigate the possibility that loss of DDAH activity may lead to the induction of other methylarginine metabolic enzymes we used HPLC techniques to measure the metabolic products of L-NMMA. In control cells we observed 3 peaks with radioactive counts and they were identified as L-NMMA, L-arginine and L-citrulline. The formation of radiolabeled L-citrulline is likely from the metabolism of L-NMMA by DDAH.
while radioactive L-arg is generated from citrulline recycling through ASS and ASL. In contrast, results from DDAH-1 and DDAH-2 silenced cells indicated the presence of 4 radioactive peaks including L-NMMA, L-arginine, L-citrulline and a yet unidentified peak. The concentration of this unidentified peak increased 2 fold in the dual silencing group as compared to the levels in either the DDAH-1 or DDAH-2 silencing groups alone. Initial mass spec analysis has been unsuccessful in identifying the unknown species and is currently an area of active investigation in our lab. Regardless, the results clearly indicate that the endothelium possesses alternate inducible pathways for metabolizing methylarginines.

Subsequent studies were carried out to assess the role of DDAH-1 and DDAH-2 in the regulation of endothelial NOS activity. Results demonstrated that adenoviral mediated over-expression of both DDAH-1 and DDAH-2 increased cellular endothelial NO production. These initial studies were done in the presence of basal methylarginine levels and demonstrate that normal endogenous levels of these NOS inhibitors are present at concentration sufficient to regulate eNOS activity. It had previously been proposed that ADMA may be responsible for the “arginine paradox” and these studies would appear to support the hypothesis. However, subsequent studies using L-arg supplementation with DDAH over-expression demonstrated an additive effect which clearly indicates that ADMA is not involved in the “arginine paradox”.

Studies were then performed using siRNA to silence both the DDAH-1 and DDAH-2 genes in BAECs. It was anticipated that silencing of DDAH would lead to increased cellular methylarginines and decreased endothelial NO production. Results supported this prediction and demonstrated that DDAH-1 silencing reduced endothelial NO production by 27% while DDAH-2 silencing reduced it by 57%. These studies were then repeated with L-arg supplementation in order to establish the ADMA dependence of the DDAH effects. The addition of L-arg (100 µM)
was able to restore ~50% of the loss of endothelial NO generation observed with DDAH-1 silencing. Although it may be predicted that L-arg supplementation should completely restore NO production given that ADMA is a competitive inhibitor of NOS, these result are consistent with previously published studies and suggest that DDAH-1 silencing may lead to ADMA accumulation in sites that are not freely exchangeable with L-arg. In support of this hypothesis it has been demonstrated by Simon et al. that within the endothelial cell exists two pools of arginine both which eNOS has access to. Pool I is largely made up of extracellular cationic amino acids transported through the CAT transport system, however Pool II does not freely exchange with extracellular cationic amino acids. Furthermore they also demonstrated that Pool II is separated into two components. Pool II A participates in the recycling of citrulline to arginine, while Pool II B is occupied by protein derived by-products. It is within this Pool II B where the methylarginines are likely to accumulate, thus rending its inhibitory effects on eNOS [280]. Furthermore, the studies were only done using one concentration of L-Arg, it would be interesting to see what effects higher doses would have endothelial NO production. Alternatively, ADMA and/or DDAH may elicit effects that are independent of NOS, this appears to be the most plausible explanation with regards to DDAH-2 wherein loss of activity reduced endothelial NO production by greater than 50% and the loss was unaffected by L-arg supplementation. This is strong evidence that DDAH may elicit effects that are independent of ADMA. Although this may represent an overall paradigm shift with regards to the role of DDAH in the endothelium, it is not with out support. Specifically, Cooke et al. have demonstrated that DDAH-1 transgenic mice are protected against cardiac transplant vasculopathy [241, 242]. Using in-vivo siRNA techniques, Wang et al. demonstrated that DDAH-1 gene silencing increased plasma levels of ADMA by 50% but this increase had no
effect on endothelial dependent relaxation. Conversely, in vivo DDAH-2 gene silencing had no effect on plasma ADMA, but reduced endothelial dependent relaxation by 40% [237]. These latter findings are particularly intriguing and demonstrate that elevated plasma ADMA is not associated with impaired endothelial dependent relaxation while loss of DDAH-2 activity is associated with impaired endothelial dependent relaxation, despite the fact the plasma ADMA levels are not increased (40). This provides strong evidence that DDAH effects are not limited to ADMA dependent regulation of eNOS.

The most convincing evidence that DDAH may regulate cellular function through mechanisms independent of ADMA mediated NOS inhibition come from data on the DDAH-1 knockout mouse. Homozygous null mice for DDAH-1 are embryonic lethal while the NOS triple knockout mice are viable [240]. This provides strong evidence that DDAH effects are not limited to ADMA dependent regulation of eNOS. Using DDAH1 heterozygous mice, which are viable, Leiper et al. demonstrated that reduced DDAH-1 activity leads to accumulation of plasma ADMA and a reduction in NO signaling. These animals exhibited a 50% decrease in DDAH activity which was associated with a 20% increase in plasma and tissue ADMA levels [240]. This in turn was associated with vascular pathology, including endothelial dysfunction, increased systemic vascular resistance and elevated systemic and pulmonary blood pressure. Given that the intracellular concentrations of ADMA are 1-3 μM, it is unlikely that a 20% increase in ADMA could be responsible for the 40% reduction in endothelial dependent relaxation observed with the DDAH+/− mice. Moreover, the addition of exogenous L-arg to the organ chambers only partially restored the loss in endothelial relaxation [240]. These results further support the hypothesis that DDAH modulates endothelial function through both ADMA-NOS dependent
pathways as well as independent. Although this represents an overall paradigm shift, it is not surprising given the lethality of the DDAH-1 knockout mouse.

Together, these results demonstrate that both DDAH-1 and DDAH-2 are involved in the regulation of endothelial NO production; however, while DDAH-1 effects are largely ADMA-dependent, DDAH-2 effects appear to be ADMA-independent. In this regard, elevated plasma ADMA may serve as a marker of impaired methylarginine metabolism and the pathology previously attributed to elevated ADMA may be manifested, at least in part, through altered activity of the enzymes involved in ADMA regulation, specifically DDAH and PRMT.

Although increased plasma levels of ADMA are associated with cardiovascular disease, it is the endothelial ADMA levels that are implicated in the regulation of NOS activity. It is therefore surprising that, to date, there have been no studies examining the cellular kinetics of ADMA synthesis and metabolism in the endothelium. It is generally accepted that PRMT’s synthesize methylarginines on proteins using the methyl donor SAM and L-arg as the terminal methyl acceptor. It is then believed that normal protein turnover releases free methylarginines which are then metabolized to citrulline by DDAH. In this regard, loss of DDAH activity has been implicated as the molecular trigger for ADMA accumulation and subsequent endothelial dysfunction. It is our hypothesis that there is crosstalk among these pathways and that the levels of both free and protein incorporated methylarginines play important roles in regulating endothelial function, including but not limited to eNOS regulation. In summary, dysregulation of the PRMT-DDAH-ADMA axis has now been shown to contribute to the pathogenesis of several cardiovascular disorders, in experimental animal models as well as human disease. Causal relationships between dysregulated arginine-methylation and the initiation, progression, or therapy of disease, however, remain to be dissected. Future investigations into arginine-
methylation and DDAH dynamics in disease states are clearly needed in order elucidate the role of this post-translational modification in the pathogenesis of cardiovascular disease.

The results from chapter 3 clearly demonstrated that loss of DDAH activity was associated with NOS impairment. This raises a critical question regarding the mechanisms of DDAH regulation in disease. Among the mechanisms proposed for the loss of DDAH activity associated with cardiovascular disease is redox modification of DDAH in response to oxidative stress. In support, Leiper et al. have demonstrated that NO inhibits the activity of DDAH-1 through a mechanism involving the formation of S-nitrosyl complexes within the catalytic domain of DDAH-1. Therefore, in chapter 4 we carried out a series of studies to investigate the effects of altered redox state and oxidative stress on DDAH activity and endothelial NO production. Using purified human recombinant DDAH-1, our lab and others have previously demonstrated that hDDAH-1 was largely resistant to oxidants (ONOO⁻, H₂O₂, OH⁻, O₂⁻) as concentrations exceeding 100 µM were needed to elicit any significant inhibition [228, 249]. However, significant inhibition was seen with the lipid peroxidation product 4-HNE and the inhibition occurred at concentrations associated with pathological conditions.

Results demonstrated that the exposure of BAECs to 4-HNE caused a dose-dependent inhibition of cellular NO production. The observed 4-HNE effects were independent of changes in either NOS expression or phosphorylation state, as the Western blotting analysis revealed no changes in either endpoint. These results suggested that the observed NOS impairment involved mechanisms other than those related to protein expression. As such, subsequent experiments were performed in order to determine whether alterations in NOS cofactors or substrate may be involved in the decreased NO bioavailability. In this regard, oxidant stress, which has been shown to occur following exposure to lipid peroxidation products, has been shown to reduce the
bioavailability of the critical NOS cofactor, \( \text{H}_4\text{B} \) [290, 311]. Loss of this cofactor results in NOS uncoupling evident by impaired NO synthesis and enhanced superoxide production from the enzyme [310]. Moreover, oxidant injury has also been demonstrated to increase the cellular levels of the endogenous methylarginine, ADMA [35]. Therefore, cellular studies were carried out to investigate the effects of adding both an antioxidant (GSH) to prevent \( \text{H}_4\text{B} \) oxidation as well as the eNOS substrate L-Arginine to overcome endogenous methylarginine-mediated NOS inhibition. Our data demonstrate that the addition of either GSH or L-Arginine alone had only modest NO-enhancing effects, however, co-incubation with both GSH and L-Arg was able to almost completely restore endothelial NO production. These data suggest that the observed NOS impairment involves both oxidant induced NOS inhibition (alleviated by the addition of GSH) as well as methylarginine inhibitory effects (alleviated by the addition of excess substrate).

Direct measurement of ADMA levels and DDAH activity within cells by HPLC demonstrated that following 4-HNE challenge intracellular ADMA levels were increased greater than 2-fold. Based on previously published studies demonstrating the kinetics of ADMA mediated cellular inhibition, a 2 fold increase in methylarginine levels would be expected to inhibit NOS dependent NO generation by 20-30 \% [96]. The additional inhibition observed could be due to compartmentalization or NOS uncoupling and increased NOS derived superoxide production in the presence of ADMA. To determine whether the increased levels of ADMA observed following 4-HNE exposure resulted from changes in the activity of the ADMA metabolizing enzyme DDAH, its activity was measured. Studies of DDAH activity demonstrated a 40\% decrease in hydrolytic activity, suggesting that the mechanism for the observed 4-HNE-directed NOS impairment was via an inhibition of DDAH. Additional studies were performed on purified recombinant hDDAH-1 in order to determine whether 4-HNE effects
were through direct interaction with the enzyme. Results demonstrated that incubation of hDDAH-1 with 4-HNE (50 μM) resulted in a > 40% decrease in enzyme activity. These effects were specific to 4-HNE as incubation with the non-oxidized carbonyl hexanol (10-500 μM) had no effect on DDAH activity. Similar studies were performed with purified recombinant eNOS and no inhibition was observed following 4-HNE exposure. 4-HNE forms Michael adducts with histidine and cysteine residues on proteins. In this regard, the catalytic triad of DDAH contains both cysteine and histidine residues and mutation of either amino acid has been demonstrated to render the enzyme inactive. [314-316].

As further support to the role of DDAH in mediating the inhibitory effects of 4-HNE on endothelial NO production, studies were performed using DDAH over-expressing BAECs. Over-expression of DDAH should lead to a decrease in cellular methylarginines with the concomitant increase in NOS-derived NO. DDAH over-expression was induced using an adenoviral construct carrying the human DDAH-1 gene. DDAH over-expression increased cellular DDAH activity in control cells by 50% and resulted in a 22% increase in cellular NO production. If one then considers the 2-fold increase in the levels of ADMA observed following the 4-HNE treatment, a ∼40 % inhibitory effect would be predicted [96].

Subsequently, a series of studies were performed using this same transduction protocol to examine the effects of DDAH over-expression on 4-HNE mediated endothelial NO inhibition. Although DDAH over-expression did increase DDAH activity and decrease endogenous methylarginines, the over-expression of the enzyme alone was not sufficient to prevent the 4-HNE-induced decrease in NO production. In fact, our results demonstrated that exposure of DDAH over-expressing cells to 4-HNE resulted in worsened outcome as NO levels were significantly lower than that in the control cells exposed to 4-HNE. Although these results may
appear contradictory to our hypothesis, they in fact support it and demonstrate that NOS uncoupling is likely occurring. The mechanism involved methylarginine mediated regulation of eNOS derived superoxide. These findings are consistent with studies presented in chapter 5 wherein using electron paramagnetic resonance spin trapping techniques we measured the dose dependent effects of ADMA and L-NMMA on \( \cdot \text{O}_2^- \) production from eNOS under conditions of \( \text{H}_4\text{B} \) depletion. In the absence of \( \text{H}_4\text{B} \), ADMA dose dependently increased NOS derived \( \cdot \text{O}_2^- \) generation, with a maximal increase of 151 % at 100 \( \mu \text{M} \) ADMA. L-NMMA also dose dependently increased NOS derived \( \cdot \text{O}_2^- \), but to a lesser extent, demonstrating a 102 % increase at 100 \( \mu \text{M} \) L-NMMA. Moreover, the native substrate L-arginine also increased eNOS derived \( \cdot \text{O}_2^- \), exhibiting a similar degree of enhancement as that observed with ADMA. Measurements of NADPH consumption from eNOS demonstrated that binding of either L-arginine or methylarginines increased the rate of NADPH oxidation. Spectrophotometric studies suggest, just as for L-arginine, that binding of ADMA and L-NMMA shift the eNOS heme to the high-spin state, indicative of a more positive heme redox potential, enabling enhanced electron transfer from the reductase to the oxygenase site. These results demonstrate that the methylarginines can profoundly shift the balance of NO and \( \cdot \text{O}_2^- \) generation from eNOS. These observations have important implications with regard to the therapeutic use of L-arginine and the methylarginine-NOS inhibitors in the treatment of disease. While these studies were done using purified enzyme, in the cell, eNOS is known to have various cofactors that may play a role in eNOS derived superoxide. Currently, the effects of methylarginines on cellular eNOS derived superoxide are an area of active investigation in our lab.

Based on the results presented thus far, our hypothesis would predict that treatment of DDAH over-expressing cells with an antioxidant would restore NO to levels similar to those
observed with L-Arg and GSH treatment, if in fact methylarginines are contributing to the inhibition in NO generation seen with 4-HNE challenge. Indeed, we demonstrated almost complete protection of cellular NO production following 4-HNE challenge using a combination of viral over-expression of DDAH and treatment with GSH, when compared to the respective control. These results would indicate that GSH alone reduces NOS uncoupling, but not the methylarginine accumulation, while L-Arg supplementation and/or DDAH over-expression overcomes the 4-HNE-induced increase in methylarginines but not the NOS uncoupling.

The research presented demonstrates for the first time that the lipid peroxidation product 4-HNE can inhibit the endothelial NO production. The doses used in this study represent pathological levels of this highly reactive lipid peroxidation product and suggest that this bioactive molecule may play a critical role in the endothelial dysfunction observed in a variety of cardiovascular diseases. The inhibitory effects of 4-HNE appear to be mediated through both oxidant stress and elevated levels of the endogenous NOS inhibitors ADMA and L-NMMA, as either L-Arg supplementation or DDAH over-expression in the presence of an anti-oxidant were able to restore NO production. Together, these results represent a major step forward in our understanding of the regulation, impact, and role of methylarginines and lipid peroxidation in cardiovascular disease.

Altered redox status of the endothelium has been implicated as a central mechanism in the endothelial dysfunction associated with cardiovascular diseases. Based on the results described in these studies, we propose that loss of DDAH activity under conditions of oxidative stress contributes to the pathogenesis of cardiovascular disease through its effects on NOS derived NO and superoxide production. Specifically, we believe that decreased DDAH activity inhibits eNOS derived NO production through both ADMA-dependent and independent pathways.
Moreover, the ADMA accumulation that occurs as a result of loss of DDAH activity is also involved in the perpetuation of eNOS derived superoxide which likely contributes to the NO inactivation observed in cardiovascular disease.

Biochemical studies using recombinant NOS have demonstrated that eNOS, in the absence of H₄B, has the potential to be a major source of superoxide with catalytic rates approaching those of NADPH Oxidase and Xanthine Oxidase [371]. However, cellular studies of eNOS derived superoxide have revealed that H₄B depletion alone does not significantly increase superoxide fluxes [372, 373]. Instead, it appears that increased levels of the H₄B oxidation product, H₂B, is the molecular trigger for eNOS uncoupling. Evidence for this hypothesis is supported by our data as well as work from Gross et al. in which they demonstrated 48-h exposure to diabetic glucose levels (30 mM) caused H₂B levels to increase from undetectable to 40% of total biopterin. This H₂B accumulation was associated with diminished NO activity and accelerated superoxide production. However, it is unclear why H₂B accumulates in cellular and animal models of diabetes. Bioaccumulation of H₂B or quinoid H₂B following oxidation of H₄B 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 H₄B. Given that numerous studies have clearly identified increased H₂B formation in diabetes suggests that these conditions are likely associated with impaired pterin salvage or recycling pathways. Therefore, we hypothesized that in diabetes, enzymes involved in either the H₄B salvage or recycling pathways are impaired resulting in an inability to maintain adequate H₄B levels. The result is eNOS uncoupling and altered NO and ROS signaling which leads to diabetic vascular dysfunction.
Therefore, the studies described in chapter 6 were carried out to investigate pterin regulation in diabetes. Initial studies were conducted in order to determine the effect of ROS and RNS on DHFR activity. These studies demonstrated that the DHFR enzymatic activity is sensitive to ROS with significant inhibition observed with pathophysiologically relevant doses of superoxide, NO and H$_2$O$_2$. In contrast, OONO- at pathophysiologically relevant levels induced a significant increase DHFR activity. This is an intriguing finding given that ONOO- has been shown to be the most potent oxidizer of H$_4$B and may represent a novel compensatory mechanism for the cell to maintain adequate H$_2$B / H$_4$B ratios.

Although previous studies have clearly demonstrated increased oxidative stress and NOS uncoupling in diabetes, no studies to date have examined the role of DHFR in this process. Therefore, we carried out in vivo studies in order to determine the effects of the diabetic state on tissue DHFR activity. We observed that in the kidney of db/db mice, DHFR activity was significantly inhibited when compared to wt age matched controls. This decrease in DHFR activity resulted in increased H$_2$B levels in db/db mice. Functional studies were also performed to determine the effect of the loss of DHFR activity on aortic vascular relaxation. Results demonstrated a 35% impairment of the NO mediated vascular relaxation in db/db mice when compared to the wt age matched controls. This decreased endothelial dependent relaxation was associated with increased eNOS derived O$_2^-$ in the aorta as measured by EPR. In contrast to the wild type mice, eNOS derived O$_2^-$ was detectable in the isolated aorta of db/db mice. These findings are in line with previous reports of increased H$_2$B levels in diabetic mice [361, 364] and implicate DHFR as a key regulatory element involved in eNOS dysregulation.

In summary, the data presented in this thesis demonstrate a critical role for the DDAH-ADMA axis in the pathogenesis of endothelial dysfunction associated with cardiovascular
diseases. Evidence suggests that DDAH is capable of modulating both eNOS derived NO and superoxide generation through ADMA-dependent as well as independent pathways.


BIOGRAPHICAL SKETCH

Arthur Pope was born in 1982 in Chicago, IL. He graduated from the Illinois Mathematics and Science Academy in 2001. Following graduation, he attended the University of Illinois at Urbana Champaign and obtained a B.S. degree in Chemistry in 2005. He then enrolled in the Integrated Biomedical Science Program at The Ohio State University College of Medicine in June of 2005 to obtain his doctorate of philosophy. In January 2006 he joined Dr AJ Cardounel’s lab and in June of 2007 he relocated with his mentor to the University of Florida joining the Interdisplenary Program in Biomedical Sciences where he obtained his doctorate of philosophy in August of 2009. During his graduate training he received a pre-doctoral fellowship award from the NIH National Heart Lung and Blood Institute. Arthur has also been the first author on three publications, and co-author on two others. He also has presented his research at several conferences and has had two invited talks.