STUDIES ON THE INFLUENCE OF FOLIC ACID AND RIBOFLAVIN ON NITRIC OXIDE PRODUCTION IN CULTURED MURINE MACROPHAGE CELLS

By

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ABSTRACT OF THE THESIS

Studies on the influence of folic acid and riboflavin on nitric oxide production in cultured murine macrophage cells

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Methylenetetrahydrofolate reductase (MTHFR) is a flavin adenine dinucleotide (FAD) - dependent, folate-metabolizing enzyme. The main product of MTHFR is methylenetetrahydrofolate, an important substrate for homocysteine metabolism and synthesis of methionine and the universal methyl donor, S-adenosylmethionine. MTHFR also catalyzes the reduction of dihydrobiopterin to tetrahydrobiopterin (BH4). BH4 is an essential cofactor for nitric oxide synthase (NOS) that produces nitric oxide (NO) for multiple functions including vasodilation and blood pressure (BP) regulation. Genome-wide

association studies have linked a common polymorphism, C677T, in MTHFR with BP, and riboflavin supplements have been shown to reduce BP in individuals homozygous for the 677TT variant form of the enzyme. We hypothesize that this relationship between MTHFR, riboflavin, and BP is mediated through nitric oxide synthesis and that deficiency in either folic acid or riboflavin will result in decreased NO output.

We investigated this hypothesis *in vitro* in stimulated murine macrophage RAW cells, which express the inducible form of NOS (iNOS). The cells were exposed to standard and reduced levels of folic acid and riboflavin. They were then stimulated with LPS. The cells were grown in medium with 0.4 mg/L riboflavin and 4.0 mg/L folic acid (control), 0.04 mg/L riboflavin (LowB2), or 0.4 mg/L folic acid (LowFA) for 48 hours, and then exposed to 100 ng/ml or 1000 ng/ml lipopolysaccharide (LPS) for 24 hours. Media was then analyzed for nitric oxide production by chemiluminescence assay using a Nitric Oxide Analyzer. Quantitative PCR was used to analyze gene expression of iNOS and arginase. In all three media conditions, no differences in RAW cell proliferation rates were observed over 48 hours. After LPS exposure, nitric oxide production in the LowB2 and LowFA cells was 30-35% and 35-40% of the control cells, respectively ($p \leq 0.001$). Expression of iNOS after LPS induction increased in all three media conditions.

Based on the findings above we further hypothesized that the decreased NO output from the B vitamin-deficient cells was due to insufficient BH4 availability to the cells. To test this hypothesis, a precursor of BH4, sepiapterin, was provided to the LowFA and LowB2 cells

before stimulating NO production with LPS. Cells were treated with the same level of deficiency in folic acid and riboflavin as above, but were supplemented with 10 umol/L of sepiapterin at 0 hour and at the time of stimulation with 100 ng/mL LPS. Cells receiving sepiapterin before and during LPS exposure did not increase NO output when compared to those exposed only to LPS ($p>0.8$).

LPS-induced nitric oxide production is reduced in RAW cells grown in either riboflavin or folic acid deficient media independent of iNOS expression. These results demonstrate the importance of the folate cycle in maintaining NOS function, and indicate a potential mechanism for the effects of MTHFR polymorphisms on BP. They also show that providing substrate for BH4 production is not sufficient to overcome the decreased NO output caused by deficiency.

Acknowledgments

I would like to acknowledge the God of heaven as the only supreme God and Ruler.

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Dedication

I would like to dedicate this Master's Thesis to Caity potaty and Ma and Beya. Even though my research made all of you sleepy, I praise God for your support in the years past and in the years to come.

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A. Introduction

A1. Transition from Infectious Disease to Non-Communicable Disease

In the 1900s there was an epidemiological transition (Omran, 1977) that saw a decrease in infectious diseases and the rise of non-communicable diseases. A nutritional transition came later (early 1980s) that saw a decrease in under-nutrition and an increase of over-nutrition, but with underlying hidden hunger from micronutrient deficiencies at subclinical levels (Kuczmarski & Flegal, 2000). This new status of over-nutrition resulted in what is known as diseases of lifestyle. Namely: diabetes mellitus type 2, cardiovascular disease, and cancers of various types. As research turned its eyes from deficiency to excess it became clear that the above-mentioned diseases are end results of processes that started more insidiously. Diabetes starts with increasing levels of insulin and cardiovascular disease starts with a number of factors such as high cholesterol or high blood pressure. However, the nutrition transition was just loading the gun through the genetic undertone that turns just a "bad diet" into a killer. Polymorphisms of genes, a deviation in the way a gene is coded, coupled with dietary inefficiencies, results in higher risk of disease. One such disease state that has been found to have both genetic and nutritional components is hypertension.

A2. Hypertension

Cardiovascular disease (CVD), which encompasses several risk conditions, is known to begin with a persistent increase in blood pressure known as hypertension. Hypertension has earned the name "silent killer" as it can go undetected for many years before a stroke or myocardial infarction results from the ongoing damage. Undetected or poorly managed

hypertension can lead to kidney failure, cardiac failure and emboli. Hypertension is classically defined as persistent blood pressure >140/90 mmHg (World Health Organization, 2013). In 2008 1 billion people were estimated to be hypertensive. Worldwide this was 40% of people over the age of 25 (World Health Organization, 2013). In the United States alone the estimated prevalence is 50 million people with hypertension. The financial cost of hypertension is projected to grow to $2.52 trillion by 2025 (World Health Organization, 2013). The magnitude of the problem resulted in increased research that implicated lifestyle factors such as smoking, excessive alcohol use, unhealthy diet – low in fruits, vegetables and whole grains – and lack of physical activity (World Health Organization, 2013), as well as genetic predispositions (Abbate et al, 2008). One of the lifestyle factors that received significant attention was high sodium intake as a major contributor to hypertension development. However, as research continued it was determined that certain people were more sensitive to salt due to likely genetic predisposition. Thus, primary hypertension was not idiopathic as previously thought, but was the consequence of a combination of lifestyle and genetic factors.

A3. Genome-Wide Association Studies

The magnitude of hypertension diagnosis worldwide and the complexity of gene-environment interactions meant traditional approaches would no longer suffice in order to tackle this epidemic. This led to using gene databases to connect hypertension via genome-wide association studies (GWAS). Searches were conducted in a case-control manner. Genetic variants of biallelic genes were compared in those with hypertension to controls,

and alleles commonly found in case subjects were singled out as risk alleles (Dubé & Hegele, 2013). The search criteria looked at variants associated with hypertension, whether it had a hypertension-related trait or if it was an established risk factor (Dubé & Hegele, 2013). This led to determining eight gene loci associated with high blood pressure (Newton-Cheh et al., 2009), namely Cytochrome P450 17A1, Phospholipase C Delta 3, Fibroblast Growth Factor 5, Chromosome 10 Open Reading Frame 107, SH2B Adaptor Protein 3, Cytochrome P450 Family 1 Subfamily A Member 2, Zinc Fingered Protein 1 and Methylenetetrahydrofolate Reductase (MTHFR) (Newton-Cheh et al., 2009). Of all these genes, MTHFR became a source of interest due to its high variant prevalence in various populations (Newton-Cheh et al., 2009).

A4. Methylenetetrahydrofolate Reductase and Blood Pressure

Methylenetetrahydrofolate reductase is a gene that codes for an enzyme by the same name. MTHFR converts methylenetetrahydrofolate to methyltetrahydrofolate, a biologically active form of folate. This conversion is occurs with riboflavin in the form of FAD as a cofactor. Methyltetrahydrofolate serves as substrate in the conversion of homocysteine to methionine. This is the precursor for S-adenosylmethionine (SAM), often referred to as the "universal methyl donor" because of its essential role in various methylation reactions. There are several MTHFR polymorphisms, but the variant allele that was linked to hypertension from GWAS was the base thymidine at locus 677 (677T), which is very common in Asian, Caucasian and Hispanic populations. It has been shown that individuals with this variant form have elevated total plasma homocysteine, a risk factor for CVD.

Elevated homocysteine in these individuals can be lowered with folic acid and riboflavin supplements (McAuley, McNulty, Hughes, Strain, & Ward, 2016). Intervention studies discovered that young to middle aged adults that have two T alleles (MTHFR 677TT) have higher blood pressure, even if they were not diagnosed with hypertension, than those homozygous for the wild-type allele (677CC), i.e. no T allele (Horigan et al., 2010). Since giving folic acid to those with the variant allele had been shown to lower homocysteine, it was hypothesized that a lowering of blood pressure would be seen with the same intervention. The results were that high doses of folic acid did not lower blood pressure, and the authors came to the conclusion that there was a folate independent mechanism that was causing increased blood pressure in those homozygous for the T allele. (McNulty, Strain, Hughes, & Ward, 2017). MTHFR's function is disrupted by the 677T variant through reduced affinity for its substrate, methyltetrahydrofolate, but it also decreases affinity for its co-factor, FAD. This same research team conducted riboflavin intervention studies that did lower blood pressure in normotensive homozygous 677TT subjects (Horigan et al., 2010)(Ashoori & Saedisomeolia, 2014). The role of riboflavin supplementation appears to have effects on blood pressure even in normotensive patients, but only those who have the 677TT variant. However, the mechanism by which riboflavin works to lower blood pressure is unclear.

B. Endothelial Function

B1. Endothelial Function Basics

The endothelium is a single layer of cells that lines the vessels of the vascular system and are the first responders to internal changes due to being in direct contact with both blood and lymph. The multiple functions of the endothelium make it a barrier and first line of defense against factors and conditions that disturb vascular homeostasis. These functions include fluid filtration, hemostasis, neutrophil recruitment, and maintenance of blood vessel tone (Rajendran et al., 2013). The endothelium also responds to stimuli such as hormones, platelet derived substances, and sheer stress (Rajendran et al., 2013).

Endothelial tone is regulated through relaxation and constriction of the smooth muscles underlying the endothelium, which are achieved through a variety of chemical signals. To achieve relaxation of the vessels the following are released: endothelial derived relaxation factors (EDRFs), nitric oxide (NO) and prostacyclin. These function to facilitate dilation of vessel walls to accommodate the pressure and sheer stress induced by blood flow against the endothelial surface see **(Figure 1)**. Blood flow typically is in a laminar pattern, which is smooth with little turbulence (Baratchi et al., 2017). However, in areas of narrowing (stenosis) or where larger vessels tend to split into smaller vessels or branch off (bifurcations), there is what is known as turbulent flow. This is swirling and tumbling of the blood as it passes through the vessels (Baratchi et al., 2017). In addition to stenosis and bifurcations, changes in flow can be influenced by blood viscosity, vessel diameter and blood density (Baratchi et al., 2017). The sheer stress created by blood flow, whether

laminar or turbulent, is an important signaling mechanism by which the endothelium regulates vessel dilation.

When there are areas of turbulent flow this tends to create low sheer stress or in areas of bifurcation or stenosis, there may be excessively high sheer stress. Both of these conditions signal for the endothelial cells to release superoxide and recruit molecules that have thrombogenic effects, such as cell adhesion molecules and interleukins (Baratchi et al., 2017). Sheer stress activates calcium receptors causing a biphasic response of intracellular calcium release and extracellular calcium intake **(Figure 2)** (Abe, Pan, Krovic, & Fujiwara, 2010). Glycolax is a receptor on the edge of the cell membrane, that when triggered by sheer stress starts a signal cascade resulting in the uptake of calcium. Calcium is the main substrate by which endothelial cells are “switched on”, being normally quiescent cells, to respond to blood flow changes. Calcium binds to calmodulin (CaM) allowing for the electron flow through nitric oxide synthase (NOS) to oxidize arginine into NO and citrulline. Approximately 0.7 pmol/pg/min of NO is needed to allow for blood vessel dilation (Lorin et al., 2014). Nitric oxide not only assists with vessel dilation, but the superoxide produced by the endothelial cells in response to sheer stress results in recruiting of adhesion molecules. These adhesion molecules are used to secure leukocytes. Nitric oxide modulates the expression of adhesion molecules by down regulating their expression and reducing their ability to adhere to the leukocytes (Carreau, Kieda, & Grillon, 2011).

Figure 1: Endothelial cell

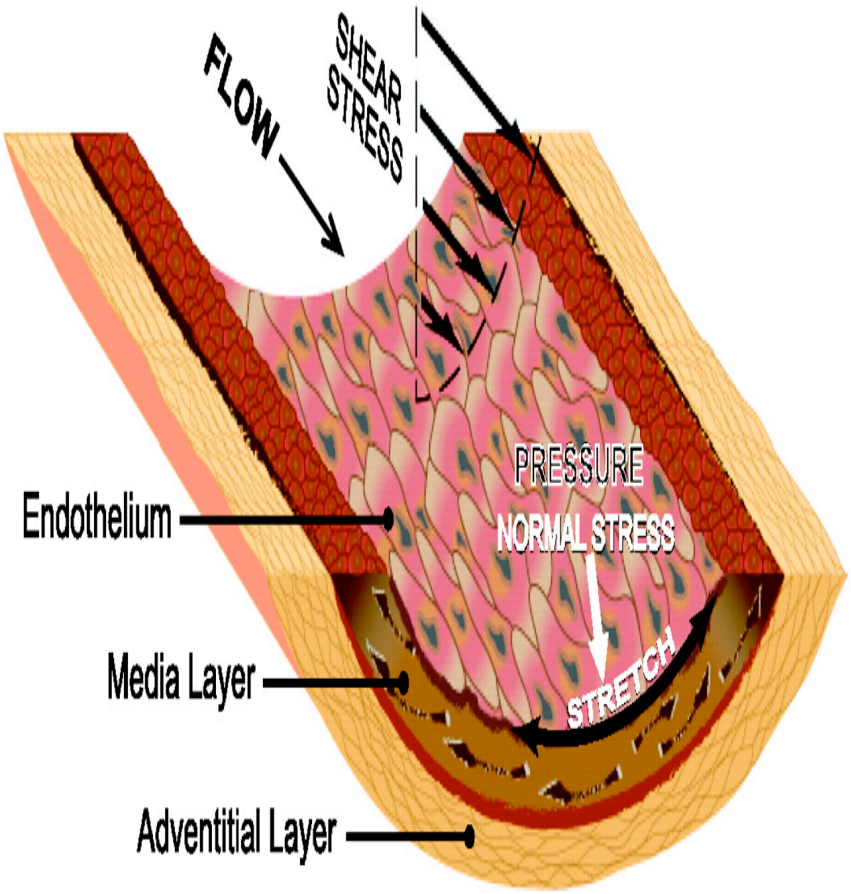

Figure 1: A blood vessel with it layers and a coating of endothelial cells. Shown is normal flow of blood and its impact on the endothelium. (www.giveyouropinion.com)

Figure 2: Stimulation of NO

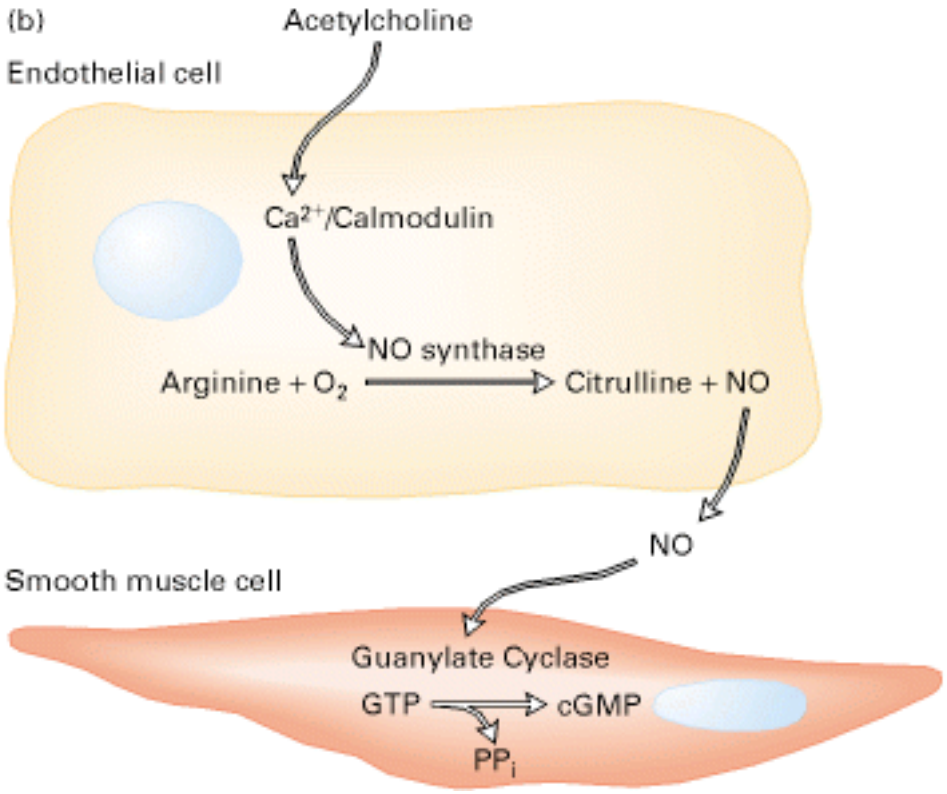

Figure 2: Calcium binds to calmodulin to activate nitric oxide synthase to release nitric oxide, which diffuses into the smooth muscle and activates guanylate cyclase resulting in relaxation. (Lodish 4th Edition)

B2. Endothelial Dysfunction and Pathology

When there is a persistence in low sheer stress due to turbulent flow or there is excessively high sheer stress due to stenosis or increased blood flow, the production of superoxide becomes more than can be overcome by super oxide dismutase (SOD). This results in peroxynitrite (ONOO) creation and oxidation of tetrahydrobiopterin, a necessary cofactor for NO production (Hirase & Node, 2012). This has an effect of creating more peroxynitrite through oxidation of BH4 that uncouples NOS and starts production of superoxide by NOS itself. The cell adhesion molecules and interleukins are then free to start the process of thrombogenesis and atherosclerosis. This combination of plaque formation and radical oxygen species damaging cell walls results in the beginning of the cardiovascular disease process of which hypertension is one of the initiating pathologies.

One of the genetic predispositions for cardiovascular disease is the above-mentioned MTHFR 677T polymorphism (Abbate, Sticchi, & Fatini, 2008). The remainder of this narrative focuses on the connection of this gene, its product (methyltetrahydrofolate), and its riboflavin cofactor (FAD), with blood pressure through the nitric oxide system.

C. Nitric Oxide

C1. Nitric Oxide Synthesis and Function

NO was discovered in 1775 by Joseph Priestly and today is recognized as a multifunctional cell messenger (Priestly, J. 1775.). In the endothelial cell, NO is synonymous with hemostasis as blood pressure control is one of NO's critical functions. The ability of the endothelium to adapt to second-by-second changes in pressure is largely dependent on the release of NO allowing for dilation of vessels as blood passes through **(Figure 3)**. It is clear that such vasodilation depends mainly on the sufficient production of NO, and that any malfunction through disease or genetic mutations may lead to dysfunction in hemostasis. Dysfunction in hemostasis can lead to a number of cardiovascular morbidities including hypertension. NO is synthesized by the enzyme NOS, of which there are three isoforms. Neuronal NOS (NOS1 or nNOS) was first discovered in nerve cells, but is also found in muscle tissue (Lorin et al., 2014). eNOS (NOS3) was the third to be discovered in endothelial cells. Inducible NOS (NOS2 or iNOS) gets its name from being only present when induced. Both eNOS and nNOS are constitutive and dependent on high intracellular concentrations of calcium (200 nM to 400 nM) (Förstermann & Sessa, 2012). In contrast, iNOS is induced by inflammation and once active needs very little calcium, below 40 nM, to remain active (Förstermann & Sessa, 2012). NO's half-life is anywhere between 4-50 sec, and it can move freely into and out of cells like oxygen (Liu & Hotchkiss, 1995).

Figure 3: eNOS stimulation

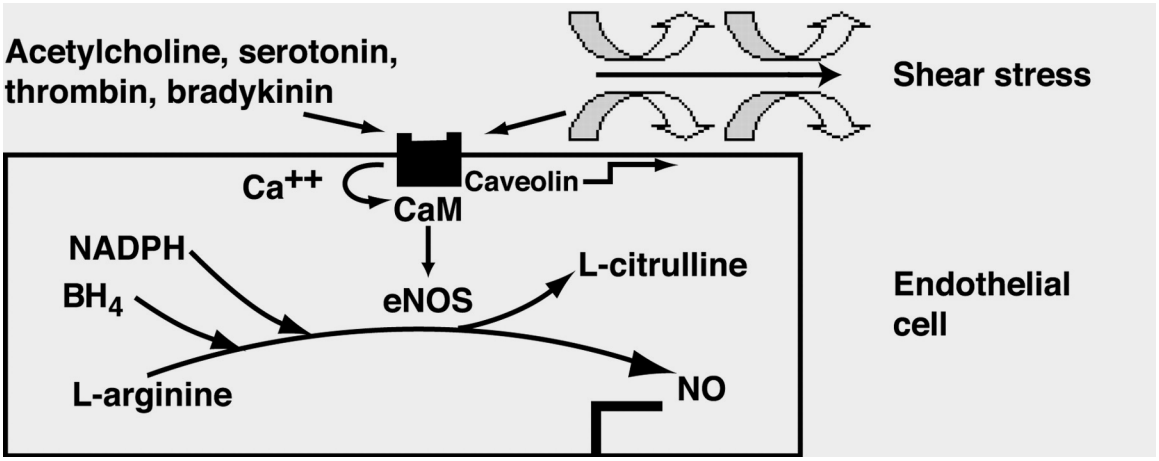

Figure 3: Factors that stimulate NO production in the endothelial cell. (Davignon &Ganz, 2004)

The NOS enzymes catalyze the synthesis of NO and citrulline from arginine and oxygen. Several co-factors are required, including heme iron, BH4, nicotinamide adenine dinucleotide phosphate (NADPH), FAD, and flavin adenine mononucleotide (FMN). The NOS enzymes are obligate homodimers with N-terminal oxidase and C-terminal reductase ends. The N-terminal oxidase contains the heme prosthetic group, the BH4 cofactor-binding site, and a calmodulin binding site. The C-terminal reductase has the binding sites for NADPH, FAD and FMN. The N-terminal end is the active site for the binding of the arginine substrate. An atom of zinc binds NOS monomers in a tetrahedral manner and this allows for the binding of arginine and BH4 (Förstermann & Sessa, 2012)

NADPH serves as the electron donor in NOS reactions in which 2 electrons are first passed to FAD, and then are passed onto FMN in a sequential manner. The electrons also pass through calcium bound calmodulin, and then reduce the heme bound iron from the ferric to the ferrous state, and in the process provide the O_2 for the binding of arginine. This binding of arginine and oxygen occurs at the first hydroxylate, a terminal guanidino nitrogen of arginine, to generate N-hydroxy-L-arginine (NOHA) as an enzyme-bound intermediate. NOHA is then oxidized further by the enzyme to generate NO and L-citrulline. This is achieved by electrons being passed from the reductase terminal of one monomer to the oxidase terminal of the second monomer in an x-shaped manner (Bendall et al., 2005). NOS homodimers are stabilized by the binding of the BH4 cofactor, which also acts as an allosteric modulator for arginine binding, i.e. by increasing the affinity of the enzyme for the

substrate (Bendall et al., 2005). The production of NO and L-citrulline ultimately costs 5 electrons with 2 NADPH donating 2 electrons each and BH4 donating a half electron before being regenerated and donating the second half electron.

C2. Arginine: The NOS Substrate

Arginine is an aliphatic amino acid that is conditionally essential during infancy, injury, and disease. Apart from being essentially conditional it is an active participant in endothelial health. It is found in the diet from meat, seafood, nuts and seeds (Popolo, Adesso, Pinto, Autore, & Marzocco, 2014). Arginine is also endogenously produced from glutamate, glutamine and proline that are precursors for citrulline. This is then converted into arginine in the intestinal-renal axis (G. Wu & Morris, 1998). Most of the endogenous arginine provision is from degraded proteins that release arginine. Arginine has a relatively long half-life in the blood stream of approximately 1 hour (Popolo et al., 2014). It has an essential role in hemostasis and vascular tone. It also functions in platelet aggregation and in allowing the heart muscles to contract (Moncada & Higgs, 1993). Therefore, when there is deficiency in its availability or there is a mutation in the enzymes that use it, this can lead to cardiovascular disease, (Lorin et al., 2014).

Arginine has a 3-carbon aliphatic side-chain completed with a guanidium at the end **(Figure 4)** (Stuehr, 2004). Arginine is the substrate for NOS, as well as for the enzyme, arginase, and produces NO and L-citrulline when oxidized by NOS **(Figure 5)**. It is the guanidino group that interacts with heme iron, both in its native and intermediate forms, that allows for binding

to NOS through hydrogen bonds (Stuehr, 2004). These bonds then extend into the BH4 binding site resulting in cooperative binding (Stuehr, 2004). When arginine binds to NOS it stabilizes the ferric heme iron into a high spin state, it increases the affinity of NOS for BH4, and increases the reduction potential of the heme iron. This makes the reduction of heme by the flavins thermodynamically viable. Arginine also stabilizes the NOS dimer and stabilizes heme-NO complexes that form during the conversion of arginine to NO and L-citrulline. When there is insufficient arginine available for NO production (Km for eNOS is approximately 3 μmol/L) (Förstermann & Sessa, 2012), NOS will continue to bind and activate dioxygen at its heme and then release reactive oxygen species like hydrogen peroxide (H_2O_2) and superoxide, **(Figure 6)** (Stuehr, 2004)(Hoang, Padgham, & Meininger, 2013). Under these conditions, NO may still be produced, albeit at a deficient rate, but the leaking of superoxide intermediate will contribute to the uncoupling of the NOS dimer and further reduction of NO synthesis.

Figure 4: Arginine Structure

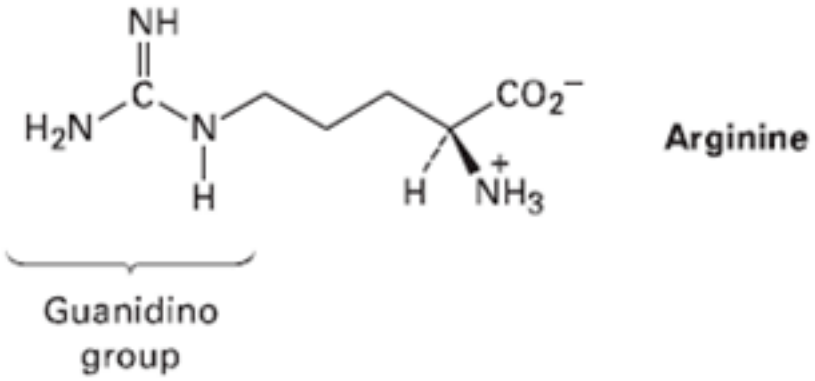

Figure 4: The structure of arginine showing the active guanidine group that binds with BH4 in NOS. (transtutors.com)

Arginase 1 uses arginine as substrate for the synthesis of ornithine in the urea pathway, and thus directly competes with NOS for arginine, preventing NO production (Hoang et al., 2013). Some studies have shown that the arginine:ornithine ratio is a good predictor of endothelial dysfunction as it has been shown that intima-media thickness is inversely correlated with the ratio (Hoang et al., 2013). In diabetic patients with cardiovascular co-morbidities, arginase was found to overlap with eNOS location in the endothelial cells resulting in competition for available arginine (Hoang et al., 2013). Another source of arginine is the conversion of citrulline to arginine via arginosuccinate synthase. This conversion is driven by insulin's stimulation of arginosuccinate synthase in the endothelial cells. Therefore, disturbance in insulin control has been shown to result in dysfunction of arginase in endothelial cells reducing availability of substrate for NO production (Hoang et al., 2013). Supplementation of L-arginine has been shown to improve blood pressure in hypertensive patients (Förstermann & Sessa, 2012). Reduced transport of arginine can also affect its availability for NOS, a condition which has been associated with cardiovascular disease (Lorin et al., 2014).

Figure 5: Conversion of Arginine into NO

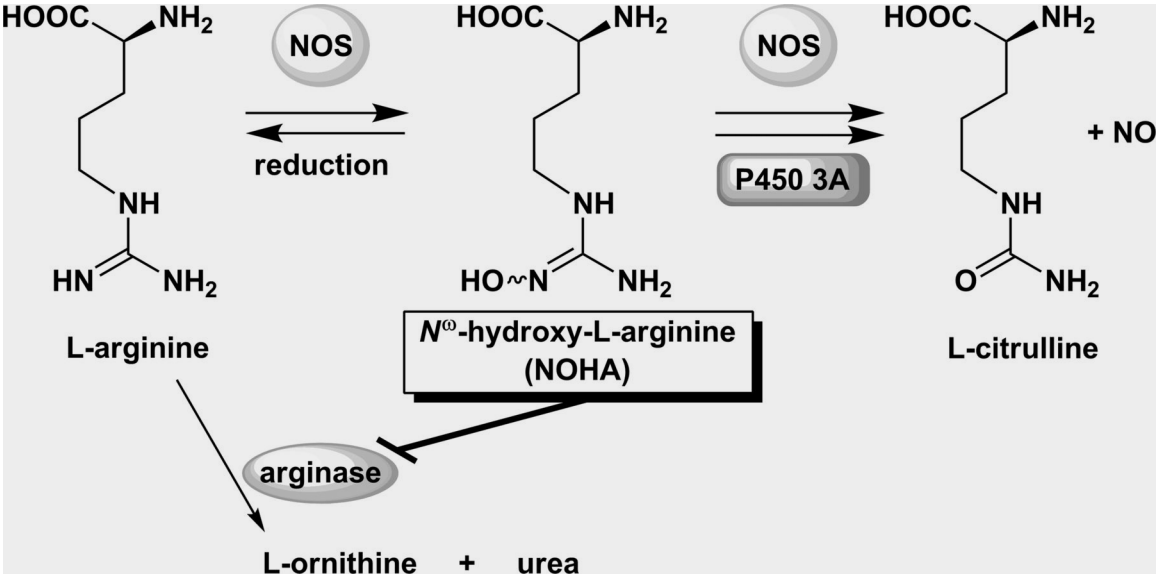

Figure 5: The conversion of arginine into citrulline via its intermediate N-hydroxy-L-arginine. (Kotthaus et al., 2011)

Figure 6: Reactive Oxygen Species

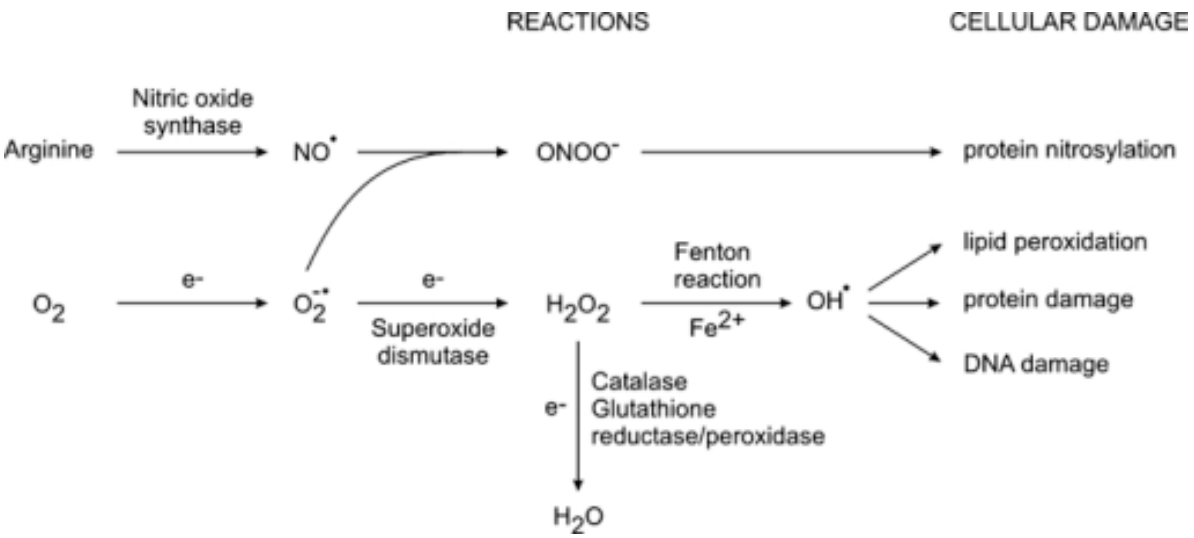

Figure 6: Production of superoxide and hydrogen peroxide and the consequences of their production. Superoxide is reduced by superoxide dismutase and hydrogen peroxide is neutralized to water by glutathione. (/openi.nlm.nih.gov).

C3. Tetrahydrobiopterin: The Essential NOS Cofactor

BH4 is a pteridine that has a heterocyclic ring structure. Biopterin is a pteridine analogue that has an amino group, a carbonyl oxygen and a 1,2-dihydroxypropyl structure at the 4,6 positions that replace the ring structure. The redox state is either fully oxidized (biopterin), partially reduced dihydrobiopterin (BH2), or fully reduced (BH4), **(Figure 7)**. BH4 is an essential co-factor for metabolism of phenylalanine to tyrosine, as well as NO production via NOS (Crabtree & Channon, 2011). In these reactions, it is an important donor of electrons and scavenger of oxidants. Due to it being labile in physiological solution it is very prone to reacting with oxygen to form radical oxygen species such as superoxide and peroxynitrite (Kirsch, Korth, Stenert, Sustmann, & de Groot, 2003).

BH4 is required for NO production, and without BH4, the system becomes uncoupled even in the presence of arginine. Despite electrons being donated by NADPH, lack of BH4 or the presence of its oxidized form BH2 results in production of reactive oxygen species (ROS). While ROS are present when there is inflammation as part of the defense mechanism against bacterial invasion, superoxide dismutase usually disarms these radical species once they have accomplished their mission of disrupting bacterial cell membranes (Bleakley, Hamilton, Pumb, Harbinson, & McVeigh, 2015). O_2^- and NO however have affinity for each other three times greater than ROS affinity for superoxide dismutase (Rajendran et al., 2013). When there is excessive production not due to bacterial invasion, the formation of peroxynitrite, an oxidant that damages cells, can cause a cyclic mechanism of continued

uncoupling. Therefore, the BH4 co-factor is clearly a vital cog in the stabilization of the NOS homodimer and the production of NO.

BH4 was first discovered by Hopkins in 1895 and then described by Watt in the 1960s (Bendall, Douglas, McNeill, Channon, & Crabtree, 2014). Its synthesis is 2-fold in that is has both a *de novo* synthesis pathway and a salvage pathway that recycles oxidized BH4 from BH2 using sepiapterin as substrate **(Figure 8)**.

Figure 7: Various Oxidation States of Pterins

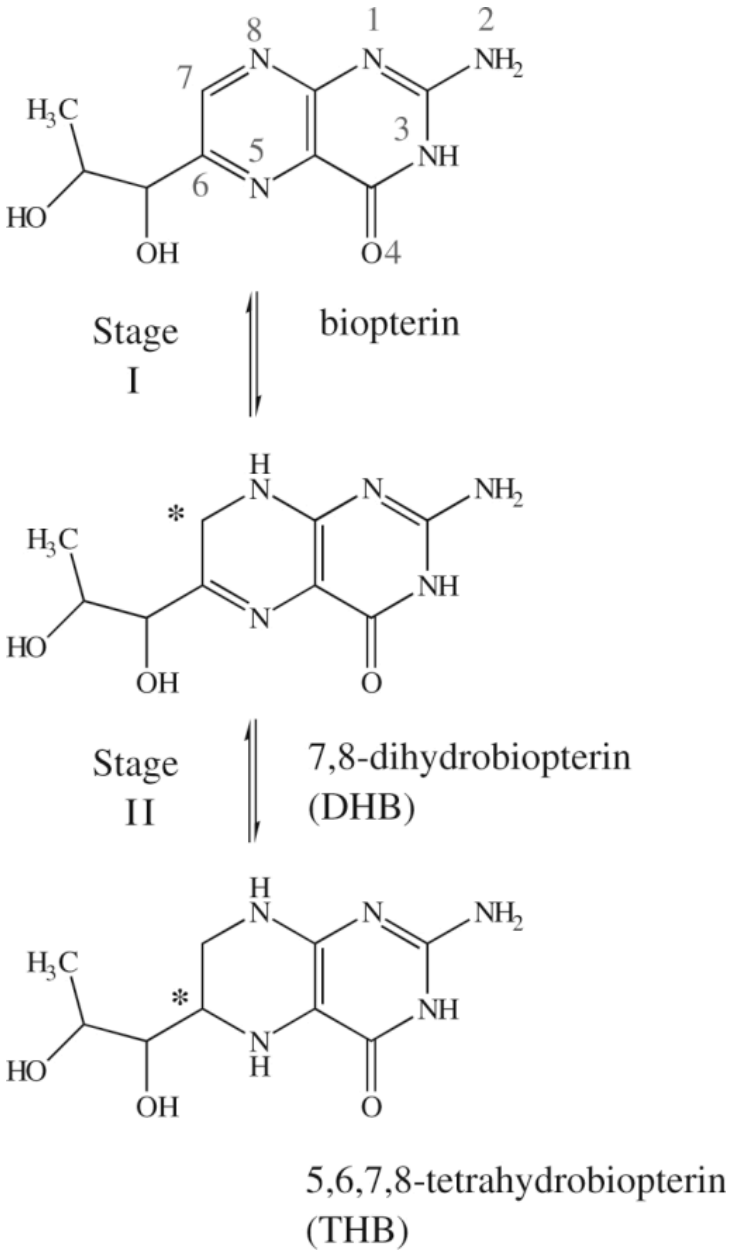

Figure 7: The stages of biopterin reduction. Biopterin is fully oxidized. Dihydrobiopterin is partially reduced and tetrahydrobiopterin fully reduced. (Dawson et al, 2006)

Guanosine triphosphate (GTP) is the substrate for *de novo* synthesis of BH4. GTP cyclohydrolase 1 (GTPCH), with co-factor zinc, catalyzes GTP into dihydroneopterin, which is the first and rate limiting step of the production of BH4 (Werner, Blau, & Thöny, 2011). This is a common step in the production of pterins, folates and riboflavin (Crabtree & Channon, 2011). Synthesis is subject to feedback inhibition by BH4 and other reduced pterins via a mechanism that requires a regulatory protein known as GTPCH feedback regulatory protein (GFRP). This inhibition can be reversed by high levels of phenylalanine (Bendall et al., 2014).

Activation of GTPCH can occur via a number of factors such as increased H_2O_2, lipopolysaccharide, interferon-γ and tumor necrosis factor-α. Dihydroneopterin triphosphate is the first intermediate that is then catalyzed by pyruvoyl tetrahydropterin synthase with magnesium and zinc as cofactors to form 6-pyruvoyl tetrahydropterin. Two NADPH are oxidized by donating 2 electrons each resulting in catalysis by sepiapterin reductase to form BH4 (Bendall et al., 2005; Werner et al., 2011). The salvage pathway for BH4 after being oxidized for NO production uses sepiapterin that is reduced by sepiapterin reductase with NADPH donating an electron to become BH2. The further reduction to BH4 is completed with another NADPH sourced electron **(Figure 9)**. This completion of the recycling process is facilitated by dihydrofolate reductase (DHFR) and possibly by MTHFR **(Figure 9)** (Matthews & Kaufman, 1980). BH4-dependent reactions are known as mixed monoxygenase reactions because they require oxygen as a co-substrate. One oxygen is incorporated into the product, such as NO, and the other oxygen is then released as water (Werner et al., 2011). In the case of NOS, BH4 does not activate the oxygen that is provided

by the heme. The BH4 provides an electron to form the intermediate NOHA that is regenerated by NADPH before a second donation occurs to complete the reaction to produce NO and citrulline. BH4 is also part of the allosteric conformational change that NOS undergoes in order to increase binding affinity for arginine (Crabtree & Channon, 2011). Because of its oxidative properties BH4 can readily merge with radical oxygen species like superoxide or peroxynitrite, which results in a radical species of trihydrobiopterin BH3*, which leads to the uncoupling of NOS.

Figure 8: De novo and Salvage Pathway of Tetrahydrobiopterin

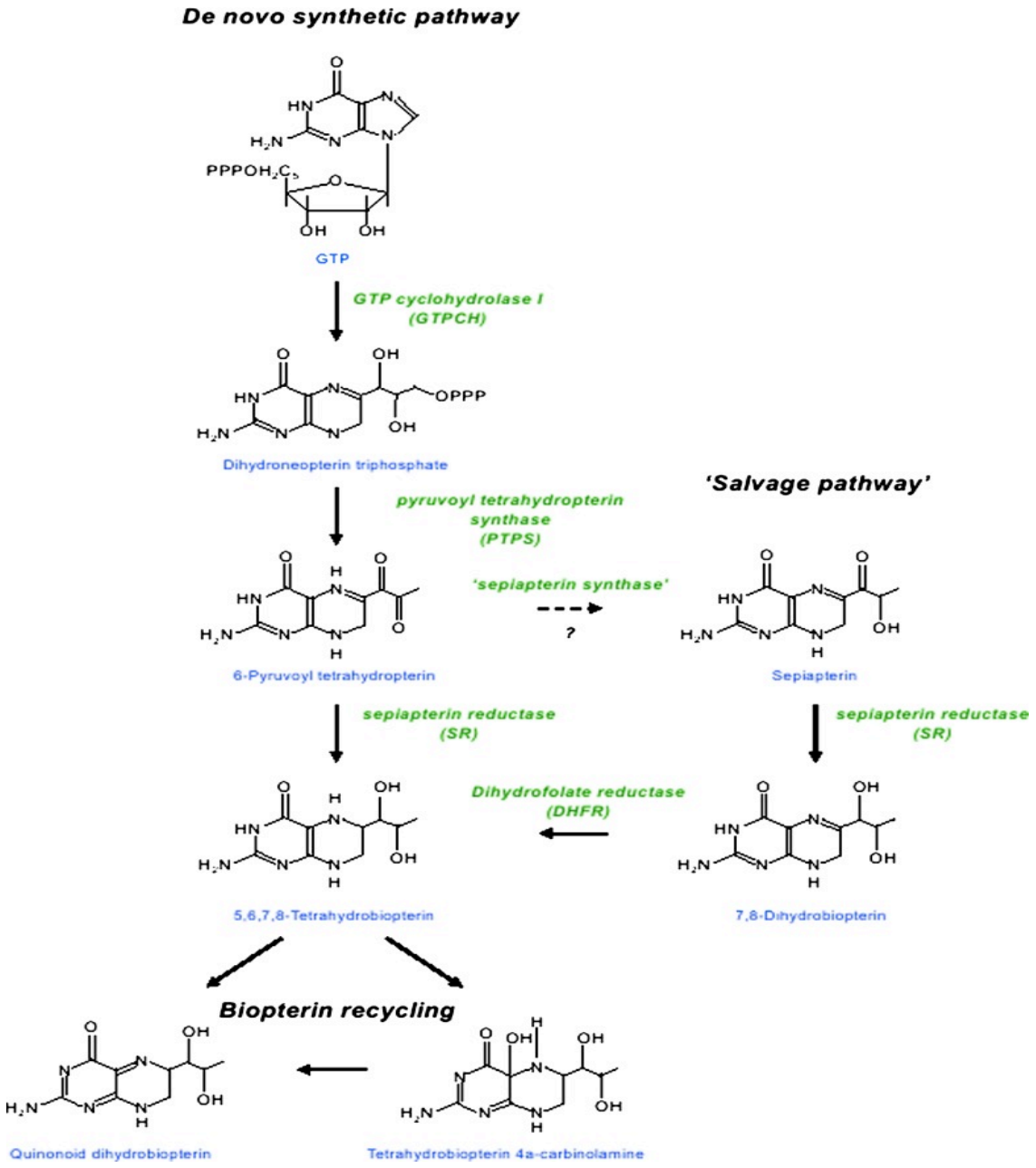

Figure 8: Three pathways for tetrahydrobiopterin production. De novo synthesis produced from GTP. The salvage pathway used in the production of NO. The recycling of carbinolamine intermediate for phenylalanine production. (Bendall et al., 2014)

This has a twofold result: a) reduction of availability of BH4 to be regenerated via the salvage pathway, and b) further uncoupling of NOS leading to increased leaking of superoxide and the formation of peroxynitrite (Crabtree & Channon, 2011; Lorin et al., 2014; Stuehr, 2004). The ratio of BH4/BH2 is an indicator of uncoupling of NOS (Bendall et al., 2005).

Folate is needed for the conversion of BH2 to BH4, and it was found that uncoupled eNOS was able to produce NO when folic acid was administered (Imamura et al., 2010). While folic acid supplementation has been shown to increase NO production in hypertensive rats studies, oral administration of BH4 directly has not been so successful (Youn, Gao, & Cai, 2012)(Hoang et al., 2013). Providing BH4 orally did not provide increased NO production, but rather had the effect of increasing BH2 plasma levels and not being able to improve the BH4:BH2 ratio. No effects were seen on the reduction of reactive oxygen species (Hoang et al., 2013). However, studies where sepiapterin was provided to cultured aortic rings did show improved NO output (Antoniades et al., 2007). Besides being an important co-factor for NOS, BH4 with its antioxidant properties also provides protection to the vasculature by serving as a growth factor for endothelial cells and endothelial progenitor cells (Hoang et al., 2013).

C4. Regulation of NOS and NO Function

The body highly regulates the production of NO and the expression of the NOS enzymes. This strict regulation is also necessary due to the highly variable capabilities of NO to regulate and induce cellular and metabolic processes. The host of functions NO has in cardiac physiology alone includes angiogenesis, platelet function, vascular tone, mitogenesis and dilation (Kolluru, Siamwala, & Chatterjee, 2010). Depending on which NOS is being discussed control starts at gene expression and goes all the way down to NO regulation once it is produced. The mechanisms for genetic regulation vary depending on the NOS in question. This narrative focuses on the regulation of eNOS as it pertains to hypertension.

eNOS is controlled through both chemical signaling and mechanical forces, specifically sheer stress. All mechanisms regulating eNOS are interdependent and can be either calcium dependent or independent. Chemical signal molecules that activate and modulate eNOS include vascular endothelial growth factor, estrogen receptor modulators and sphingosine-1-phosphate. In endothelial cells, while there are a wide variety of activators and suppressors that phosphorylate and complex to produce NO, here the focus is on what happens during sheer stress to activate eNOS and produce NO. When there is turbulent flow in the blood vessels as the red blood cells pass through at a higher pressure scraping against the endothelial cells, sheer stress is created. Serine residue 1177 in eNOS is the facilitator of the Ca-independent mechanism by which sheer stress activates eNOS by making it more sensitive to calcium, thus allowing maintenance levels of eNOS in the cells to

be sufficient for activation (Förstermann & Sessa, 2012). This informs the endothelial cells that there is not enough space for the blood to pass through, and thus several mechanisms are activated to produce NO to allow for relaxation and dilation of the entire vessel (one endothelial cell at a time) giving more space for blood to flow freely and a reduction in blood pressure (Bleakley et al., 2015).

The increase in intracellular calcium is one of the first steps needed to activate eNOS. However, it has been discovered that sheer stress is one of the few calcium independent activators of eNOS. It directly stimulates eNOS mRNA transcription and protein translation (Kolluru et al., 2010). Sheer stress activates several sites on eNOS resulting in phosphorylation. These sites are serine residues 615, 617 and 1177. When there is phosphorylation of these sites there is activation of eNOS. In contrast, tyrosine residue 116 phosphorylation has been shown to suppress activity (Mount, Kemp, & Power, 2007).

Ser1179 (a porcupine version) in the canonical pathway is activated by rise in intracellular calcium which leads to activation of calmodulin, then activation of CaM kinase II, and then phosphorylation by serine/threonine kinase Akt (protein kinase B) (Kolluru et al., 2010). This important action of Akt is facilitated by heat shock protein 90 (Hsp90), which likely acts as as a chaperone protein (Mount et al., 2007). There is a complexing between Akt, Hsp90 and CaM, with Hsp90 sitting between the 2 proteins to allow for interaction. This dislocates eNOS (Förstermann & Sessa, 2012) and allows it to translocate to the Golgi, cytosol and perinuclear structures within cells. eNOS location in the caveolae hinders NO production

until such time that it is phosphorylated as discussed above. Movement away from the caveolae allows for production of NO in the various intracellular locations depending on what stimulus started the phosphorylation. Finally NOS is regulated by its relatively short half-life of 1.6 hours (Kolodziejski, Koo, & Eissa, 2004).

D. One-Carbon Metabolism, Folate, Riboflavin, and Blood Pressure

One-carbon metabolism refers to the biochemical transfer of one-carbon units originating in the amino acid, serine, for the synthesis of thymidine and purines, and for the conversion of homocysteine to methionine and subsequent synthesis of the universal methyl donor, SAM. The B vitamin, folate, is the primary carrier of the one-carbon units and as such plays a critical role in DNA and RNA synthesis, as well as the recycling of methionine and SAM-dependent methylation reactions. The riboflavin (FAD)-dependent MTHFR enzyme is a central player in one-carbon metabolism, and as delineated below also connects folate and riboflavin to the NOS system and blood pressure regulation.

D1. Folate

Folate or vitamin B9 was first discovered by Lucy Wills in 1931. Wills used brewer's yeast to cure folate deficiency in pregnancy, and folate was then later extracted from the same product (Moat & McDowell, 2005). Active folate in the body contains a para-aminobenzoic acid moiety attached to a pteridine ring **(Figure 9)**. This combination allows for the binding of one-carbon units (Ducker & Rabinowitz, 2017; Luciano-Mateo et al., 2017). Folate also contains a tail consisting of one or more glutamate residues. Polyglutamation (2 or more glutamates in the tail) is required in order for the body to be able to retain folate within tissues of which the liver is the largest store (Pietrzik, Bailey, & Shane, 2010). The oxidation state of the pteridine ring determines what type of one carbon substitute is at the N5 or N10 position (Lucock, 2000).

Folate-mediated one-carbon metabolism requires vitamin B2 (riboflavin in the form of FAD), and vitamin B12 (cobalamin in the form of methylcobalamin). Active folate comes in various reduced forms namely tetrahydrofolate (THF), 10-formyltetrahydrofolate (10-formylTHF), 5-methyltetrahydrofolate (5-methylTHF), 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) and 5,10-methenyltetrahydrofolate (5,10-methenylTHF), (Ducker & Rabinowitz, 2017; Lucock, 2000; Newman & Maddocks, 2017). Each of the various oxidized forms is used to produce the varying products of the one-carbon metabolism. 10-FormylTHF is used for the formation of purines and formate. 5,10-MethyleneTHF is used to produce thymidine. 5-MethylTHF is used for the conversion of homocysteine to methionine, and 5,10-methenylTHF is an intermediate of the 10-formylTHF conversion process, **(Figure 10)**, (Ducker & Rabinowitz, 2017).

Folate cannot be synthesized in the body and therefore has to be consumed in the diet. Folate can be found in many sources such as green leafy vegetables, citrus fruits, yeast extracts, kidney, and liver. Folate deficiency has been indicated in a host of diseases and conditions, such as anemia, cancer and hyperhomocysteinemia, as well as neurological-disorders, such as Alzheimer's disease, neural tube defects, trisomy 21 and cleft palate. With the serious implications of deficiency mainly during pregnancy, mandatory folic acid fortification has been instituted in many countries around the world. This is done through the addition of the synthetic form of folate, folic acid. Folic acid is a fully oxidized, unsubstituted form of folate that is relatively stable when added to foods as a fortificant

and is readily converted to the active reduced form of folate (tetrahydrofolate) by the enzyme dihydrofolate reductase.

Figure 9: Folate Structure

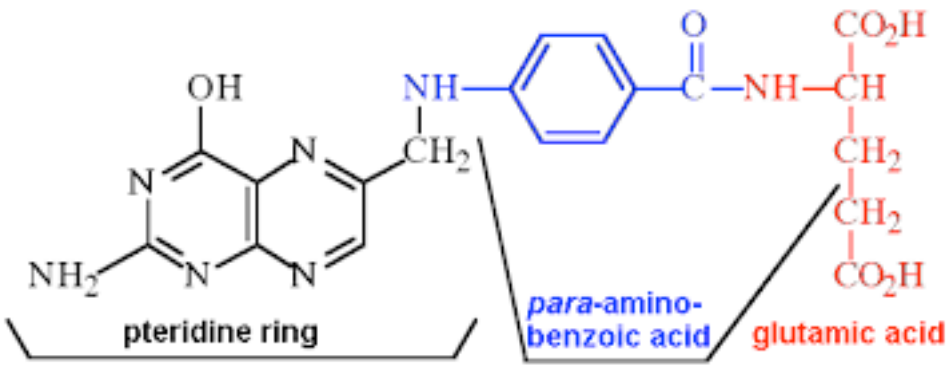

Figure 9: The components of the folate molecule. The pteridine ring is the reactive moiety of folate. The benzoic acid serves as a bridge between the pteridine ring and the glutamic acid. Addition of glutamic acid molecules to the primary glutamic acid allows for retention of folates within cells. (Gianpiero Pescarmona, 2007)

Figure 10: Various Forms of Tetrahydrofolate

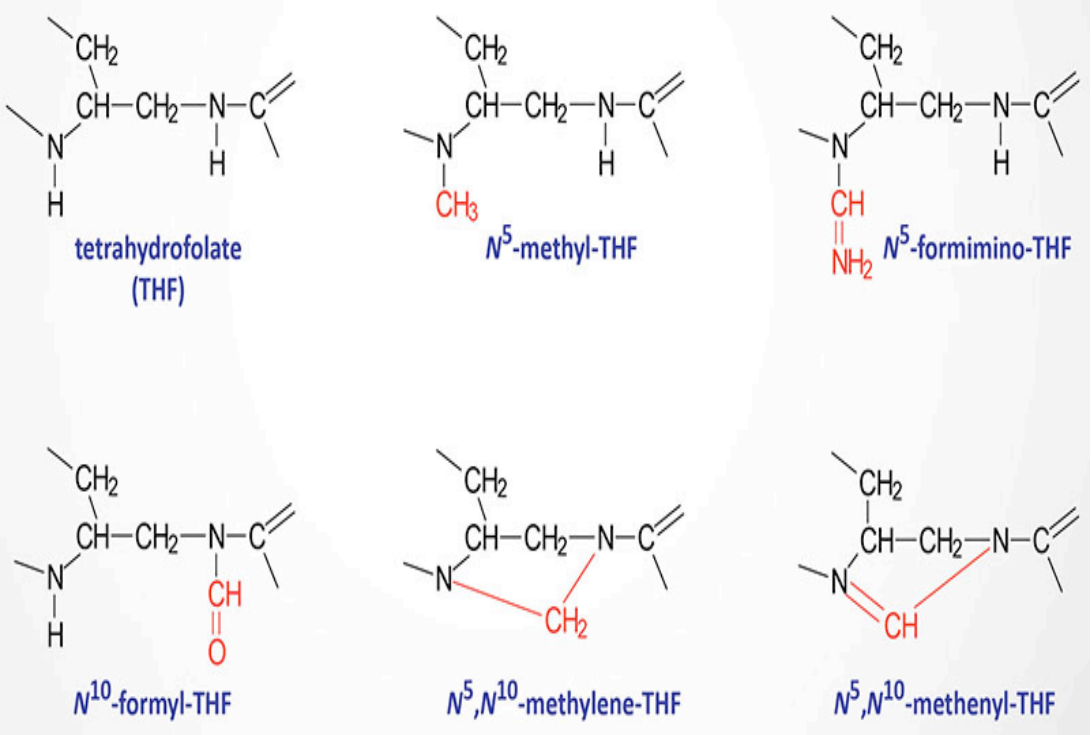

Figure 10: The various forms of tetrahydrofolate the active form of folate. Methyl-THF is the form that has antioxidant properties. Methylene-THF is the substrate for MTHFR. (Sherin and Fahmy, 2009)

Folic acid fortification has significantly reduced the prevalence of folate deficiency. However, low or sub-clinical folate status remains a problem in many populations particularly in the face of any genetic mutations that may result in inadequate uptake or utilization of folate.

D2. Methylenetetrahydrofolate Reductase

MTHFR is a flavoprotein that has 2 identical subunits. The N-terminus is where the NADPH-dependent conversion of 5,10-methyleneTHF to 5-methylTHF takes place, and the C-terminus has a binding site for SAM, an allosteric inhibitor of the enzyme (Fox & Stover, 2008). The redox reaction of MTHFR requires a proton (H^+) from NADPH that is then released to be received by FAD. Once FAD has the H^+, $NAPD^+$ is released and 5,10-methyleneTHF binds and is protonated at the 10-N position allowing for the passing of the H^+ from FAD to the C11 of the methylene group, thus forming 5-methylTHF (Fox & Stover, 2008). Once MTHFR has converted 5,10-methyleneTHF it is has committed folate to the production of methionine and SAM, thus making folate unavailable for DNA production until it can be recycled back to 5,10-methyleneTHF. With MTHFR being an important junction at which folate is committed to either thymidine (DNA) synthesis or methionine and SAM synthesis, a genetic variation in the enzyme predictably has significant biochemical ramifications **(Figure 11)**. While there are several known genetic mutations in MTHFR, the most studied is the MTHFR 667C→T variant (McNulty et al., 2017). This is due to the polymorphism being fairly common among Caucasian, Mexican and Asian populations, with the prevalence of the homozygous 677TT variant between 10-32% within these racial

groups. It was first discovered by Kang and colleagues that some individuals had a thermolabile form of the MTHFR enzyme that is associated with coronary artery disease. Later Frosst et al determined that the thermolabililty of the enzyme corresponded to a cytosine replaced by a thymidine at nucleotide position 677 (Fox & Stover, 2008; Y.-L. Wu et al., 2014)(Kang et al., 1991)(Frosst et al., 1995). This results in an alanine instead of a valine at amino acid 222 which is in the catalytic site of the enzyme (Shivapurkar, Tang, Frosst, & Alabaster, 1995). The change of amino acid brings about a 50-70% reduction in function due to the co-factor FAD not able to bind as efficiently because of the displacement of the α5 helix (Fox & Stover, 2008). This substitution may be either homozygous with both alleles having the substitution, or it may be heterozygous having one of each allele. This means that those with the variant, either hetero- or homozygous, require increased levels of folate and riboflavin in order to maintain normal MTHFR function and 5-methylTHF status. If folate and riboflavin status are insufficient, decreased availability of 5-methylTHF reduces the amount of available methionine and thus SAM. This leads to a buildup of homocysteine in the blood because of inefficient conversion to methionine. Also, because SAM is an allosteric activator of cystathionine β-synthase, the enzyme that initiates homocysteine catabolism through cystathionine synthesis, homocysteine metabolism is further impaired, which contributes to the accumulation of homocysteine in the blood (Selhub & Miller, 1995).

Figure 11: One Carbon Metabolism

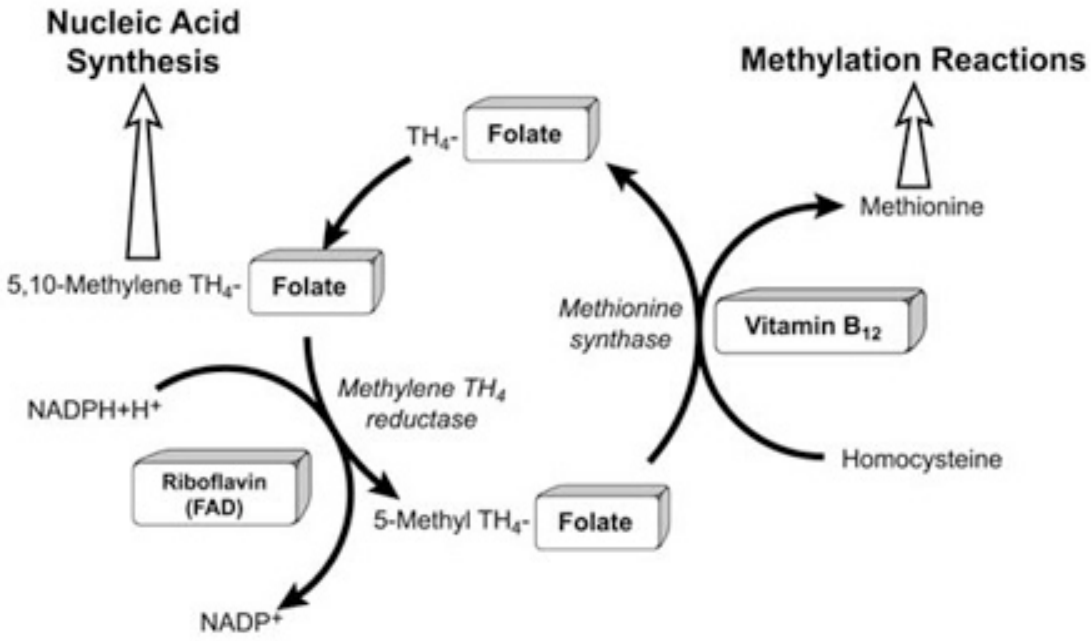

Figure 11: One carbon metabolism pathways. Methylene-THF can be used for nucleic acid synthesis, or when converted to methyl-THF it is used for the methylation of homocysteine to form methionine. (Higdon, 2003)

This buildup of homocysteine has been long researched for its implications in cardiovascular disease. Hyperhomocysteinemia is now considered to be an independent risk factor for cardiovascular, peripheral vascular, and cerebrovascular disease, as well as Alzheimer's disease and other dementias. Because the MTHFR 677T allele is associated with hyperhomocysteinemia, it has been postulated that this provides the mechanistic connection between MTHFR and CVD. The link of MTHFR to hypertension is more recent, but the role of the MTHFR polymorphism in CVD has been well documented in populations in which the phenotype is associated with increased risk of CVD ranging from 14-21% (McNulty, Pentieva, Hoey, & Ward, 2008). However, hyperhomocysteinemia may not explain the association of MTHFR with CVD, as the association of MTHFR with blood pressure is likely independent of homocysteine.

In the endothelium it has been shown that folate increases NO production and also synergistically improves binding of BH4 to NOS, which is not only necessary for heterodimer stabilization of the enzyme, but is also part of NO production as described above (Antoniades et al., 2006). This suggests that there is modulation of the inflammation process that leads to atherosclerosis that is independent of homocysteine and directly related to decreased availability of 5-methylTHF (Antoniades et al., 2009). Several studies have found that giving high levels of folic acid induce a homocysteine-independent improvement in vessel dilation. Clarke et al found that giving folic acid to a cystathionine β-synthase deficient mouse induced mesenteric bed relaxation after induced constriction by methoxamine or prostaglandin-F2α (Clarke et al., 2006). A rat study by Elmadbouh et al had

2 groups receiving folic acid as a preventative or as a treatment for post-adrenocorticotrophic hormone-induced hypertension. They found that the treatment group showed no improvement in blood pressure, but those who had the preventative dose did see improvements (Elmadbouh, Elodemi, Nabih, Elfiky, & Omar, 2016). Human studies in elderly versus young (median age 22 and 70) subjects showed that both intravenous and oral intake of methylTHF and folic acid improved cutaneous vascular conductance, but only in the elderly subjects (Stanhewicz & Kenney, 2017). Under conditions of repressed DHFR and inhibited NO under hypoxic conditions, folic acid was able to restore DHFR function, improve NO output and restore BH4 (Chalupsky, Kračun, Kanchev, Bertram, & Görlach, 2015). A meta-analyses by McRae et al showed that hypertensive patients receiving 5000 μg/day folic acid for at least 6 weeks had improved flow mediated dilation, but little improvement in blood pressure (McRae, 2009).

While folate intake appears to have an effect on the vascular system at large, studies have also looked at what low folate levels will do to subjects with CVD. Moat et al reviewed several studies that found lowered plasma folate and red blood cell folate was present in those with CVD compared with control subjects. They also found that there was an inverse association with the same parameters of folate status and myocardial infarction and carotid stenosis. Furthermore, low red blood cell folate was associated with vascular disease while low plasma levels were associated with coronary artery disease. A dose-dependent association with decrease in risk of venous thromboembolism was also noted. Folate supplementation either prevented impaired flow mediated dilation or improved it. Finally,

5-methylTHF showed evidence of having an antioxidant effect (Moat et al., 2004). With the increasing evidence of the link of the MTHFR C677T polymorphism with CVD, Wu et al found that the polymorphism was directly linked to essential hypertension whereas another common polymorphism, A1298C, was not (Wu et al., 2014). With the clear effect of folate on vascular health Antoniades et al, after having investigated the function of folates in restoring NO output when NOS was inhibited, turned to look at MTHFR's effect on vascular health. They found that those with the 677T variant had increased vascular reactive oxygen species, and lower vascular BH4. Providing 5-methylTHF resulted in increased vascular NO availability and a decrease in superoxide (Antoniades et al., 2009). All this evidence points to MTHFR's role in vascular disease either due to low folate or due to its polymorphism. However, MTHFR may play another role that has been attributed to DHFR. This is the conversion of BH2 to BH4 in the salvage pathway (Matthews & Kaufman, 1980). As previously discussed this is an important short cut to provide BH4 for production of NO. In the 1980s it was found in pig liver that MTHFR was able to produce BH4 and that SAM inhibited the production of BH4 *in vitro* (Matthews & Kaufman, 1980). Thus, MTHFR may have a dual role in regulating blood pressure by maintaining cellular BH4 for NOS function. This may occur in two ways: 1) production of 5-methylTHF which scavenges free radicals and inhibits the oxidation of BH4, and 2) direct synthesis of BH4. Because MTHFR is FAD-dependent, this also implicates riboflavin in the maintenance of BH4 and NOS function.

D3. Riboflavin

Riboflavin (vitamin B2) is a water-soluble B vitamin that is the precursor for the cofactors FAD and FMN. The primary function of these cofactors is the exchange of electrons in redox reactions, often with the niacin cofactor, NADPH, that are required for adenosine triphosphate (ATP) production, neurotransmitter metabolism, synthesis or activation of vitamins, and many other biochemical functions (~170 enzymatic reactions in total) (McAuley et al., 2016). It also interacts with iron in order to help with iron absorption and utilization for hemoglobin synthesis (McAuley et al., 2016). Relevant to this thesis, FAD serves as a cofactor for MTHFR, and both FAD and FMN serve as cofactors for NOS. Its chemical structure is a 7,8-dimethyl-10-1'-D-ribityl isoalloxazine (McNulty et al., 2008) **(Figure 12)**. FAD and FMN are the principle forms of riboflavin found in food (Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline, 1998)

Figure 12: Riboflavin Structure

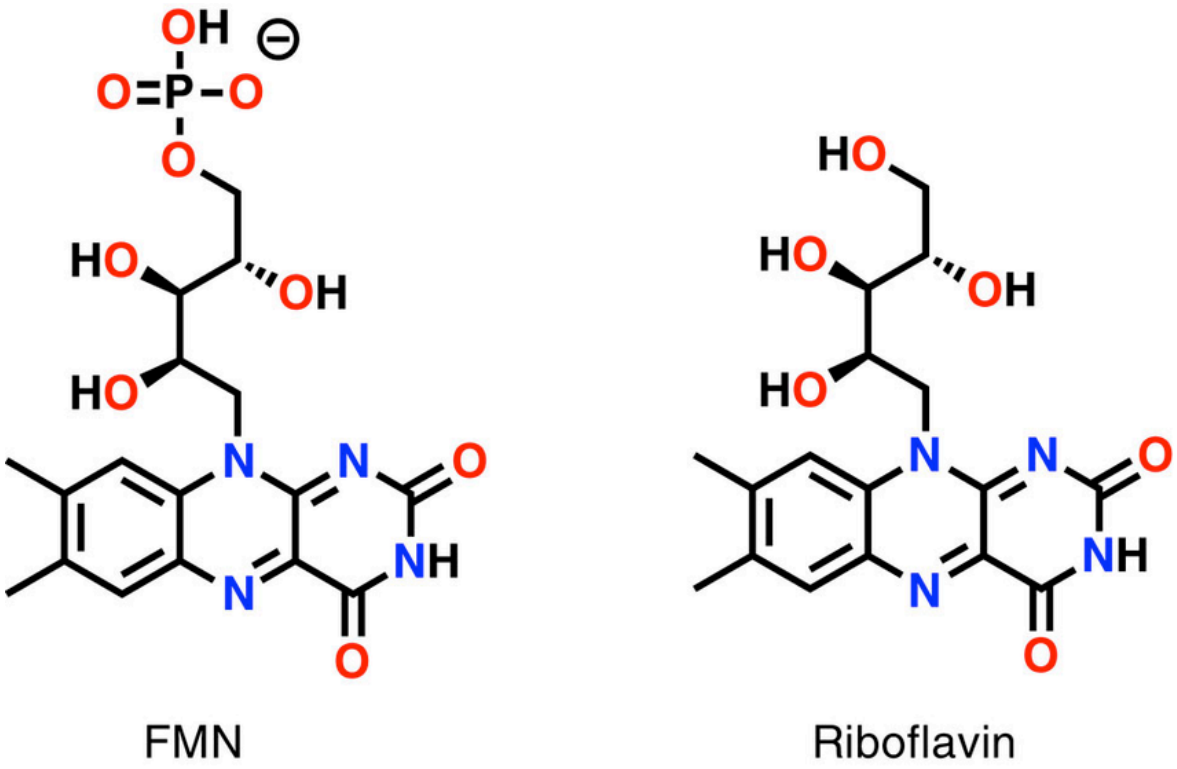

Figure 12: Riboflavin chemical structure and its structure in its cofactor form FMN (Roux et al, 2015).

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Clinical deficiency of riboflavin generally occurs during instances of overall malnutrition in conjunction with multiple micronutrient deficiencies. Thus, it is difficult to discern pathology that is specific to riboflavin deficiency as it rarely occurs in isolation. Likely manifestations include muscle weakness, cheliosis and stomatitis in and around the mouth, and glossitis. Modern flour production typically leads to losses of riboflavin (along with many other vitamins and minerals), and therefore commercial flours are enriched with riboflavin in many countries including the U.S. Therefore, it is rare to see outright deficiency of many micronutrients including riboflavin. However, sub-clinical deficiency is likely very prevalent, but is insidious as it may have far reaching effects without overt manifestations. In particular, low riboflavin status has been linked with hypertension.

A specific link between riboflavin and hypertension is in pre-eclampsia. It was shown that riboflavin deficiency during pregnancy increases the risk of pre-eclampsia (Wacker et al., 2000). Pre-eclampsia is defined as hypertension, proteinuria and edema (Wacker et al., 2000). In the same vein, the MTHFR C677T polymorphism has also been implicated in the risk of pre-eclampsia (McAuley et al., 2016). Not only is there risk of hypertension through pre-eclampsia in riboflavin deficient women, Chan et al found that MTHFR deficient mice fed low riboflavin diets have poor cardiac development, linking riboflavin to heart development in addition to function (Chan et al., 2010). In sodium induced hypertension in mice it was found that feeding riboflavin analogs lowered blood pressure when compared to a placebo group (Trachewsky, 1981). A similar study was done in hypertensive stroke prone rats and it was found that providing riboflavin supplementation lowered blood

pressure (França & Vianna, 2010). None of these studies, however, linked the NOS system as the mechanism by which riboflavin has an effect on blood pressure.

At Ulster University in Northern Ireland, a group of researchers supplemented individuals who had the MTHFR 677T variant with riboflavin and found that it reduced total plasma homocysteine levels independently of folate (McNulty et al., 2017). Moreover, when subjects with the 667TT variant were given riboflavin, there was lowering of their blood pressure despite having resting blood pressure in the normotensive range. This effect was not seen in those who had the wild-type 677CC variant or who were heterozygous (677CT). This improvement in blood pressure in a folate sufficient population indicates that the importance of riboflavin as a cofactor for MTHFR has been underestimated. Studies with folic acid supplements have not shown a similar effect on blood pressure as observed for riboflavin. Also, improvements in blood pressure due to riboflavin were mainly seen in younger individuals that have not yet contracted CVD. Similar studies in older individual with diagnosed CVD yielded mixed results that ruled out riboflavin as an intervention for established CVD (McAuley et al., 2016; McNulty et al., 2008) (Horigan et al., 2010). Importantly, the riboflavin-induced decrease of blood pressure, even in normotensive individuals, is of sufficient magnitude to make supplementation of this vitamin in line with newer recommendations that call for reduction of blood pressure beyond the standard 120 mmHg/80 mmHg to see a true minimization of stroke risk (McNulty et al., 2017).

In summary, a clear link has been established between riboflavin and hypertension, with the effect of riboflavin supplementation mainly seen in those with the MTHFR 677TT variant and in young to middle-aged adults. What remains unclear is the mechanism by which riboflavin exerts its effect. Based on what is known about the roles of riboflavin in MTHFR and NOS function, as well as the influence of 5-methylTHF on BH4, the following mechanisms are proposed: 1) riboflavin promotes the synthesis of 5-methylTHF by MTHFR, which in turn protects BH4 from oxidation by superoxide, 2) riboflavin promotes the reduction of BH2 to BH4 by MTHFR, and 3) riboflavin serves as a cofactor for NOS function.

Based on these proposed mechanisms, the overall goal of the work described in this thesis is to determine the effects of folate and riboflavin deficiencies on NO production in an *in vitro* model system.

Specific Aims and Hypotheses

Hypertension affects approximately 1 billion people around the world and 50 million people in the U.S. alone. Due to the severity of the problem much research has been done to understand the mechanisms that control blood pressure. A distinct player in blood pressure control is nitric oxide, a ubiquitous messenger and product of nitric oxide synthase that has the ability within seconds to induce vasodilation and change vascular tone. In addition to NOS, genome-wide association studies have linked at least 8 genes directly to blood pressure. One of these genes encodes for MTHFR, an FAD-dependent enzyme that catalyzes the conversion of methylenetetrahydrofolate to methyltetrahydrofolate. This enzyme also converts dihydrobiopterin to tetrahydrobiopterin, a necessary co-factor for NOS. Both these reactions are dependent on dietary riboflavin as the biochemical precursor for FAD.

It was discovered that young to middle aged adults who have a common variant form of the MTHFR gene, 677TT, have a higher normotensive blood pressure than those who have the wild-type form (677CC). Methyltetrahydrofolate works as an antioxidant that mops up radical oxygen species that may be produced when NOS is uncoupled. It has also been shown to help BH4 bind more tightly to the NOS dimer. Therefore, methyltetrahydrofolate has a role in NO production through its interaction with BH4. A finding that was unexpected was riboflavin's role in blood pressure. When provided with riboflavin supplementation, individuals with the MTHFR 677TT variant had a reduction in their blood pressure, an effect that was not seen in those with the 677C allele. What is not clear is the mechanism by

which this effect of riboflavin is mediated. With the knowledge that NO is a hemostatic regulator and MTHFR's role in NOS function we hypothesized that any deficiency in folate as an antioxidant and riboflavin as a cofactor would impair NO production. Therefore, the overall goal of the research presented herein was to determine the effects of folate and riboflavin deficiencies on cellular NO synthesis.

Specific Aim 1:

To determine the effects of folate and riboflavin deficiencies on NO production in murine macrophage RAW cells. RAW cells express the inducible form of NO synthase when stimulated by lipopolysachharide. The hypothesis tested was that deficiencies of folate and riboflavin decrease the synthesis of methyltetrahydrofolate and FAD, and consequently decrease BH4 availability, thus reducing nitric oxide synthesis by iNOS after exposure to LPS

Specific Aim 2:

To determine if supplementation of RAW cells with sepiapterin will enable NO production to be restored despite deficiency in folate or riboflavin. Sepiapterin is a precursor for the salvage pathway of BH4. The hypothesis tested was that providing sepiapterin will allow NO production to be restored to the level of non-deficient cells after exposure to LPS.

Methods

Time course measuring viability

RAW murine macrophage cells were cultured in Advanced Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 200 mM L-glutamine, and pencillin streptomyocin combination (Pen/Strep). Cells were cultured until 80% confluent. Cells were split to be cultured for 96 hours in either low folic acid (LowFA), low riboflavin (LowB2), or control medium. Advanced DMEM with no riboflavin and folic acid was made complete with 10% FBS, 1% Pen/Strep and L-glutamine. 0.4 mg/L of folic acid with 0.4 mg/L of riboflavin was added to create a low folic acid medium. Advanced DMEM with no riboflavin and folic acid was made complete with 10% FBS, 1% Pen/Strep and L-glutamine. Four mg/L folic acid and 0.04 mg/L riboflavin was added to create a low riboflavin medium. Every 24 hours via hemacytometer using trypan blue exclusion, cells were counted and checked for viability. It was determined that 48 hours was the optimum time at which deficient cells were still viable and proliferating at a similar rate compared with controls.

Induction with LPS for nitric oxide production

Cells were cultured in LowFA or LowB2 or control for 48 hours before adding 100 ng/mL or 1000 ng/mL of lipopolysaccharide (LPS). All conditions had a control to which no LPS was added. Cells and media were collected after 24 hours of LPS induction and frozen in liquid nitrogen before being kept at -20°C.

Nitric Oxide Analyzer (GE Systems) was used to analyze medium for nitric oxide content. Cell lysates were kept in trizol before extracting messenger ribonucleic acid (mRNA) for polymerase chain reaction (PCR) to determine iNOS expression in all 3 conditions. PCR was conducted using Taqman reagents (ThermoFisherScientific) on the ViiA™ 7 Real-Time PCR System (ThermoFisherScientific).

Sepiapterin supplementation and LPS induction for nitric oxide production

RAW BLUE murine macrophage cells were given 100 µL 10 µM Sepiaterin or 100 µL Phosphate Buffer Solution (PBS) at 0 hour and then grown for 48 hours in 10 mL control, LowFA or LowB2 medium. Cells were counted at 48 hours post collection by hemacytometer with trypan blue to measure cell viability. Sepiapterin solution (Sigma Aldrich) was made using 2.37 mg in 1 mL dimethyl sulfoxide (DMSO) 99.9% (Sigma Aldrich) that was argon purged for 10 minutes to make a stock solution of 10,000 µM. Two hundred and fifty µL of sepiapterin stock was mixed with 2.25 mL of argon purged PBS (pH 7.2) and filtered to make a final solution of 1000 µM.

A summary of experimental condition is as follows:

Condition	**0 Hour**	**48 hour**
Control no LPS no Sepiapterin	100 uL of 250 uL DMSO in 2.25 mL PBS filtered	100 uL of 250 uL DMSO in 2.25 mL PBS filtered
Control Sepiapterin	10 µM/L Sepiapterin	10 µM/L Sepiapterin

Control LPS	100 uL of 250 uL DMSO in 2.25mL PBS filtered	100 ng/mL LPS
Control LPS and Sepiapterin	10 µM/L Sepiapterin	10 µM/L Sepiapterin and 100 ng/mL LPS
LowFA no LPS no Sepiapterin	100 uL of 250 uL DMSO in 2.25 mL PBS filtered	100 uL of 250 uL DMSO in 2.25 mL PBS filtered
LowFA Sepiapterin	10 µM/L Sepiapterin	10 µM/L Sepiapterin
LowFA LPS	100 uL of 250 uL DMSO in 2.25 mL PBS filtered	100 ng/mL LPS
LowFA LPS and Sepiapterin	10 µM/L Sepiapterin	10 µM/L Sepiapterin and 100 ng/mL LPS
LowB2 no LPS no Sepiapterin	100 uL of 250 uL DMSO in 2.25 mL PBS filtered	100 uL of 250 uL DMSO in 2.25 mL PBS filtered
LowB2 Sepiapterin	10 µM/L Sepiapterin	10 µM/L Sepiapterin
LowB2 LPS	100 uL of 250 uL DMSO in 2.25 mL PBS filtered	100 ng/mL LPS
LowB2 LPS and Sepiapterin	10 µM/L Sepiapterin	10 µM/L Sepiapterin and 100 ng/mL LPS

Cells and media were collected after 24 hours of LPS induction and sepiapterin exposure and frozen in liquid nitrogen before being analyzed. Nitric Oxide Analyzer (GE systems) was used to determine the nitric oxide content of each condition medium. Secretory alkaline

phosphatase (SEAP) was measured in the medium using spectrophotometer at 625 mm over 2 hours at 5 minute intervals. Extraction of mRNA was done for PCR to detect iNOS and arginase1 expression using Taqman reagents (ThermoFisherScientific) on the ViiA™ 7 Real-Time PCR System (ThermoFisherScientific).

Statistics

The mean NO produced over 24h after exposure to 100 or 1000 ng/mL LPS (mean of 4 separate experiments) was compared among control, LowFA and LowB2 cell cultures by ANOVA followed by Scheffe's post-hoc test. The interaction of the concentration of LPS (100 or 1000 ng/mL) with B vitamin status of each culture (control, LowFA or LowB2) on NO production was assessed by 2-Factor ANOVA. The interaction of B vitamin status (control, LowFA or LowB2) with exposure to sepiapterin (0 or 10 µl/L) on NO production also was assessed by 2-Factor ANOVA. Statistical significance was defined as $p < 0.05$. Statistics were carried out using STATVIEW for Macintosh (version 5.0.1) and SigmaPlot (version 11.1.0).

Results

To test the capacity of RAW cells to proliferate in folate and riboflavin deficient media, we first conducted a time course to see at what point deficiency hindered growth in comparison to control cells. After 96 hours of being either deficient in folate or riboflavin, cell proliferation was severely reduced compared with replete controls **(Figure 13)**. The lower rate of cell proliferation began at approximately 48 hours after the initiation of the cultures.

Figure 13: Cell Count Time Course

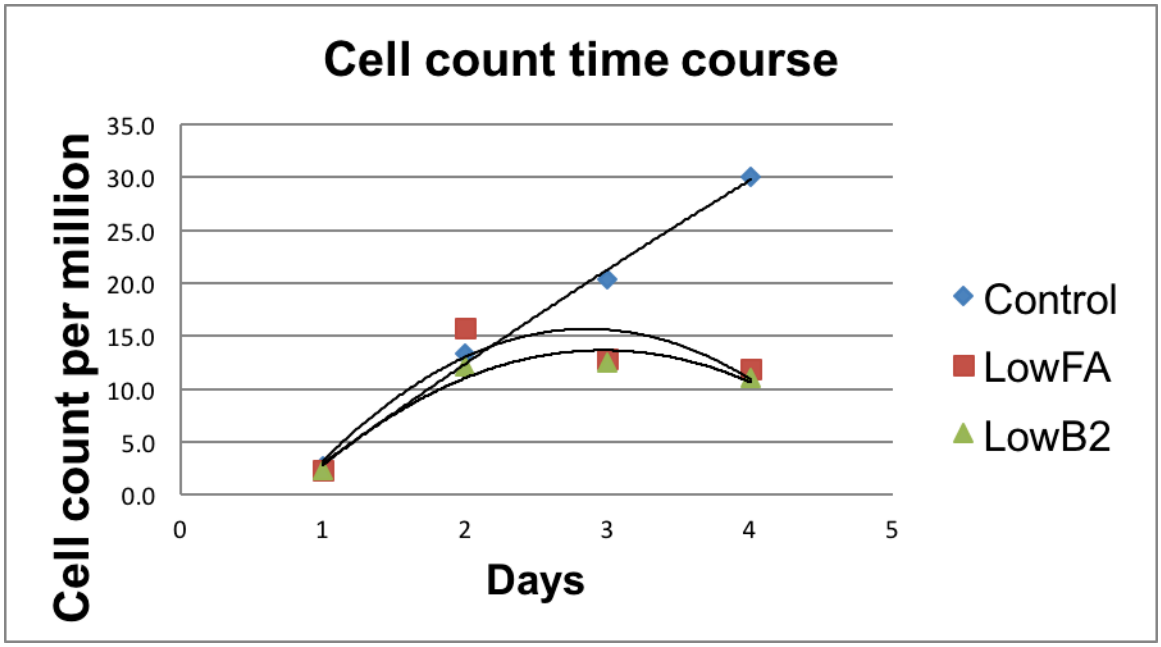

Figure 13. X-axis: Number of days the cells were made deficient of either folic acid or riboflavin. Y-axis: cell count per million. Cell counts approximately doubled from day 1 to day 2 but the deficient cells in both conditions remained stagnant over the 3rd day and started to drop off in count by day 4.

Once it was established that at 48 hours cells were still viable and proliferating at a similar rate in all 3 conditions (control, LowFA and LowB2), all subsequent experiments of LPS

exposure were conducted at the 48 hour time point. After exposure to LPS (100 ng/mL or 1000 ng/mL) for 24 hours, NO production was 60-70% lower in the LowFA and LowB2 cultures compared with controls ($P \leq 0.001$) **(Figure 14)**. No significant interaction was found between the level of LPS (100 ng/mL or 1000 ng/mL) with B-vitamin status (control, LowFA or LowB2). Regardless of the amount of LPS or the B-vitamin status, iNOS expression was increased after LPS in all conditions **(Figure 15)**.

Figure 14: Decrease in NO Output

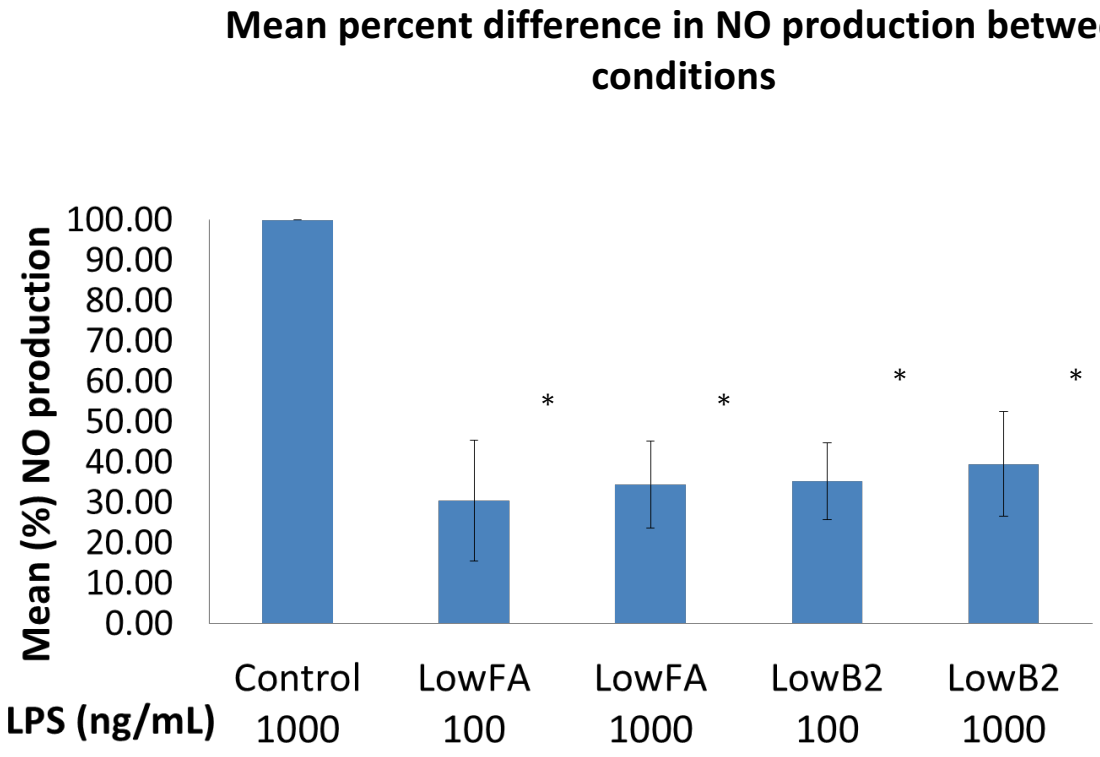

Figure 14. X-axis: Conditions and ng/mL of LPS received. Y-axis: Mean % NO production with control as 100%. LowFA cells produced 30-35% less NO than controls. LowB2 cells produced 35-40% less NO than control cells. * $P<0.001$ compared to control. There was no statistical difference between deficient groups.

Figure 16 shows the effect of sepiapterin on NO production after LPS exposure. Consistent with the first experiment, low B-vitamin status was associated with lower NO production after LPS exposure compared with the control condition (P=0.014). However, sepiapterin had no effect on NO production in control, LowFa or LowB2 cells (p=0.8). As shown in **Figure 17**, nuclear factor kappa-β (NF-Kβ) activation, which reflects iNOS expression, was increased by LPS in all conditions.

Figure 15: iNOS expression

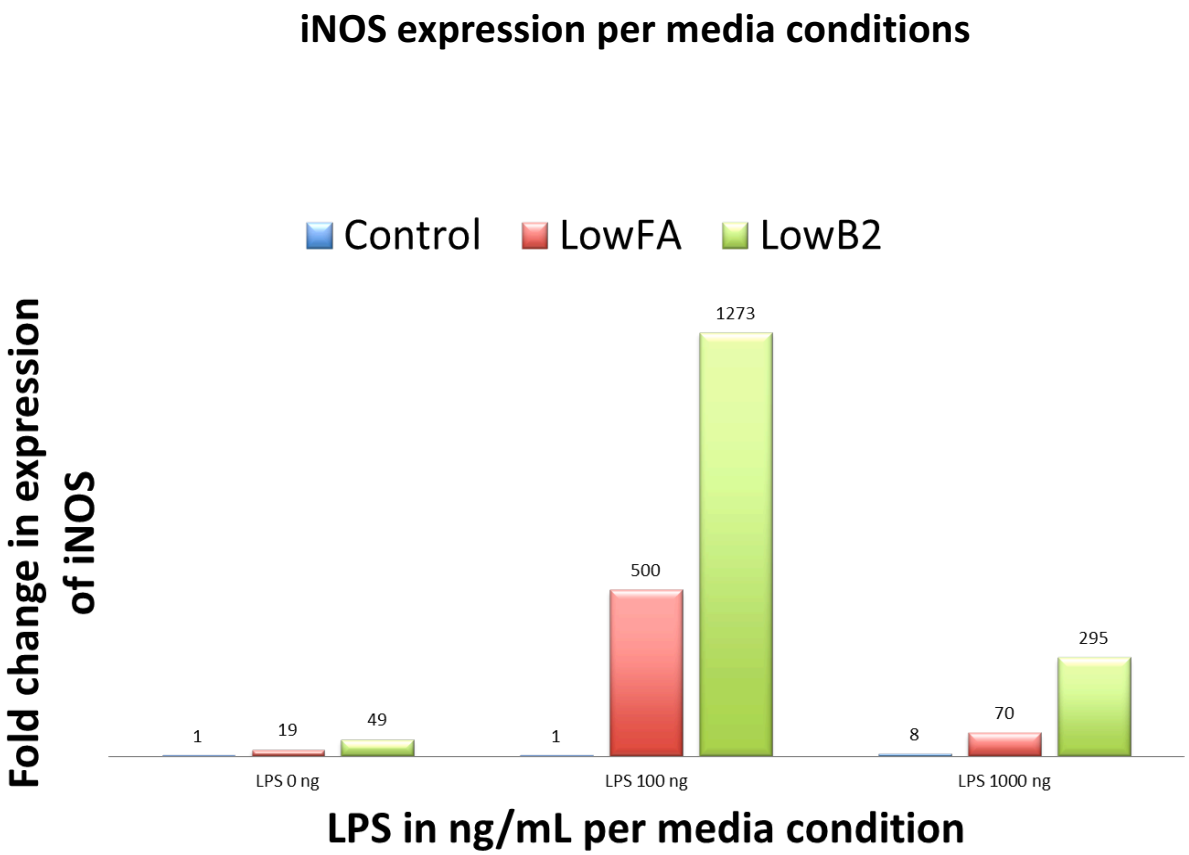

Figure 15: Increases in iNOS expression in cells low in folic acid or riboflavin. Regardless of condition, iNOS expression increased after LPS exposure.

Figure 16: NO output in LPS and sepiapterin stimulated cells

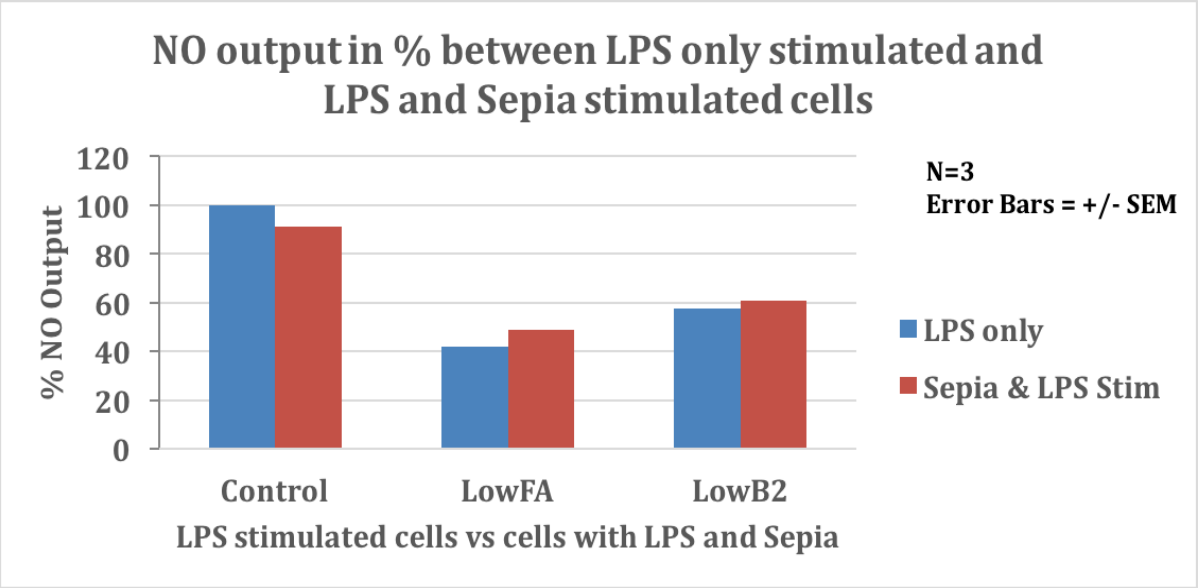

Fig. 16: Nitric oxide output with and without supplemental sepiapterin. Cells made deficient in folate and riboflavin and then given LPS produced lower amounts of NO compared to the control cells. Sepiapterin supplementation had no effect on NO production in all conditions.

Figure 17: SEAP detection

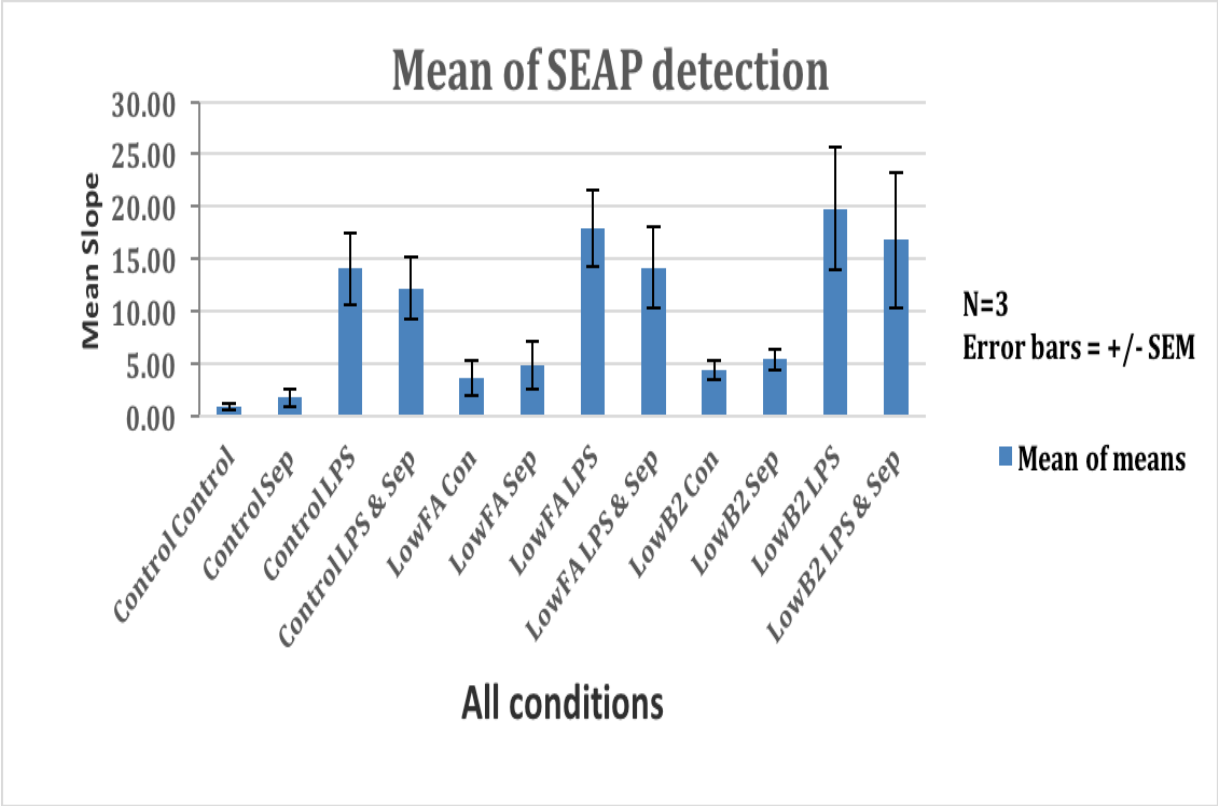

Figure 17: SEAP assay in all culture conditions. NF-Kβ activation is detected through secretory alkaline phosphatase. Those cells deficient in folate and riboflavin but receiving sepiapterin did not have increased activation when compared to those who only received LPS.

Discussion

We hypothesized that depriving murine macrophage RAW cells of folate or riboflavin will result in reduced NO output after exposure to LPS. Our findings are consistent with this hypothesis: We saw that after exposure to LPS for 24 hours, NO production under low folate or low riboflavin culture conditions was 60-70% less than B vitamin replete cells. This was observed despite increased iNOS expression in all conditions. Our second hypothesis was that adding sepiapterin, a precursor for BH4 production, would increase NO output despite B vitamin deficiency. Our findings were not consistent with this hypothesis as there was not an increase in NO output after LPS exposure in control, LowFA or LowB2 cells that received sepiapterin supplementation.

Our hypothesis was based on 3 plausible mechanisms: 1) MethylTHF has antioxidant properties that work to protect BH4 from oxidation and decreased availability of methylTHF allows for increased oxidation of BH4 thereby causing BH2 to bind with NOS resulting in uncoupling. 2) MTHFR with FAD and its cofactor is part of the salvage pathway that converts BH2 back into BH4 for binding with NOS. This salvage pathway is important because de novo production of BH4 is not sufficient to meet NOS requirements quickly enough during NO production. Thus, impairing the salvage pathway through impairing MTHFR function would result in a decrease in BH4 increasing available BH2 resulting in eventual uncoupling. Our third mechanism looks at the impairment of both MTHFR and NOS due to a lack of FAD, a necessary electron donor. Decreasing FAD availability will result in both MTHFR not being

able to convert BH2 to BH4 and NOS itself not being able to pass along the electrons received from NADPH needed for the oxidation of arginine into NO and citrulline.

The findings of the Irish group from Ulster University showed that increasing riboflavin in MTHFR 677TT variant subjects showed a reduction of blood pressure in normotensive subjects (Horigan et al., 2010). In the case of those with the 677TT variant there is increased need for FAD because the enzyme has decreased affinity for its cofactor. Our results show that riboflavin deficiency leads to reduced NO production after LPS exposure provide a potential mechanism for the findings of the Ulster group, i.e. that riboflavin supplements promote NO production and thus lowers blood pressure. Other researchers found that without the presence of BH4, folic acid was unable to overcome the effect of the NOS inhibition, and therefore it appears to be a synergistic effect rather that folic acid having an effect on its own (Moat, Clarke, Madhavan, Lewis, & Lang, 2006). This synergistic effect is consistent with our experimental results that show lowered NO production in the presence of folate deficiency. A reduced amount of BH4 uncouples NOS producing peroxynitrite (ONOO) and superoxide (O_2-). This results in the scavenging of available NO to create more radical species and also further oxidize the available BH4 to BH2. Thus, a cycle of oxidation is created, which leads to uncoupling of NOS. Another result of having the salvage pathway impaired is that there is increased BH2 to compete and therefore inhibit NO production. While BH2 displacement of BH4 in the homodimer does not prevent NOS from functioning altogether it does result in the production of 0_2^-. This has a further cyclic effect because the present NO that is available is converted to ONOO or there is production of hydrogen

peroxide (H_2O_2). While these radical nitrogen and radical oxygen species have their function in the macrophage, when induced by LPS in endothelial cells this results in damage to cell lining and organelles.

Another hypothesis as to why the results showed consistent decrease in NO production is that NOS is not able to homodimerize under conditions of decreased BH4, which is a necessary component not only in NO production, but also in stabilizing the homodimer itself. However, since there was some NO production in the experiments (30-40% of the control level) this may not have been the case. BH4 not only plays a role in the NOS enzyme itself, but its presence in the cell is opportunity for antioxidant removal. Decreased availability of BH4 means less arsenal against free radicals that are produced, which can potentially bind with NO to reduce its availability. In this experiment and based on Moat et al's finding of a synergistic effect of folate and BH4 to protect NO production, the lack of available folate to assist BH4 in its antioxidant role could be lowering NO output. In the present experiment PCR quantification did show that NOS was being expressed under both control and B vitamin deficient conditions, and therefore lack of gene expression was not what was causing the decrease in NO output. This may be due to B vitamin deficiency not having any effect on iNOS expression. Alternatively, B vitamin deficiency may inhibit iNOS expression but due to a lack of feedback inhibition on expression because of lowered NO production, these two mechanisms balance the level of expression.

Our second experiment was modelled on the experiment by Antoniades et al's (2007) in which eNOS function, inhibited by the non-specific inhibitor of NOS, L-NAME (L-nitroarginine methyl ester), was restored in aortic rings by supplementation with sepiapterin (Antoniades et al., 2007). Our findings, however, were not consistent with their findings. This may be due to the differences in the models used. Macrophages do not have eNOS and the RAW cells used in the present study is an immortalized tumor cell line that has a set phenotype, and it is unknown if these cells maintain a fully functional sepiapterin salvage pathway **(Figure 8)**. Alternatively, the production of peroxynitrite after LPS exposure, which is expected to oxidize BH4 back to BH2 **(Figure 8)**, may be so high that the salvage pathway is overcome despite sepiapterin supplementation.

Limitations

One of the limitations of these studies is the use of immortalized macrophages, which do not have eNOS, but rather iNOS. The reasoning for the use of this particular cell line is that iNOS is not constitutive and when induced produces a large amount of NO which is easy to detect. eNOS does not physiologically make large amounts of NO. The iNOS system provided a clear mechanism to turn NO production on and off without a base level of NO being present. Raw cells also quickly proliferate and allow for passaging with relative ease. However, while the present study results demonstrate proof-of-principle that low folate and low riboflavin can reduce NO production, RAW cells are not endothelial cells, and therefore may not reflect what occurs in the vascular endothelium. Differences in iNOS and eNOS that are pertinent to this experiment is that BH4 bind to iNOS with higher affinity than

it does to eNOS. In a similar manner, its oxidized form BH2 also binds more tightly to iNOS than it does to eNOS (Stuehr, 1997). With this in mind when supplementing with sepiapterin, the salvage pathway first produces BH2 before it produces BH4. The enzymes that produce BH4 (DHFR and MTHFR) as part of the final step in the salvage pathway, in our experiment are likely impaired by a lack of FAD. This results in increased BH2 availability and binding to NOS, which leads to NOS uncoupling and lowering of the amount of NO produced. Another limitation of our study was that we did not measure ROS or the BH4/BH2 ratio. Measurement of ROS would have provided evidence for assessing the validity of the methylTHF antioxidant hypothesis. Measurement of the ratio of BH4/BH2 would have provided evidence of the extent of NOS uncoupling.

Future Directions

With proof-of-principle demonstrated that low folate and low riboflavin can impair NO production, future studies should address the following issues:

- Determining if folate and riboflavin deficiencies affect NO production in endothelial cells (in vitro and in vivo).
- Determining the role of the MTHFR enzyme in NO production using siRNA or other methodologies to knock down gene expression in in vitro models, or utilizing MTHFR deficient mice.

In conclusion, our study has found that deficiency of folate or riboflavin in macrophage cells impairs NO production, and that supplementation of sepiapterin does not restore NO production in deficient cells.

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