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EPIGENETIC MODIFICATIONS ASSOCIATED WITH AORTIC
REMODELING IN HYPERHOMOCYSTEINEMIA

By

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B.Tech., Anna University, 2009

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A Dissertation

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DEDICATION

This dissertation is dedicated to my parents Mrs. Radha Narayanan & Mr. M. Narayanan and my grandparents Mrs. Swarna Krishnamurthi & Mr. R. Krishnamurthi for believing in me and encouraging me throughout my life.

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ABSTRACT

EPIGENETIC MODIFICATIONS ASSOCIATED WITH AORTIC REMODELING IN HYPERHOMOCYSTEINEMIA

Nithya Narayanan

April 16, 2014

Background: Hyperhomocysteinemia (HHcy) is prevalent in hypertensive patients and is an independent risk factor for aortic pathologies. HHcy is known to cause an imbalance between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) leading to accumulation of collagen in the aorta resulting in stiffness and development of hypertension. Although the exact mechanism of extracellular matrix remodeling (ECM) is unclear, emerging evidence implicates epigenetic regulation involving DNA methylation. The purpose of the study was to investigate whether inhibition of DNA methylation reduces high blood pressure by regulating aortic ECM remodeling in HHcy.

Methods: In the first set of experiments, we studied the effect of HHcy in causing epigenetic changes leading to aortic remodeling resulting in hypertension. Cystathionine beta synthase heterozygous knockout (CBS^{+/-}) mice of C57BL/6J background were used

as HHcy model and their Wild type (WT) littermates were used as controls. Hcy level in plasma was measured by HPLC-UV. Blood pressure was measured using tail-cuff method. The mRNA expression levels of ECM proteins and epigenetic modifications enzymes in the aorta were measured by RT-PCR. Global methylation level was measured by ELISA.

In the second set of experiments, we studied effects of an epigenetic DNA methyltransferase inhibitor, 5-Aza-2'-deoxycytidine (Aza) in HHcy. For in vivo studies, we used 8-12 weeks old WT and CBS^{+/-} mice and administered them with Aza for four weeks. During the treatment period, blood pressure was measured using tail-cuff method. Aorta wall thickness, lumen diameter and resistive index were measured before and after treatment using ultrasound. Aortic responses to vasoconstrictor and vasodilators were measured using tissue myobath. Collagen accumulation in the aortic walls was measured by Mason Trichrome and Picrosirius red staining. ECM proteases, Hcy metabolism enzymes and DNMT1 expression were measured using immunostaining. Global methylation levels were assessed using ELISA. For cell culture studies, mouse aortic smooth muscle cells were treated with Aza and Hcy for 48 hrs. MMP9 activity was measured using gelatin zymography and DNMT1, 3a and 3b expression was measured using immunoblotting.

Results: In CBS^{+/-} mice, increased plasma Hcy levels were associated with an increase in blood pressure. There was an increase in the mRNA levels of collagen and decrease in elastin in HHcy mice. Global hypermethylation was accompanied by an increased expression of methylation proteins in HHcy. Aza treatment normalized plasma SAM, SAH and Hcy levels and blood pressure in HHcy mice. Thoracic and abdominal aorta

ultrasound revealed reduction in resistive index and wall-to-lumen ratio. Vascular response to vasoactive agents improved following Aza treatment in HHcy. Histology showed a marked reduction in collagen deposition in the aorta. Aza treatment decreased the expression of DNMT1, 3b, MMP9, TIMP1 and S-adenosyl homocysteine hydrolase (SAHH) and upregulated methylene tetrahydrofolate reductase (MTHFR)

Conclusion: We conclude that reduction of DNA methylation in HHcy reduces adverse aortic remodeling to mitigate hypertension.

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CHAPTER I

INTRODUCTION

Cardiovascular diseases cause to about 13.4 million deaths annually worldwide and are a major global economic concern [1, 2]. Although arterial diseases are not the most common of the cardiovascular diseases (CVD), they cause 3.4% of deaths due to CVD (Figure 1) [3]. Data from the Centers for Disease Control and Prevention (CDC) state that diseases of the aorta and its branches account for 43,000 to 47,000 deaths annually in the United States [4]. Many risk factors including age, lifestyle, hypertension and elevated plasma homocysteine (Hcy) levels contribute to the occurrence of aortic diseases.

Homocysteine (Hcy) is a non-protein forming, non-essential amino acid that is an intermediate in methionine metabolism. Plasma Hcy levels varies between individuals and elevated plasma Hcy (hyperhomocysteinemia, HHcy), is associated with increased risk of CVD. In 2004, Giusti et al., report an increase in total plasma Hcy levels in patients with aortic dissection and abdominal aortic aneurysm [5]. The biological effects of HHcy relevant to aortic diseases include increased oxidative stress, pro – inflammatory effects, impaired endothelial function, altered lipid metabolism and extracellular matrix remodeling. [6-9]

Extracellular matrix (ECM) provides physical support and triggers various signaling pathways within all tissues and organs. The production of ECM proteins such as collagen, or proteases such as matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs) play a crucial role in determining the structure and function of the aorta. In aortic diseases, the balance between expressions of different ECM components is disrupted, leading to remodeling.

Emerging evidence suggests that epigenetic modifications play an important role in controlling a wide spectrum of biological processes. DNA methylation regulates gene expression during fetal reprogramming and contributes to the development of an extensive range of chronic pathologies. DNA methylation is facilitated by the enzyme, DNA methyltransferase (DNMT) that catalyze the transfer of a methyl group to previously unmethylated DNA. Several studies suggest that changes in epigenetic modifications can be caused by age, environmental factors/lifestyle, or various diseases [10-13]. Hence, the US Food and Drug Administration has approved drugs that specifically target epigenetic modifications such as DNA methylation and histone modification molecules which are currently in therapeutic use for diseases such as stroke and cancer [14, 15]. Nevertheless, it is unknown whether epigenetic inhibitors have a therapeutic role in HHcy-induced hypertension. In the current study, we examined the epigenetic modifications involved in aortic ECM remodeling in HHcy induced hypertension and the effect of a DNMT1 inhibitor in mitigating this condition.

Figure 1: Percentage breakdown of deaths attributable to cardiovascular diseases (United States: 2010). Source: National Heart, Lung, and Blood Institute from National Center for Health Statistics reports and data sets.

CHAPTER II

BACKGROUND

Aortic diseases

The aorta, which is the largest artery in the body, supplies oxygenated blood from the left ventricle to systemic circulation. The aorta can be divided into different sections based on areas that its branching arteries supply: the ascending aorta supplies blood to the heart, the arch of the aorta supplies the head, neck and arms, and the descending aorta supplies the lower half of the body (Table 1). A study of the thoracic aortic diameters using computed tomography reveals that the diameter of the ascending aorta is larger than the diameter of the descending thoracic aorta and the aorta in a normal healthy adult dilates with age at a rate of 0.1cm per decade [16]. The aorta is composed of three tunics or layers: the intima, media and adventitia. The innermost layer or intima consists of a layer of squamous endothelial cells, an acid mucopolysaccharide rich extracellular matrix composed of fibroblasts and macrophages, and elastic lamella. The media comprises of elastic fiber rings separated by layers of collagen and elastin fibers with undifferentiated smooth muscle cells. The concentric elastic lamellae contribute to the radial strength and elasticity of the aorta. The outermost layer or adventitia, consists of a thin collagen rich layer of fibroblasts, smooth muscle cells and vasa vasorum. Vasa vasorum are a network of arterioles that provide a vascular supply to the aorta [17]. Aortic diseases can be classified as aortic valve diseases such as those that affect the bicuspid aortic valve, or

aortic disorders due to changes in its wall composition such as atherosclerosis, aortic aneurysm and aortic dissection.

Aorta	Branches
Ascending aorta	Coronary arteries
Arch of the aorta	Innominate Left common carotid Left subclavian
Descending aorta Thoracic aorta	Visceral – Pericardial, Bronchial, Esophageal, Mediastinal Parietal – Intercostal, Subcostal, Superior phrenic
Abdominal aorta	Visceral – Celiac, Superior mesenteric, Inferior mesenteric, Middle suprarenals, Renals, Internal spermatics, Ovarian (in females) Parietal – Inferior phrenics, Lumbars, Middle sacral Terminal – Common iliacs

Table 1: The aorta and its branches

Bicuspid aortic valves

Bicuspid aortic valves are characterized as two unequally sized leaflets instead of the normal tricuspid aortic valve [18]. This congenital abnormality is most commonly identified as a systolic ejection click during exam. It is present in about 1-2% of the population and the anomaly often has no symptoms, although the coronary arteries may be abnormal and sometimes the aortic root is dilated. A causal relationship between aortic aneurysm and bicuspid aortic valves has been hypothesized [19].

Atherosclerosis

Atherosclerosis can affect any artery of the body. Aortic plaques are a common feature of atherosclerosis which is found in elderly people. Plaques are formed due to deposition of fat, cholesterol and calcium in the intimal layers of arteries. These plaques harden and narrow the vessel diameter causing obstruction to blood flow. Plaques with thickness ≥ 4 mm are termed severe plaques and are susceptible to embolization. The plaque can disintegrate, forming a blot clot and can result in pathophysiological complications such as heart attack, stroke and peripheral artery diseases. Some risk factors associated with atherosclerosis are hypertension, hypercholesterolemia, age, smoking, diabetes, increased levels of homocysteine, thrombotic diathesis markers such as prothrombin and activated protein C resistance, and inflammatory markers like white blood cell count and C-reactive protein [20-24]

Aortic aneurysm

An aneurysm is an abnormal, irreversible dilation caused by weakness due to a decrease in elastin in the arterial wall. Abdominal aortic aneurysms (AAA) are known to be associated with atherosclerosis, hypertension, hyperhomocysteinemia and smoking [25-28]. The most common site for an aneurysm to develop is in the abdominal aorta. Most AAA occurs below the level of the renal arteries. They may affect the bifurcation of the aorta as well as the iliac arteries. Aneurysms often increase in size; they can rupture and lead to hemorrhage and even death. If an aneurysm is greater than 5 cm, surgery is generally recommended. In the descending thoracic and abdominal aorta, aneurysms result from severe intimal atherosclerosis, increased elastin and collagen degrading enzymes and pro-inflammatory cytokines, chronic inflammation, and remodeling of the elastic media [5]. Important risk factors of aortic aneurysm include cigarette smoking, age, gender, hypertension and elevated levels of homocysteine [29].

Thoracic aortic aneurysms (TAA), like AAA, are caused due to atherosclerosis, trauma, and hypertension. Other unique factors which have been found to play a major role in causing TAA are syphilis [30], Marfan syndrome (MFS), and bicuspid aortic valves. Aneurysms in the ascending aorta are due to degeneration of the aortic media by cystic medial necrosis (CMN): loss of smooth muscle cells and degeneration of elastic fibers. Accelerated CMN has been known to be associated with genetic syndromes, like MFS [5]

MFS is an autosomal dominant condition affecting connective tissue that is characterized by skeletal, ocular, and cardiovascular problems. MFS patients have

thoracic aortic aneurysms and dissections (TAA/TAD) as well as valvular abnormalities, like bicuspid aortic valve [31]. CMN in MFS patients may be caused by abnormal fibrillin-1 which is a part of the elastic fiber that surrounds an elastin core. This results in a weakened aortic wall due to loss of intact elastin.

Aortic Dissection

Dissection is a condition which is distinct from aneurysm and may affect the thoracic or abdominal aorta. An intimal tear leads to separation of layers of the aortic wall and forms a passageway for blood flow. This diverts blood flow throughout the aorta in the chest and abdomen and results in ischemia [32].

Homocysteine

Homocysteine (Hcy) is a non-protein coding amino acid derived from dietary methionine. In the plasma, 70% of Hcy is bound to albumin, 1% exists as free thiols and the remainder is bound to cysteine by disulfide linkages, or as Hcy dimers. Dietary methionine gets activated by adenosine triphosphate (ATP) and three isoenzymes of methionine adenosyl triphosphate (MAT I, II and III) to form S-adenosyl methionine (SAM), a universal methyl donor. The methyl group of SAM is activated by the positive charge of its sulfur atom and gets removed from SAM to form S-adenosyl homocysteine (SAH). This highly reactive methyl group methylates various substrates such as proteins, RNA, phospholipids, myelin, catecholamines, polysaccharides, creatine, carnitine and DNA. SAH is hydrolyzed by SAH hydrolase (SAHH), in a reversible reaction, to yield Hcy. Total plasma Hcy levels are sometimes denoted as tHcy. Normal plasma Hcy levels in humans are between 5 and 15 μ M [33].

Hcy gets metabolized through two pathways – trans-sulfuration and remethylation (Figure 2). In most tissues, Hcy is remethylated to methionine by vitamin B12 dependent methionine synthase (MS). MS uses a methyl group from the de-methylation of methyl tetrahydrofolate (methyl THF). Methyl THF is one of the intermediates in the folate metabolism pathway, which is synthesized from 5, 10 methylene-tetrahydrofolate catalyzed by methylene tetrahydrofolate reductase (MTHFR). In liver and kidneys, Hcy remethylation is carried out by betaine Hcy methyltransferase (BHMT). The transsulfuration pathway is active in liver, kidney, small intestine and pancreas. Hcy condenses with serine and gets converted to cystathionine in the presence of vitamin B6 activated enzyme, cystathionine beta synthase (CBS). Cystathionine is hydrolyzed by γ

cystathionase to cysteine and α ketobutyrate. CBS removes Hcy from the methionine cycle permanently by catalyzing the synthesis of cystathionine from Hcy and serine [34].

Hyperhomocysteinemia (HHcy)

Plasma levels greater than 15 μM are termed hyperhomocysteinemia (HHcy). Based on plasma Hcy levels, HHcy can be categorized as mild (16-30 μM), moderate (31-100 μM) and severe (>100 μM) [33]. Literature, however, suggests that plasma Hcy levels above 10 μM , increases the risk for incidence of cardiovascular diseases [35]. A clinical study done in 2004 reported an increase in the plasma Hcy levels of patients with abdominal aortic aneurysm (15.7 ± 6.5 μM) [28] (Figure 3) and aortic dissection (Figure 3) [5, 36]. In patients with AAA, there was an increased trend in the expression of SAHH, MTHFR and the expression of CBS when compared to healthy subjects [37]. A similar trend was observed in patients with TAA. These results suggest a compensatory increase in the Hcy metabolism proteins, MTHFR and CBS to combat HHcy during aortic aneurysm (Figure 4) [37].

Genetic mutations and polymorphisms

Although several causative factors are responsible for HHcy, genetic mutation is one of the most important influences (Figure 5). Mutations in the genes involved in the Hcy metabolism pathway, inhibits their expression, resulting in changes in Hcy levels in the body. Since folate is a cofactor in remethylation of Hcy, without MTHFR, plasma Hcy levels increase. MTHFR polymorphisms impair the ability to process folate, resulting in a defective Hcy remethylation pathway. The most common MTHFR polymorphism is C677T (thermolabile mutation). In this mutation, the C->T conversion

in the gene code at nt 677 results in an alanine to valine substitution. This change in the amino acid sequence decreases the activity of MTHFR at higher temperature (thermolability). *Wong et al.*, report that in AAA, plasma Hcy levels were greater in MTHFR TT homozygote individuals than in wild type CC controls [38]. Another MTHFR polymorphism is A1298C, where the A->C conversion in the genetic code at nt 1298 leads to aglutamate to alanine substitution. This polymorphism also results in decreased MTHFR activity; however, its impact on tHcy is lesser than that of the C677T mutation. Individuals heterozygous for both of these mutations exhibit decreased MTHFR activity and plasma folate levels and increased tHcy [39]. In 1966, *Mudd et al.*, report that people with HHcy due to a severe genetic defect of CBS, are affected with premature arteriosclerosis and frequent thromboembolisms [40]. Since mutations in the Hcy metabolism genes lead to accumulation of Hcy, knockout mice models for MTHFR and CBS are predominantly used to study the pathogenic mechanisms of HHcy [9, 41].

Figure 2: Overview of the homocysteine formation and metabolism pathway

Figure 3: Total plasma Hcy (tHcy) levels are increased in patients with abdominal aortic aneurysm (AAA) and aortic dissection. The above data are reproduced from a 2004 article by Dr. Giusti et al., [5]. The above data show an increase in tHcy levels in aortic aneurysm and aortic dissection. (B – Patients with mild cardiovascular system involvement C – Patients with major criteria in cardiovascular system)

Figure 4: CBS expression was increased in aortic aneurysm [37]. RNA extracted from various human aortic tissue samples were reverse transcribed and amplified using primers designed for CBS, MTHFR, SAHH. Data are shown as mean $\pm$ SEM. * $p<0.05$ vs. normal controls, † $p<0.05$ vs. AAA (n=4). Ctrl – controls, AAA – abdominal aortic aneurysm, TAA – thoracic aortic aneurysm, CBS – Cystathionine beta synthase, MTHFR – Methylene tetrahydrofolate reductase, SAHH – S-adenosyl homocysteine hydrolase, bp – base pairs

Figure 5: Factors affecting plasma Hcy levels

Hyperhomocysteinemia and vascular dysfunction

In 1969, Kilmer McCully report subjects with homocystinuria, an inherited disorder of HHcy and increased Hcy concentration in urine associated with CBS deficiency, exhibit vascular complications including increased smooth muscle proliferation, progressive stenosis and hemostatic changes [42, 43]. Several mechanisms are proposed by which HHcy may lead to vascular damage. Hcy can induce oxidative stress due to increased production of reactive oxygen species (ROS) [44, 45]. ROS decreases the bioavailability of nitric oxide, a primary mediator of vasorelaxation, reacting with nitric oxide and forming peroxynitrate. Hcy impairs endothelium-dependent vasodilation due to reduced bioavailability of nitric oxide [44, 46], increased levels of asymmetric dimethylarginine (an intermediate formed during proteolysis of methylated proteins) [47] and endothelial dysfunction [48]. Hcy triggers the expression and release of cytokines such as monocyte chemoattractant protein 1 (MCP1) and IL-8, specific for neutrophils and monocytes which cause inflammation [49-52]. Hcy can also induce mobilization of intracellular calcium, causing platelet aggregation in type 2 diabetes mellitus [53]. Other mechanisms suggest that Hcy enhances LDL oxidation and lipid peroxidation [54-56], increases smooth muscle proliferation [57], causes abnormal platelet function [58-60] and causes extracellular matrix remodeling in arteries [61] and veins [62].

Extracellular matrix remodeling

The structural design of different components of the extracellular matrix (ECM) is necessary for understanding the mechanical properties of tissues. ECM is the non-cellular component which provides physical scaffolding for the cellular constituents and initiates crucial biochemical and biomechanical signaling mechanisms. ECM consists of different types of components – Fibrous proteins like collagen and elastin; matrix metalloproteinases and tissue inhibitor of matrix metalloproteinases.

Fibrous proteins - Collagen and Elastin

The fibrous proteins provide the structural support for vessels. Both collagen and elastin make up the wall of the aorta. Collagen provides rigidity and is responsible for the mechanical strength of the vessels. There are about 16 different types of collagen known in the body. Col I is about 90% of all human collagen and is present in most tissues. Col I, III, IV and V are abundantly found in arterial wall. Both Col I and III are the major constituents of the intima, media and adventitia. There was a decrease in the expression of Col I in AAA (Figure 6) [37]. Col IV and V are localized to the endothelial basement membrane and basement membranes of smooth muscle cells of the intima and media. In TAA, decreased expression of Col I and IV have been reported (Figure 6) [37]. Diffusely distributed type V collagen was also observed in the intercellular space of the intima [63].

Elastin is responsible for the reversible extensibility during cyclic loading of the cardiac volume. Elastic fibers are organized in the medial layer of the aorta as an interconnecting, fenestrated network. Each layer of elastic fibers is associated with

circumferentially oriented smooth muscle cells and collagen fibers and constitutes a lamellar unit. The number of units is established during development and is directly related to the tension in the arterial wall, so that the tension/lamellar unit is constant across the body and the arterial tree [64]. Elastin undergoes degradation and alterations with increasing age and various pathologies resulting in arterial wall stiffness [65]. Increased arterial stiffness is an important, independent predictor of cardiovascular mortality in hypertension, end-stage renal failure, diabetes, and elderly adults [66]. Arterial stiffness triggers a negative feedback cycle that increases the mechanical load on the heart and leads to heart failure [67].

Patients with homozygous CBS deficiency showed an increased internal and external diameter of the common carotid arteries [68]. Arterial histological analysis of homocystinuric patients showed medial changes and internal elastic laminal fragmentations [69, 70].

Figure 6: Collagen I was decreased in AAA and TAA tissues. RNA extracted from various human aortic tissue samples were reverse transcribed and amplified using primers designed for Collagen I, Collagen IV, Elastin. Data are shown as mean $\pm$ SEM. * $p < 0.05$ vs. healthy controls (n=4), ‡ $p < 0.05$ vs. healthy controls and TAA samples (n=4); AAA – abdominal aortic aneurysm, TAA – thoracic aortic aneurysm, Ctrl – controls, Col1 – Type 1 collagen, Col4 – Type 4 collagen

Matrix metalloproteinases (MMPs)

Regulated ECM synthesis and degradation is necessary for development, morphogenesis, wound healing, and tissue repair and remodeling. Matrix metalloproteinases (MMPs), also known as matrixins, are responsible for the timely degradation of ECM under normal physiological conditions. MMPs play a major role in tadpole tail metamorphosis by Gross and Lapiere in 1962 [71]. MMPs are zinc and calcium dependent endopeptidases which are primarily secreted in cells such as fibroblasts, vascular smooth muscle, macrophages, neutrophils and mast cells. Based on the molecular structure, substrate specificity and mechanism of activation, MMPs are classified into four groups: archetypal MMPs, matrilysins, gelatinases and furin-activated MMPs [72].

Archetypal MMPs are further divided into three subgroups: *collagenases*, *stromelysins* and *other archetypal MMPs*. *Collagenases* are classified as: collagenase-1 (MMP-1), collagenase-2 (MMP-8), and collagenase-3 (MMP-13). Collagenases cleave native collagens into characteristic N-terminal $\frac{3}{4}$ and C-terminal $\frac{1}{4}$ fragments. MMP-1 initiates cleavage of fibrillar collagens at a single site so that other MMPs can further degrade collagen [73]. The main sources of collagenases are activated fibroblasts (MMP-1), neutrophils (MMP-8) and vascular smooth muscle cells (MMP-1 and MMP-13). Collagenases are abundantly present in aortic aneurysm tissues [74]. *Stromelysins* are classified as stromelysin-1 (MMP-3) and stromelysin-2 (MMP-10). Stromelysins are structurally similar to collagenases, but they do not possess the ability to cleave native collagens. MMP-3 is more proteolytic than MMP-10 and activates various pro-MMPs [75, 76]. Although stromelysin is primarily produced by vascular smooth muscle cells

and fibroblasts, in aortic aneurysm they are derived from macrophages. *Other archetypal MMPs* include MMP-12, MMP-19, MMP-20 and MMP-27. MMP-12, macrophage metalloelastase, is primarily expressed and secreted by activated macrophages and mainly cleaves elastin.

Matrilysins lack a hemopexin domain and are represented by matrilysin-1 (MMP-7) and matrilysin-2 (MMP-26, or endometase). Matrilysins play an important role in degradation of ECM molecules, including Col IV, laminin and may be responsible for apoptosis and inhibition of angiogenesis. MMP-26 is the smallest known MMP and is involved in the activation of MMP-9 [77].

Gelatinases consists of gelatinase A (MMP-2) and gelatinase B (MMP-9). Both are constitutively expressed by cells, including fibroblasts, keratinocytes, endothelial cells, polymorphonuclear leukocytes, monocytes, alveolar macrophages and osteoclasts. When compared to other MMPs, gelatinases possess three type II fibronectin-like repeats within their catalytic domain. Gelatinases may cleave various ECM substrates such as gelatin, collagen types I, IV, V, VII, IX, X, elastin, fibronectin, aggrecan, vitronectin, laminin, as well as non-ECM molecules, including pro-TNF, TGF- β , pro-IL-1 β and pro-IL-8 [78]. MMP-9 is the most abundant elastase in human AAA tissue. It is also expressed by aneurysm-infiltrating macrophages at the site of damaged tissue. MMP-9 is correlated with higher aneurysm diameters (Figure 7) and is present in high amounts in the plasma of patients with AAAs [79].

Furin-activated MMPs have a unique sequence inserted between the prodomain and the catalytic domain which is cleaved by furins (pro-protein convertases or serine

proteinases), thereby activating the enzymes. These MMPs are divided into two subgroups: secreted MMPs, (membrane-type I and type II MMPs) and GPI-anchored MMPs. The furin-activated secreted MMPs include MMP-11, MMP-21 and MMP-28. Type I transmembrane MMPs include: MT1-, MT2-, MT3-, and MT5-MMP (MMP-14, -15, -16, and -24, respectively). Type II transmembrane MMPs consist of MMP-23A and MMP-23B and GPI-anchored MMPs include MT4-MMP and MT6-MMP (MMP-17 and -25, respectively). MT-MMP-1 plays an important role in the pathogenesis of aortic aneurysm. Besides degradation of ECM components, MT1-MMP activates MMP-2 and facilitates migration of macrophages, thus promoting inflammatory infiltration of the aortic wall [74].

Tissue inhibitors of MMPs

Tissue inhibitors of matrix metalloproteinases (TIMPs) prevent aortic wall destruction and prevent aneurysm development by inhibiting MMPs [80]. TIMPs are secreted by cells, including macrophages, VSMC and platelets. TIMPs bind to the catalytic domain of MMPs and block their enzymatic activity. TIMPs possess differential affinity to MMPs, for example, TIMP-1 preferably binds to MT-MMPs. The presence of MMP-neutralizing agents such as α 2-macroglobulin prohibits a direct interaction between TIMPs and active MMPs [81]. TIMP-1 is a collagenase inhibitor while TIMP-4 is secreted extracellularly primarily from heart tissue and may be involved with extracellular matrix homeostasis [82]. TIMP-2 plays a major role in the activation of MMP2. TIMP-2 binds to the hemopexin domain of pro-MMP-2 which is necessary for MT1-MMP to activate MMP-2 [83].

Functional polymorphisms in genes encoding for TIMPs could influence the activity of MMPs in the aneurysm wall. Several single nucleotide polymorphisms (SNPs) which could affect TIMP transcription were assessed in patients with aortic aneurysms. The genotyping results were analyzed for male and female patients separately since the TIMP-1 gene is located on the X chromosome. The literature suggests that two polymorphisms for TIMP-1 (434 C/T and rs2070584 T/C) [84] and two for TIMP-2 (a promoter SNP -479 C/T, and 573 G/A, but in male patients group only) [85, 86] are associated with aortic aneurysm. Although TIMP-1 mRNA expression levels did not change significantly, TIMP-4 mRNA levels were higher in abdominal aortic aneurysm when compared to normal healthy controls (Figure 7).

Figure 7: TIMP4 expression was increased in AAA [37]. RNA extracted from various human aortic tissue samples were reverse transcribed and amplified using primers designed for MMP1, TIMP1, MMP9, TIMP4. Data are shown as mean $\pm$ SEM. * $p < 0.05$ vs. normal ($n=4$) ; AAA – abdominal aortic aneurysm, TAA – thoracic aortic aneurysm, Ctrl – control, MMP – matrix metalloproteinase, TIMP – tissue inhibitor of metalloproteinase

Epigenetic Modifications

In a eukaryotic nucleus, the basic unit of chromatin consists of 147 base pairs of DNA wrapped around two copies of four histone proteins H2A, H2B, H3 and H4 [87]. Histone proteins consist of a globular carboxyl-terminal domain and an amino-terminal tail which project from the nucleosome core. The amino tails of the histone proteins are susceptible to post translational modifications such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation and ADP-ribosylation [88]. These inheritable histone modifications affect gene transcription, and maintenance of epigenetic information by making a histone modification signature or a “histone code” [89].

Epigenetics can be described as the inheritable changes occurring beyond the genetic makeup, without affecting the actual nucleotide sequence. Every organism carries a certain epigenetic pattern throughout its pedigree. These epigenetic patterns are translated to phenotypic expression due to the regulation of gene transcription exerted by the epigenetic modifications. However, this epigenetic blueprint can be altered by various environmental factors like diet, hormonal imbalance during physical stress and pollutants [90-92]. The most commonly studied epigenetic modifications are – DNA methylation and histone modifications (Figure 8). Normally, histone modifications have short-term gene expression effects and DNA methylation is considered to be long-term silencing of gene expression where both of the mechanisms are interdependent.

DNA Methylation

DNA methylation is an epigenetic modification involving an addition of methyl group to the cytosine residues at the 5-carbon position. Methylated cytosine (5-mC), was first described in 1925 by Johnson and Coghill [93]. In mammals, 5-mC is primarily found in cytosine-guanidine rich regions in the gene called CpG islands. They account for approximately 7% of CpG dinucleotides genome-wide and are associated with the 5'-regulatory regions of $\approx 40\%$ to 60% of human genes [94, 95]. These islands are found mostly at the upstream promoter regions of the genes. Since promoter region modifications are essential in regulating the binding of transcription factors, CpG island methylation plays a major role in controlling gene expression. In healthy somatic cells, 70% to 90% of CpG dinucleotides, representing 3% to 6% of all cytosines, are methylated [95]. DNA methylation is facilitated with the help of DNA methyltransferases (DNMTs). There are mainly three active DNMTs : DNMT1, DNMT3a and DNMT3b. DNMT3a and 3b facilitate de novo methylation which is responsible for methylation patterns during early embryo development [96]. DNMT1 is primarily found in somatic cells and is localized to the DNA replication fork, where it is specific to hemi-methylated DNA. It is involved in maintenance methylation.

DNA methylation is associated with transcriptional silencing in a growing number of cellular functions, such as X chromosome inactivation, genomic imprinting, mammalian embryonic development, and lineage specification [94, 95]. Aberrant DNA methylation patterns have been associated with various diseases. For example, the cancer genome is characterized by genome-wide hypomethylation and paradoxical hypermethylation of CpG islands associated with tumor-suppressor genes.

DNA methylation can affect gene expression in two different ways. First, since methyl groups of CpG dinucleotides project into the major groove of the DNA helix, it prevents the binding of transcription factors such as Myc, activator protein-2, -19, hypoxia-inducible factor -1 α , -20 and the insulator protein CTCF21, -22 to CpG dinucleotide-containing cis-DNA binding elements. However it is likely that this mechanism is not relevant for a majority of transcription factors whose target genes are regulated by DNA methylation [95]. Second, a group of methyl-CpG binding proteins are capable of specifically recognizing the mammalian methylated CpG rich regions which act as transcriptional repressors. These include proteins containing a homologous methyl-CpG-binding domain (MBD1, MBD2, MBD4, and MeCP2) and nonhomologous protein, Kaiso, which is capable of binding methylated CpG dinucleotide doublet [97]. These proteins can directly repress transcription, prevent the binding of activating transactors, or alter the structure of chromatin and promote transcriptional repression.

Histone Modifications

Although the nucleosome is a tight structure, histone post-translational modifications can affect the chromatin structure and function and hence modulate the accessibility of transcriptional regulators to cis-DNA binding elements. Histone modifications at a certain promoter region determine the epigenetic state of the genome, and one of the most important types of histone modifications is acetylation [98]. Histone acetylation neutralizes the positive charge of the lysine residues, thereby disrupting the electrostatic attraction between histone proteins and negatively charged DNA. This interaction results in a change from heterochromatin to euchromatin, allowing the transcription initiation complex to access DNA promoter. Thus, euchromatin is the transcriptionally active region and is associated with acetylated histones and hypo-methylated DNA. Heterochromatin, however, is transcriptionally repressed and bound to non-acetylated histones and hyper-methylated.

Histone acetylation is regulated by the balanced activities of two key enzymes - histone acetyl transferase (HATs) and histone de-acetylase (HDACs) [99]. HATs acetylate lysine residues and result in an open chromatin promoting gene transcription, whereas HDACs remove acetyl groups and condense the chromatin causing transcriptional repression [100]. Some proteins function as transcriptional activators such as p300 which possess intrinsic HAT activity [99]. A previous report suggests that HAT and HDACs play a major role in the development of atherosclerosis and restenosis due to their regulatory role in processes such as inflammation, proliferation of VSMCs and matrix remodeling [101]. HDACs aid in maintaining endothelial function and structural integrity of the

vessels by regulating the expression of proteins involved in angiogenesis such as vascular endothelial growth factor [102, 103]

Histones methylation takes place on either lysine (K) or arginine (R) residues providing binding sites for recruitment of other regulatory proteins [104]. Methylated histones such as H3K9, H3K27, H3K79 and H4K20 are associated with gene repression, whereas activated genes are associated with methylated H3K4 and H3K36 residues [104]. Histone methylation is carried out by histone methyl transferase (HMT), whereas histone demethylases (HDMs) remove methylation. Proteins involved in DNA methylation mechanism such as DNA methyl transferase and methyl-binding proteins interact with histone-methylating enzymes, resulting in cross talk between the histone and DNA methylation pathways [104]

Figure 8: Illustration showing epigenetic modifications which play a major role in regulating gene transcription. DNMT – DNA methyltransferase, MBD – Methyl binding domain, HAT – Histone acetyltransferase, H3K9 – Histone 3 at lysine 9, HDAC – Histone deacetylase

CHAPTER III

HYPOTHESIS AND SPECIFIC AIMS

Key Objective

To investigate effects of epigenetic modifications, such as DNA methylation, on aortic remodeling and hypertension during HHcy.

Hypothesis

Upregulation of global DNA methylation in hyperhomocysteinemia dysregulates cellular mechanisms causing endothelial dysfunction and adverse extracellular matrix remodeling leading to hypertension

Specific Aims

- **Specific Aim 1:** To determine whether HHcy increases the activity of methyltransferases and histone methylation to alter ECM metabolism in aorta.

The experimental results associated with Aim 1 are presented in chapter IV.

- **Specific Aim 2:** To determine whether DNA methylation inhibitor, 5 Aza 2' deoxycytidine (Aza) alleviates HHcy, improves smooth muscle and endothelial function and normalizes blood pressure.
- **Specific Aim 3:** To determine whether Aza mediates its effects by modulating the expression of proteins involved in ECM regulation.

The experimental results associated with Aim 2 and 3 are presented in chapter V.

Figure 9: Schematic diagram of overall hypothesis. Epigenetic modifications regulate aortic remodeling resulting in hypertension during HHcy.

CHAPTER IV

AORTIC REMODELING IN HYPERHOMOCYSTEINEMIA

Introduction

Hcy is a risk factor for diseases such as stroke, ischemic heart disease, peripheral vascular and aortic diseases [105]. Although the normal range of homocysteine in the blood is around 5–15 $\mu\text{mol/L}$, the range varies with age and sex as well as nutritional factors [106]. HHcy is found in a study of 86 hypertensive patients compared to 82 normal healthy controls [107]. Plasma Hcy levels are positively correlated with blood pressure in human subjects with methionine loading [108]. Arteriosclerosis is prevalent in patients with increased homocysteine due to genetic defects in enzymes involved in Hcy metabolism, such as cystathionine beta-synthase (CBS) and methylene tetrahydrofolate (MTHFR) and in patients with nutritional deficiency of folic acid, vitamin B6 and B12.

Hcy promotes arterial stiffness by increasing the synthesis of ECM components such as collagen. Collagen provides rigidity and structure to the arterial wall, while elastin provides distensibility by allowing blood vessels to resume their shape after stretch or contraction. During HHcy, the imbalance between elastin and collagen production destroys proper elasticity of the vessel and excessive collagen deposition causes vascular stiffness and tissue fibrosis. These effects increase vascular resistance for blood flow that results in arterial hypertension.

DNA methylation is an important epigenetic mechanism in transcriptional regulation of many genes. The methyl group is usually attached to the cytosine residue in the nucleotide sequence with the help of specific enzymes called DNA methyl transferases (DNMT) [109]. There are two types of methylation involved – *de novo* methylation by DNMT 3a and DNMT 3b and maintenance methylation, by DNMT1. Methyl binding domain (MBDs) proteins like MBD2 have been shown to bind to these methylated CpG islands and down-regulate transcription of downstream sequences by recruiting co-repressor complexes [110]. These co-repressor complexes are proteins which are bound to chromatin remodeling proteins like Histone 3 which is trimethylated at lysine 9 (H3K9) [111], and HDACs. Hcy induced DNA methylation plays an important role in atherosclerosis [112]. In the current study, aortic tissue samples from wild type mice (C57BL/6J) and CBS +/- mice (with high methionine diet) were taken and mRNA expression levels of various genes involved in methylation such as DNMTs, MBD2 and H3K9 and genes involved in ECM metabolism such as collagen and elastin were measured to study the correlation of DNA methylation and ECM metabolism in HHcy. It was hypothesized that high levels of Hcy due to defects in methionine metabolism cause increased global methylation which induces remodeling of the aortic wall.

Methods:

Mouse tissue samples

CBS +/- (B6129P2) mice and their wild type littermates (WT, C57BL/6J) were obtained from The Jackson Laboratories. CBS +/- mice were fed with a high methionine diet for three weeks in order to create the HHcy condition. Aortic tissue was isolated from WT and CBS+/- mice.

Blood Pressure Measurements

Blood Pressure was measured by a noninvasive tail-cuff method (CODA; Kent Scientific, Torrington, CT). Animals were placed on a warming platform at 37°C and allowed to acclimatize for 10 min before measurements were taken. Systolic, Diastolic and Mean arterial blood pressure were recorded in WT and CBS+/- mice.

Plasma Hcy Measurement

Plasma Hcy levels were measured using HPLC-UV as described before.[113-115]

Reverse transcription polymerase chain reaction (PCR)

Total RNA was isolated from mouse aortic tissue using Trizol reagent (Invitrogen) [116]. One microgram (1 µg) of total RNA was reverse transcribed using the Reverse Transcription System (Promega), according to the manufacturer's instruction. Primers, to amplify specific gene sequences, were designed using Primer3 [117] and obtained from Invitrogen (Carlsbad, CA). The list of primers is given in Table 2. PCR was done in a

Biorad DNAEngine Thermal cycler (Hercules, CA). The PCR thermal cycle was 95°C for 7 mins, 35 cycles of 95°C for 50 s, 55°C for 1min and 72°C for 1min, and 72°C for 5 mins. PCR products were resolved by gel electrophoresis in 1% or 1.5 % agarose gels and the images were recorded and analyzed using Imagelab software (Bio-Rad, Hercules, CA). The band intensities of the PCR product were normalized to GAPDH.

Immunostaining

Mouse aortic tissue was collected and cryo-blocks were prepared in Peel-A-Way disposable plastic tissue embedding molds (Polysciences Inc., Warrington, PA., USA) containing tissue freezing media (Triangle Biomedical Sciences, Durham, N.C., USA) and stored at -80°C until further use. Five micron (5 µm) tissue sections were made using a Cryocut (Leica CM 1850) and placed on Super frost plus microscope slides, air-dried and processed for immunohistochemistry (IHC). Slides were fixed using 3.7% paraformaldehyde, permeabilized and blocked with blocking solution (2% BSA and 0.5% Triton X-100 in PBS). Slides were incubated with mouse polyclonal anti-Histone 3 trimethyl K9 (Abcam, Cambridge, MA) overnight. Alexa Fluor 488 raised in mouse from Invitrogen (Carlsbad, CA) was used as the secondary antibody. The stained slides were mounted and visualized with a laser scanning confocal microscope (Olympus FluoView1000) with appropriate filters. Images were analyzed using Image Proplus software taking different optical fields at random into consideration.

Quantification of 5-methylcytosine

Genomic DNA was extracted (Zymo Research Corp., Irvine, CA, USA) from the aortas of mice from the experimental groups. In order to measure levels of global methylation, the amount of 5-methylcytosine (5-mC) was determined using ELISA (Zymo Research Corp., Irvine, CA, USA), as per the manufacturer's instructions. Samples were read at 410 nm and the values are represented as percent of 5-mC

Statistical Analysis

Statistical analysis was done using Primer of Biostatistics to compare data collected from groups. Differences between CBS+/- and controls were determined by t-test. A probability level ($p < 0.05$) is considered statistically significant. All values are presented as mean $\pm$ SEM.

Results:

Plasma Hcy and blood pressure measurements in CBS+/- mice

Plasma Hcy levels in CBS+/- mice increased when compared to WT mice (Figure 10). Systolic, diastolic and mean blood pressure in CBS+/- mice was also increased when compared to WT mice (Figure 11).

Expression of metabolites involved in remodeling proteins in mice aortic tissue samples

To evaluate the expression of ECM fibrillar proteins, mRNA levels of collagen and elastin were assessed in WT and CBS+/- mice fed with a methionine diet (Figure 12). On an average, the expression of *Colla1* in CBS+/- mouse aorta was increased when compared to WT but the increase did not reach statistical significance. The mRNA levels of *Col4a1* were significantly increased in CBS +/- mouse samples when compared to WT controls. In contrast, the level of Elastin was found to be decreased significantly in CBS +/- samples compared to WT (Figure 12).

Expression levels of genes involved in epigenetic modifications in mouse aortic samples

DNA methylation levels were analyzed by measuring %5-mC and mRNA levels of the enzymes, DNMT1, 3a and 3b that are involved in DNA methylation. CBS+/- mice had a higher %5-mC aortic content when compared to WT mice aorta suggesting global methylation in HHcy (Figure 13). There was an increasing trend of DNMT 1 and 3a levels in CBS +/- mice with high methionine diet when compared to WT mice (Figure

14). However, there was no change in DNMT3b levels in aortic samples from WT and CBS +/- mice. To study levels of methyl binding proteins and histone methylation, mRNA expression levels of MBD2 and H3K9 (trimethyl) respectively, were measured in CBS+/- mice. MBD2 and Histone 3 trimethylation in lysine 9 (H3K9) expression levels were increased in CBS +/- mice compared to WT (Figure 15, 16). However, there was no significant difference in the histone deacetylase 1 (HDAC1) mRNA levels between WT and CBS+/- mice (Figure 15).

Figure 10: Plasma Hcy was increased in aortic tissue from CBS+/- mice. Plasma Hcy levels in WT and CBS+/- mice were measured using HPLC-UV. Data are shown as mean $\pm$ SEM. n=4; * p<0.05 vs. WT

Figure 11: Blood pressure was increased in HHcy mice. Blood pressure in WT and CBS+/- mice was measured by the tail-cuff method. Systolic, Diastolic and Mean blood pressure data are shown as mean $\pm$ SEM. n=4; * p<0.05 vs. WT

Gene	Primer pair sequence (5'-3')	Product size (bp)
Collagen - I	AGAACTTTGCTTCCCAGATG CTATCTGTACCACCCCCTTG	162
Collagen - IV	GCTGCTAAGAACTTGCCTTC GGGGACAGAGAAGATGTCAC	105
Elastin	TGACAGTATAGGGCTGAGCA GAGTTGTTGTGGGTGAGACA	238
DNMT1	GGGTCTCACCAAGTATCTCA GGTGTGTGACTCCAGTTTTT	235
DNMT3a	GGGAGAGAGGGAAAATTCTA GGTTTTCTTCAAGGTTTCCT	298
DNMT3b	GACTGCCTGGAGTTCAGTAG ACAGGCAAAGTAGTCCTTCA	285
GAPDH	TAAATTTAGCCGTGTGACCT AGGGGAAAGACTGAGAAAAC	177
MBD2	TAGCGCATCAGATGTAACAG AGAGACTTGTCTGTGATGG	199
H3K9	CTGTCTCAAAGAAGGAGGTG GGACAGACAAACACCAAAGT	279
HDAC1	GACTGGACCCTCTGTCATTA TTTCTCCTAAAGTGCAGCTC	248

Table 2: List of primers used in murine aortic samples.

Figure 12: Collagen 4 was increased and elastin was decreased in HHcy. Representative RT-PCR image of collagen 1a1 (Col1A1), 4a1 and elastin mRNA expression. GAPDH was used as a loading control. The mRNA expressions were determined by densitometry analysis using Biorad Imagelab software. The intensity values were normalized by GAPDH intensity. n =3. Data are shown as mean $\pm$ SEM. *,# p<0.05 vs. WT.

Figure 13: Global hypermethylation in HHcy aorta. Bar graph representing percent 5-mC detected in WT and CBS+/- mice aorta genomic DNA samples. Data are shown as mean $\pm$ SEM for n=4. *p<0.05 vs. WT.

Figure 14: Increasing trend of DNMTs in aorta of HHcy mice. Representative RT-PCR image of DNMT1, 3a and 3b mRNA expression. GAPDH was used as a loading control. Graphical representation of mRNA expressions as determined by densitometry analysis using Biorad Imagelab software. Intensity values were normalized by GAPDH intensity. n =3. Data are shown as mean $\pm$ SEM.

Figure 15: Increasing trend in expression of methylation genes in aorta of HHcy mice. Representative RT-PCR image of MBD2, H3K9 and HDAC1 mRNA expression. GAPDH was used as a loading control. Graphical representation of mRNA expressions as determined by densitometry analysis using Biorad Imagelab software. The intensity values were normalized by GAPDH intensity. n =3. Data are shown as mean $\pm$ SEM

Figure 16: H3K9me3 expression was increased in HHcy mice aorta. Immunostaining of aorta sections with H3K9 (trimethyl) antibody (green fluorescence). Bar diagram indicate the intensity values as determined by Image Pro software. n =3. Data are shown as mean±SEM. Original image x60 magnification. *p<0.05 vs. WT

Discussion:

Previous studies report that Hcy is a leading risk factors which is responsible for aortic disease progression such as aortic dissection [5, 118, 119]. Elevated levels of Hcy in plasma, known as HHcy, trigger various effects such as impaired post-injury endothelial repair and function, deregulation of lipid metabolism, altered platelet activity, enhanced VSMC proliferation, activation of cellular immune response, all of which are believed to be primary characteristics in the development of vascular pathologies such as atherosclerosis and abdominal aortic aneurysm (AAA) [120-125]. Recent studies emphasize the role of epigenetic modifications such as DNA methylation in the regulation of the transcription of enzymes involved in various mechanisms including platelet activity and VSMC proliferation [126, 127]. The present study demonstrated the effect of HHcy on global DNA methylations in the aorta. Increased levels of plasma Hcy correlated with increased blood pressure in CBS[±] mice when compared to WT. Concurrent to our findings, Lim and Cassano *et al.*, previously reported that a 5 $\mu\text{mol/L}$ increase in plasma Hcy causes an increase of systolic and diastolic blood pressure by 0.7/0.5 mmHg in men and 1.2/0.7 mmHg in women [108]

Hcy increases the synthesis and accumulation of collagen in a dose dependent manner in VSMCs [124]. Atherosclerosis is characterized by increased smooth muscle proliferation and accumulation of ECM components, resulting in arterial intimal thickness [128-130]. Collagen provides rigidity to the artery wall and accounts for about 60% of the ECM components responsible for the formation of atherosclerotic plaques [131]. Our results showed an increase in Collagen 1 and 4 levels in aortic tissue samples

from HHcy CBS +/- mice. In addition, a significant decrease in elastin levels in aortic tissue from CBS +/- mice was observed when compared to WT. An increased collagen to elastin ratio due to increased collagen and decreased elastin levels in CBS +/- mice accounts to carotid arterial remodeling [132]. Elastin provides distensibility to the vessel wall and a decrease in elastin content increases the vascular resistance to blood flow resulting in hypertension. These findings suggest that adverse aortic ECM remodeling associated with HHcy, may cause hypertension.

HHcy alters the methylation patterns in the genome globally and in a gene specific manner. DNA methylation plays a predominant role in regulating gene expression. CBS knockout mice have a tissue specific fluctuation in their DNA methylation levels [133]. S- adenosyl methionine (SAM), an intermediate in the Hcy synthesis pathway, serves as the universal methyl donor for the methylation of various substrates such as DNA and proteins. Our results showed an increase in %5-mC and mRNA levels of DNMT1, and 3a in aortic tissue from HHcy mice. This finding suggested that an increase in synthesis of Hcy causes DNA hypermethylation. DNA methylation arrays in peripheral leukocytes of subjects with schizophrenia indicate a positive correlation between plasma Hcy and methylation of CpG residues in CpG islands [134]. Other epigenetic mechanisms such as histone methylation and methyl binding proteins act in conjunction to regulate genome wide transcriptional activity. The mRNA expression of MBD2 and Histone 3 trimethylated in Lysine 9 residue (H3K9) proteins suggested an increase in the expression of histone methylation and methyl binding proteins in HHcy. In addition, we studied mRNA expression of HDAC 1 but no significant change was found in the samples.

In summary, results of our study, combined with previous findings concerning the effect of Hcy on adverse ECM remodeling, delineate possible epigenetic mechanisms involved in aortic pathologies. Although evidence suggests an association between gene promoter specific hyper-methylation and atherosclerosis [29], extensive studies of the role of methylation in regulating various genes up or down in other aortic pathologies such as aortic aneurysm still need to be done.

CHAPTER V

EFFECT OF 5-AZA-2'DEOXYCYTIDINE IN AORTIC REMODELING IN HYPERHOMOCYSTEINEMIA

Introduction

Aortic disease is one of the most prevalent causes of increased mortality in the world [135-137]. Several risk factors including hyperglycemia, high serum cholesterol and elevated level of homocysteine (Hcy), known as hyperhomocysteinemia (HHcy), are associated with aortic aneurysm and atherosclerosis [138, 139]. The vascular pathology associated with HHcy is characterized by excess extracellular matrix (ECM) turnover, causing increased deposition of collagen, leading to vessel stiffness [140, 141]. Under physiological conditions, ECM homeostasis is maintained by equilibrium between matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs). Previous literature demonstrated that HHcy increases the expression and activity of MMP-9 which causes matrix degradation and accumulation of collagen in the ECM [142]. Arterial hypertension is associated with endothelial dysfunction [143]. HHcy causes auto-oxidation of sulfhydryl groups, promoting reactive oxygen species production which uncouples endothelial nitric oxide synthesis and reduces nitric oxide (NO) synthesis and bioavailability [144-146]. A reduction in NO signaling further increases superoxide generation, forming a vicious cycle and impairs endothelial function. The functional consequence of endothelial dysfunction impairs vasodilation

resulting in arterial hypertension [147, 148]. Hcy is a non-protein coding amino acid synthesized by de-methylation of methionine. The methyl group which is excised during the synthesis of Hcy is used in various methylation reactions involving DNA, proteins, amino acids and Hcy [149]. DNA methylation is one of the most intensely studied epigenetic mechanisms in the development of aortic diseases. There are three DNA methyl transferases (DNMTs) involved in DNA methylation: DNMT1 which is involved in maintenance methylation and DNMT3a and 3b which catalyze *de novo* methylation [150]. Current research is focusing on the use of DNMT inhibitors in several disease conditions. Decitabine or 5-Aza-2'-deoxycytidine (Aza), a DNMT1 inhibitor is approved by the FDA for treatment of myelodysplastic syndrome (MDS). Other inhibitors such as Vidaza (5 Aza cytidine) are currently in phase 2 and phase 3 cancer trials [151].

The purpose of the present study was to investigate the role of DNA methylation in aortic ECM remodeling and vascular dysfunction in HHcy associated hypertension. We hypothesized that increased levels of Hcy and DNMT1 result in adverse ECM remodeling and endothelial dysfunction, leading to arterial hypertension. We also examined whether DNMT1 inhibitor, Aza could modulate ECM metabolism enzymes to mitigate hypertension. The results showed that Aza treatment in HHcy mice protects the aorta by regulating epigenetic mechanism of genes involved in ECM metabolism.

Materials and Methods

Antibodies and reagents

Monoclonal antibodies to DNMT1, DNMT3b, Methylenetetrahydrofolate reductase (MTHFR) (mouse) and DNMT3a, MMP9, TIMP1, Hcy (rabbit) were purchased from Abcam (Cambridge, MA), mouse polyclonal antibody S-adenosyl homocysteine hydrolase (SAHH) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The following secondary fluorescent antibodies were used: Texas Red anti-rabbit, Alexa Fluor 488 anti-mouse from Invitrogen (Carlsbad, CA). The following horse radish peroxidase (HRP) conjugated secondary antibodies were used : anti-mouse and anti-rabbit from Santa Cruz Biotechnology (Santa Cruz, CA).

Animal Models

Male C57BL/6J (wild type; WT) and Cystathionine beta-synthase heterozygous knockout; CBS +/- (B6129P2) mice, aged 8–12 weeks, obtained from Jackson Laboratories (Bar Harbor, ME) were used in this study. The disruption of the Cystathionine beta-synthase (CBS) gene in this heterozygous model, results in mild HHcy. All mice were fed standard chow (Lab diet 5010, St. Louis, MO) and water *ad libitum*. WT and CBS+/- mice were treated with 5-Aza-2'-deoxycytidine (Aza; Sigma, St. Louis, MO) for four weeks by intraperitoneal injection (100µL, 0.5mg/kg body weight; three consecutive times every week). Control animals received saline injections only. Animals were divided into four groups: WT, WT treated with Aza (WT+Aza), CBS+/-, and CBS+/- treated with Aza (CBS+/- +Aza). At least four animals ($n \geq 4$) were used in each group. All procedures were done according to National Institutes of Health

guidelines and approved by the Institutional Animal Care and Use Committee of the University of Louisville, School of Medicine.

Cell Culture

Mouse aortic smooth muscle cells (SMCs) from ATCC (Manassas, VA) were cultured in 6-well TPP (Techno Plastic Products, Trasadingen, Switzerland) cell culture plates with DMEM medium containing 10%FBS and 0.2 mg/ml G-418. Cells were allowed to grow to about 60% confluence and treated with 80 μ M Hcy and 100 μ M Aza for 48 hours.

Blood Pressure Measurements

Blood pressure (BP) was measured in conscious mice by a non-invasive tail cuff method (CODA, Kent Scientific, Torrington, CT). Animals were allowed to acclimatize in the restraining chambers on a warm platform for short duration (20-30 min) for a few days before data was recorded. Under standard conditions (room temperature, lighting, and quiet surroundings) BP was recorded weekly before and after starting Aza treatment including systolic, diastolic, and mean pressures.

Ultrasound

Under isoflurane anesthesia, aorta ultrasound was done using a Vevo 2100 system (Visual Sonics, Toronto, ON, Canada) before and after Aza treatment. The thoracic and abdominal area was depilated and the animal was placed supine on a warm platform (37°C). Using a MS550D (22-55 mHz) transducer, the thoracic and abdominal aorta were imaged. Cross-sectional images of the aorta in the B Mode were used to measure wall thickness and lumen diameter to obtain wall-to-lumen ratio. Peak systolic velocities

(PSV) and end-diastolic velocities (EDV) values were measured in pulsed-wave doppler mode. Resistive index (RI) was calculated using the following equation: $RI = (PSV - EDV)/PSV$.

Plasma Hcy metabolite levels

Plasma levels of SAM, SAH and Hcy were analyzed using HPLC-UV as described before [113-115].

Vascular reactivity studies with aortic rings:

Aortic ring preparation – The thoracic aorta was extracted from euthanized mice and immersed in Kreb's solution (pH 7.4, 37 ° C) containing (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 12.5 NaHCO₃, and 10.9 D-glucose. Aortic rings were prepared by cutting 2 mm wide segments and attached to an isometric force transducer with two tungsten wire triangles [0.002 in. diameter, (Scientific Instruments Services, Ringoes, NJ)] in a tissue myobath (Radnoti, Monrovia, CA) which was continuously aerated with 95% O₂-5% CO₂. Rings were stretched to develop 0.5 g optimal resting tension and equilibrated for an hour to obtain baseline measurements using a DigiMed Tissue Force Analyzer (Micromed, Louisville, KY) [152].

Response to Phenylephrine, Acetylcholine and Sodium Nitroprusside - After a baseline recording, vasoconstriction was studied by adding different concentrations of phenylephrine (Phe) from 10⁻⁶ to 10⁻² M to the organ bath to make final concentrations of 10⁻⁹ to 10⁻⁵ M, respectively. Once maximal constriction was reached at 10⁻⁵M Phe, Acetylcholine (Ach) was added to the organ bath similar to Phe in gradually increasing concentrations to detect endothelial-dependent vasorelaxation. The tissue rings were

washed with fresh Kreb's solution and pre-constricted with a maximum dose of Phe followed by 10^{-5} M Sodium nitroprusside (SNP) to study endothelium-independent vasorelaxation. The tissue responses were recorded for 10 min for each drug concentration using DMSI-410 1.8.27 software (Micromed, Louisville, KY).

Cryosectioning

Aortic tissue was cryopreserved in Peel-A-Way disposable plastic tissue-embedding molds (Polysciences, Warrington, PA) containing tissue-freezing media (Triangle Biomedical Sciences, Durham, NC). Tissues were stored at -80°C until use. Serial sections of 7- μm thickness were made using a Cryocut (Leica CM 1850). Cryosections were placed on Superfrost plus microscope slides, air dried and stored at -80°C until further use.

Collagen staining

To measure the deposition of collagen in the aortic wall, sections were stained with a Masson trichrome kit (Richard Allan Scientific, Kalamazoo, MI) following the manufacturer's instructions. Collagen deposition is shown as blue color. Images were captured with a light microscope (Olympus FluoView1000; B&B Microscope Ltd, Pittsburg, PA). Sections were stained separately to differentiate type I and III collagen using Picrosirius red staining kit (Polysciences, Inc., Warrington, PA) [153]. Images were captured under polarized light filter (Olympus FluoView1000; B&B Microscope Ltd, Pittsburg, PA).

Immunostaining

Immunofluorescence staining was done on 7- μ m thick aortic sections using anti-MTHFR, MMP9, TIMP1, SAHH, DNMT1 and Hcy antibodies. Images were captured using a confocal microscope (Olympus FluoView1000; B&B Microscope Ltd, Pittsburg, PA) and analyzed by Image Proplus 7.0 software (Media Cybernetics, Inc., Rockville, MD).

Immunoblotting

Protein was extracted from cultured SMCs using RIPA lysis buffer (Boston BioProducts, Worcester, MA) with protease inhibitors. Protein concentration was determined using the Bradford assay and 50 μ g of total protein was loaded in SDS-PAGE gels. Protein was resolved by electrophoresis, transferred to a PVDF membrane and incubated with primary antibody and secondary HRP- conjugated antibody. Protein bands were detected using ECL substrate (Millipore, Billerica, MA). Bands were detected using Imagelab (Biorad, Hercules, CA) and normalized by a GAPDH control. The band intensities were quantified using ImageJ software.

Gelatin Zymography

Cultured SMCs were scraped with extraction buffer [10 mmol/l cacodylic acid, 20 ZnCl₂, 1.5 NaN₃ and 0.01% Triton X-100 (pH 5.0)] and incubated overnight at 4°C with gentle agitation. The homogenate was centrifuged for 5 min at 10000 xg and the supernatant was collected. Protein concentration in the sample was measured using the Bradford method, and 100 μ g of the protein was resolved in 10% SDS-PAGE containing 0.1% gelatin as MMP substrate. Gels were washed in renaturing buffer to remove SDS, rinsed

in water and incubated for at least 48 h in developing buffer at 37°C in a water bath with gentle shaking. Gels were stained with 0.5% Coomassie brilliant blue for 1 h at room temperature. MMP activity in the gel was detected as white bands against a dark blue background.

Quantification of 5-methylcytosine

Genomic DNA was extracted from aorta and the amount of 5-methylcytosine (5-mC) was measured by ELISA following manufacturer's instructions (Zymo Research Corp., Irvine, CA, USA). Values are presented as mean $\pm$ SEM as percent of 5-mC

Statistical Analysis

Statistical analysis was done using Primer of Biostatistics 7.0 (McGraw-Hill, New York, NY). One-way analysis of variance (ANOVA) followed by Bonferroni Correction was used for comparison between the experimental groups. Differences were considered significant when $p < 0.05$. Values are presented as mean $\pm$ SEM ($n \geq 4$).

Figure 17: Schematic representation of the experimental plan

Results

Effect of Aza on physiological parameters

The body and heart weights of CBS^{+/-} (HHcy) mice were lower than the WT groups (Figure 18). A significant increase was noticed following Aza treatment. The plasma Hcy level (Figure 19) was higher in CBS^{+/-} mice when compared to WT groups. This can be explained from decreased SAM: SAH due to decreased levels of SAM and increased levels of SAH, precursors of Hcy in CBS^{+/-} mice when compared to WT mice (Figure 20). Following Aza treatment, SAM: SAH ratio was increased causing a decrease in plasma Hcy levels in CBS^{+/-} mice. The baseline systolic, diastolic (Figure 21) and mean blood pressures (Figure 22) in CBS ^{+/-} mice were higher compared to WT groups. After 4 weeks of Aza treatment, a significant decrease was observed in the BP of CBS^{+/-} mice.

Wall-to-lumen ratio and Resistivity Index

HHcy causes aortic vessel remodeling. To analyze the structural changes in the aorta, we measured the lumen diameter and wall thickness of the ascending aorta and lumen diameter of abdominal aorta. The wall-to-lumen ratio of ascending aorta in CBS^{+/-} mice was increased compared to WT groups (Figure 23). Similarly, the diameter of abdominal aorta in CBS^{+/-} mice was significantly decreased from WT and WT + Aza mice (Figure 25). After Aza treatment, the wall-to-lumen ratio of the ascending aorta decreased and the lumen diameter of abdominal aorta increased in CBS^{+/-} mice and was similar to WT groups. Resistive index (RI) is a measure of the resistance of the vessel to blood flow. The RI of ascending (Figure 24) and abdominal aortas (Figure 26) were increased in

CBS+/- mice compared to WT groups and significantly decreased following Aza treatment.

Aortic response to Phenylephrine, Acetylcholine and Sodium Nitroprusside

To evaluate effect of Aza on aortic function, the response of aortic rings from the experimental groups to vasoconstriction and vasorelaxation in response to Phe and Ach respectively, was tested in a dose-dependent manner. Aortas from CBS+/- mice were nearly three-fold and seven-fold less responsive to Phe (Figure 27) and Ach (Figure 28) compared to WT groups. Similarly, endothelium-independent relaxation to maximum concentration of SNP was reduced by three-fold (Figure 29) in CBS+/- mice compared to WT groups. Aza treatment restored the aortic responses to Phe, Ach and SNP so that they were similar to WT groups.

Effect of Aza on collagen deposition

Collagen deposition was quantified in the aorta as an indication of vascular stiffness. WT groups without or with Aza showed normal blue intensity, whereas, increased intensity was observed in CBS+/- mice suggesting increased collagen deposition in the adventitia (Figure 30). Aza treatment reduced total collagen content by 2.4-fold compared to its untreated control. Under polarized light filter, picrosirius red stain shows type I collagen as yellow and type III as a green color (Figure 31). Type I and III collagen from WT without Aza treatment was considered normal and used for further analysis. Aorta from WT+Aza did not differ from its control (Figure 31). In contrast, CBS+/- mice showed 3.8-fold increase in type I and a 4.5-fold increase in type III collagen compared to untreated WT mice (Figure 31). Following treatment with Aza in CBS+/- mice type I

collagen decreased by two-fold and type III by 1.5-fold compared to untreated CBS+/- mice (Figure 31).

DNMT1 inhibition decreases expression of ECM remodeling and Hcy synthesis and triggers Hcy remethylation

To examine effects of Aza treatment on the expression of proteins involved in Hcy metabolism, we measured the expression of MTHFR, SAHH and Hcy by immunostaining. There was an eight-fold increase in Hcy and two-fold increase in SAHH expression (Figure 32) in CBS+/- mice compared to WT groups. MTHFR expression was decreased by 1.5-fold in CBS+/- mice (Figure 32) compared to WT groups. These results suggested an up-regulation of Hcy synthesis and down-regulation of Hcy remethylation pathway in CBS+/- mice. Aza treatment decreased the expression of Hcy and SAHH and increased MTHFR in CBS+/- mice which were similar to WT controls. To evaluate the expression of ECM remodeling genes, we measured the expression of MMP9 and TIMP1 (Figure 33). There was a three-fold increase in MMP9 and 2.6-fold increase in TIMP1 in CBS+/- mice (Figure 33) compared to WT control. Following Aza treatment their levels were reduced and were similar to WT groups. MMP9 activity in cultured SMCs was quantified using gelatin zymography. Hcy treatment increased MMP9 activity in SMCs and following Aza treatment, MMP9 activity was decreased following Aza treatment (Figure 34).

Global DNA Methylation in hyperhomocysteinemia

To better understand the effect of HHcy on maintenance methylation, we measured the expression of DNMT1 in all the groups. There was a three-fold increase in DNMT1

expression in CBS^{+/-} mice compared to WT controls, which was normalized by Aza treatment (Figure 35). Similarly there was an increase in DNMT1 and 3b expression in Hcy treated SMCs which was reduced after treatment with Aza (Figure 36). To determine the overall methylation levels in aorta during HHcy, we measured 5-methylcytosine (5-mC) from the extracted DNA. As shown in Figure 37, we observed a significant increase in methylation levels in CBS^{+/-} mice compared to WT groups. Following treatment with Aza there was a significant reduction in the CBS^{+/-} mice compared to its control. There was an increase in global methylation levels in Hcy treated SMCs. Following Aza treatment, methylation levels were decreased (Figure 38).

Figure 18: Effect of Aza treatment on gravimetric parameters. Bar graph representing gravimetric data i.e. body and heart weight. Data are shown as mean±SEM. n=6; * $p < 0.05$ vs. WT and WT+Aza; † $p < 0.05$ vs. CBS

Figure 19: Aza treatment normalized plasma Hcy levels. Bar graph representing plasma Hcy level in all groups was measured using HPLC-UV. Data are shown as mean $\pm$ SEM. n=4; * p < 0.05 vs. WT and WT+Aza; † p < 0.05 vs. CBS.

Figure 20: Aza treatment normalized SAM/SAH ratio. Bar graphs represent plasma SAM and SAH levels in all experimental mice were measured using HPLC-UV. Data are shown as mean $\pm$ SEM. n=4; * p < 0.05 vs. WT and WT+Aza; † p < 0.05 vs. CBS

Figure 21: Aza treatment normalized systolic and diastolic blood pressure in HHcy mice. Blood Pressure was measured using tail cuff method. Line graphs represent the systolic and diastolic blood pressure. Values are expressed as mean $\pm$ SEM. $n=4$; * $p < 0.05$ vs. WT and WT+Aza; $^{\dagger} p < 0.05$ vs. CBS

Figure 22: Aza treatment normalized mean blood pressure in HHcy. Blood Pressure was measured using tail cuff method. Line graphs represent the mean blood pressure. Values are expressed as mean $\pm$ SEM. n=4; * p < 0.05 vs. WT and WT+Aza; † p < 0.05 vs. CBS

Figure 23: Aza treatment normalized ascending aorta wall-to-lumen ratio in HHcy.

B-mode ultrasound image of ascending aorta. Wall thickness (mm) and lumen diameter (mm) were measured by Vevo 2100 (Visual Sonics Vevo Ultra Imaging System). Bar graphs of wall-to-lumen ratio of ascending aorta represented as mean $\pm$ SEM. $n=4$, * $p < 0.05$ vs. WT and WT+Aza; † $p < 0.05$ vs. CBS

Figure 24: Aza treatment normalized ascending aorta resistive index. Pulse Wave mode images of ascending aorta. Resistive index (RI) estimated the resistance offered by the aorta and is calculated as $(PSV-EDV)/EDV$. RI was measured by Vevo 2100 (Visual Sonics Vevo Ultra Imaging System) Bar graphs represent mean RI $\pm$ SEM in ascending aorta. n=4, * $p < 0.05$ vs. WT and WT+Aza; † $p < 0.05$ vs. CBS

Figure 25: Aza treatment normalized abdominal aorta lumen diameter. B-mode ultrasound image of abdominal aorta. Lumen diameter (mm) was measured by Vevo 2100 (Visual Sonics Vevo Ultra Imaging System). Bar graphs represent the lumen diameter of abdominal aorta represented as mean $\pm$ SEM. $n=4$, * $p < 0.05$ vs. WT and WT+Aza; $^{\dagger} p < 0.05$ vs. CBS

Figure 26: Aza treatment normalized abdominal aorta resistive index. Pulse Wave mode images of abdominal aorta. Resistivity Index (RI) was measured by Vevo 2100 (Visual Sonics Vevo Ultra Imaging System). Bar graphs represent mean RI $\pm$ SEM in ascending aorta. $n=4$, * $p < 0.05$ vs. WT and WT+Aza; † $p < 0.05$ vs. CBS

Figure 27: Aza treatment improved aortic response to Phe. Aorta was extracted from experimental mice (WT, WT+Aza, CBS, CBS+Aza) following protocol described in Materials and Methods. Aortic rings were mounted in myobath containing Kreb's solution. The rings were treated with phenylephrine (Phe) in a dose dependent manner as described in Materials and Methods. Data are shown as n=4; * p < 0.05 vs. WT and WT+Aza; † p < 0.05 vs. CBS

Figure 28: Aza treatment improved aortic response to Ach. Aorta was extracted from experimental mice (WT, WT+Aza, CBS, CBS+Aza) following protocol described in Materials and Methods. Aortic rings were mounted in myobath containing Kreb's solution. Rings were treated with acetylcholine (Ach) in a dose dependent manner as described in Materials and Methods. Data are shown as n=4; * $p < 0.05$ vs. WT and WT+Aza; † $p < 0.05$ vs. CBS

Figure 29: Aza treatment improved aortic response to SNP. Aorta was extracted from experimental mice (WT, WT+Aza, CBS, CBS+Aza) following protocol described in Materials and Methods. Aortic rings were mounted in myobath containing Kreb's solution. Rings were treated with sodium nitroprusside (SNP) in a dose dependent manner as described in Materials and Methods. Data are shown as n=4; * p < 0.05 vs. WT and WT+Aza; † p < 0.05 vs. CBS

Figure 30: Collagen expression was decreased post Aza treatment. Collagen is stained as dark blue. Original image, x 20 magnification. Bar graph represents the % mean intensity $\pm$ SEM of blue color in the aorta. n=4, * $p < 0.05$ vs. WT and WT+Aza; † $p < 0.05$ vs. CBS

Figure 31 A: Aza treatment decreased collagen I and III in HHcy. Picrosirius red staining for type I and type III collagen (yellow and green colors represent type I and type III collagen respectively), Original image, x 20 magnification

Figure 31 B: Aza treatment decreased collagen I and III in HHcy. Bar graph represents % mean intensity $\pm$ SEM of yellow (Type I) and green (Type III) color in aorta. n=4. * $p < 0.05$ vs. WT and WT+Aza; † $p < 0.05$ vs. CBS; ‡ $p < 0.05$ vs. WT.

Figure 32A: Aza treatment decreases Hcy accumulation. The protein expressions of SAHH, Hcy and MTHFR were measured. Expression of MTHFR and SAHH are shown as green fluorescence and expression of Hcy is seen as red fluorescence. Original image, Hcy x 20 magnification, SAHH, MTHFR, x 10 magnification

Figure 32B: Aza treatment decreases Hcy accumulation. Bar graph representations of the IHC images. Y-axis represents % change mean intensity $\pm$ SEM. $n=4$; * $p < 0.05$ vs. WT, WT+Aza and CBS+Aza; † $p < 0.05$ vs. WT, WT+Aza and CBS+Aza; ‡ $p < 0.05$ vs. WT, WT+Aza

Figure 33A: Aza treatment normalizes the expression of MMP9 and TIMP1 in HHcy. The protein expressions of MMP9, TIMP1 were measured. Expression of MMP9 and TIMP1 are seen as red fluorescence. Original images, x 10 magnification

Figure 33B: Aza treatment normalizes the expression of MMP9 and TIMP1 in HHcy. Bar graph representations of the IHC images. Y-axis represents % change mean intensity $\pm$ SEM. n=4; * $p < 0.05$ vs. WT, WT+Aza and CBS+Aza; † $p < 0.05$ vs. WT, WT+Aza and CBS+Aza; ‡ $p < 0.05$ vs. WT and WT+Aza.

Figure 34: Aza treatment decreased MMP9 activity in Hcy treated SMCs. Gelatin zymography was done to analyze the activity of MMP9 in Aza and Hcy treated SMCs. Bar graph is expressed as mean intensity $\pm$ SEM * $p < 0.05$ vs. Ctrl and Aza, † $p < 0.05$ vs Hcy

Figure 35: Aza treatment decreases DNMT1 in HHcy. The protein expressions of DNMT1 were measured. Expression of DNMT1 is shown as green fluorescence. Bar graph representations of the IHC images. Y-axis represents % change mean intensity $\pm$ SEM. n=4; * $p < 0.05$ vs. WT, WT+Aza and CBS+Aza; Original image x 10 magnification

Figure 36: Aza treatment decreases DNMT1, 3b in Hcy treated SMCs. Immunoblotting of DNMT1, 3a and 3b. Bar graphs show the relative expression of DNMT1, 3a and 3b over controls, after normalization with GAPDH. The data are shown as mean $\pm$ SEM. n=4; *, † p < 0.05 vs. ctrl, Aza and Hcy + Aza.

Figure 37: Aza treatment decreases global methylation levels in aorta. Overall methylation levels were measured using ELISA. The bar graphs represent the mean %5-mC $\pm$ SEM; n=4, * p < 0.05 vs. WT and WT+Aza; † p < 0.05 vs. CBS.

Figure 38: Aza treatment decreases global methylation in SMCs. Overall methylation levels were measured using ELISA. The bar graphs represent the mean %5-mC $\pm$ SEM; n=5, * p < 0.05 vs. Ctrl and Aza; † p < 0.05 vs. Hcy.

Discussion

HHcy plays a critical role in the development of various aortic diseases [154-157]. HHcy induces the expression of MMPs involved in ECM metabolism, promoting aortic remodeling resulting in arterial hypertension [141]. Epigenetic mechanisms such as DNA methylation are known to control the expression of ECM components [158]. Although various studies report aberrant DNA methylation pattern in early stages of atherosclerosis [159] and aortic aneurysm [29], the role of DNA hypermethylation on aortic remodeling and arterial hypertension in HHcy still remains unclear. In the recent years, epigenetic inhibitors are being used as therapeutic agents in various cancer drug trials [151]. Our study provides new insights into the mechanism and the use of epigenetic inhibitors as therapeutic option in hypertension associated aortic pathologies.

In the present study, we used CBS^{+/-} mice as HHcy model. In a previous report, *Gupta et al.*, demonstrate that CBS deficient mice have decreased fat mass due to reduction in lipogenesis [160]. Our observation of reduced body weight in CBS^{+/-} mice supported this earlier finding. Plasma Hcy level is an important predictor of pulse pressure in hypertensive subjects [161]. In the previous study, increased plasma Hcy in CBS^{+/-} mice was associated with elevated systolic, diastolic and mean blood pressure. Evidence from earlier studies suggest that lowering plasma Hcy level by supplemental vitamins such as folic acid, B6 and B12 is effective in delaying the formation of atherosclerotic plaques [162]. Our results showed that Aza lowers plasma Hcy in CBS^{+/-} mice to normal levels. Plasma levels of S-adenosyl homocysteine (SAH), precursor of Hcy, is correlated to Hcy levels [125]. S-adenosyl methionine (SAM) and SAH levels were decreased and increased respectively in HHcy mice. Aza treatment reversed this effect in CBS^{+/-} mice.

During HHcy, the conversion of SAM to SAH liberates excess methyl groups which are used in methylating substrates including DNA and proteins. Indeed hypermethylation of gene promoters occurred in hypertension. For example, sulfatase 1, (SULF1) gene, an endosulfatase, has been reported to be hypermethylated in hypertensive subjects [163]. In our study, we showed that Aza treatment in hypertensive CBS+/- mice restored systolic, diastolic and mean blood pressure to normal levels, which suggested an important role for hypermethylation in HHcy associated hypertension.

Excessive accumulation of ECM proteins in HHcy causes hypertrophic vascular remodeling by increasing the wall thickness and decreasing lumen diameter. [62, 164]. In the present study, ultrasound examination revealed increased wall-to-lumen ratio of ascending aorta and decreased lumen diameter of abdominal aorta in CBS+/- mice. In an earlier study from our lab, treatment of CBS+/- mice with SAH hydrolase inhibitor, (Deazaadenosine, DZA), decreased systolic blood pressure without a change in the aortic wall-to-lumen ratio within the first week of treatment [141]. This result suggested that the reduction in systolic blood pressure was secondary to reduction in cardiac output preceding structural remodeling [141]. In the present study, we show that Aza treatment decreased BP at three weeks suggesting a significant role for epigenetic mechanism in aortic remodeling during HHcy-induced hypertension. The reduction in blood pressure (systolic, diastolic and mean) was associated with a reduction in wall-to-lumen ratio of ascending aorta and increase in lumen diameter of abdominal aorta. Resistive index (RI) of aorta was measured as an indication of resistance to blood flow. In previous literature, a positive correlation was reported for carotid artery RI and atherosclerosis [165]. In our study, increased RI in CBS+/- suggested vascular stiffness and was associated with

increased MMP9 activity and TIMP1 expression. The increase in TIMP1 appears to be compensatory to increased MMP9. Consistent with our findings, increased MMP9 and TIMP1 occurs in pathologies such as neonatal encephalopathy [166] and aortic aneurysm [167]. Our results demonstrated that Aza treatment reduces collagen deposition and also decreases MMP9 and TIMP1 expression in HHcy. The reduction in the wall-to-lumen ratio and RI upon Aza treatment suggested mitigation of aortic ECM remodeling.

Although previous studies reported no significant changes in smooth muscle contraction (Phe) [168] or endothelium-independent relaxation (SNP) [169] between WT and HHcy mice, we saw a significant decrease in the aortic response to Phe, Ach and SNP in HHcy mice. Consistent with our findings, Tyagi *et al* [170] reported that HHcy mice showed decreased response to vasoconstrictor (Endothelin-1) compared to controls. In the present study, the impairment of vessel function suggests endothelial and smooth muscle dysfunction and vascular stiffness. Aza treatment improved vascular response to Phe, Ach and SNP in HHcy and also reduced vessel stiffness as indicated by reduction of collagen deposition. This suggested that DNMT1 inhibition improved aortic function during HHcy thereby mitigates hypertension.

Hcy is derived from de-methylation of dietary methionine. S-adenosyl homocysteine hydrolase (SAHH) plays a major role in the synthesis of Hcy from S-adenosyl homocysteine. Following synthesis, Hcy has two fates: a) transsulfuration to cysteine aided by CBS and b) remethylation to methionine by MTHFR in the folate metabolism pathway [149]. Previous study shows that in HHcy, betaine Hcy methyltransferase (BHMT) and formation of 5-methyltetrahydrofolate by methylenetetrahydrofolate reductase (MTHFR) is inhibited [171]. The fate of homocysteine in the body is controlled

by a balance between the remethylation and transsulfuration pathways. During high methionine diet, Hcy catabolism is increased favoring transsulfuration over remethylation. In contrast, with a low methionine diet the remethylation pathway was favored compared to transsulfuration [149]. Since CBS is a key enzyme in Hcy metabolism, genetic mutations in CBS [172] and dietary deficiency of amino acids such as methionine or taurine and vitamins (folate and B12) can affect the plasma Hcy concentrations. In a recent study, Tang *et al* demonstrated that when WT and transgenic mice expressing human CBS were fed a low methionine diet, the protein levels of CBS in the liver and the activity were decreased to conserve methionine levels in the body [173]. In the transsulfuration pathway, Hcy is catabolized to yield taurine which reduces total serum cholesterol by lowering very low density lipoprotein (VLDL) and low density lipoprotein (LDL) [174]. When CBS^{+/-} mice were fed taurine deficient diet, there was an upregulation of CBS monoallele along with a reduction in total serum cholesterol levels. These studies suggest the impact of enzymes involved in Hcy metabolism such as CBS, SAHH and MTHFR in maintaining plasma Hcy levels. In the present study, immunostaining analysis revealed an upregulation of SAHH and Hcy expression and downregulation of MTHFR in the aorta from HHcy mice. Also, a previous study suggests a negative correlation between the concentration of SAH and MTHFR expression [125]. Upon treatment with Aza, there was a reduction in the SAHH expression and increase in MTHFR suggesting increased remethylation to reduce plasma Hcy levels.

Previous reports in the literature link HHcy to both global hypermethylation [175] and hypomethylation [176]. A study by Zhang *et al.*, showed that the effect of Hcy on methylation patterns of gene promoters was dependent on Hcy concentration. HHcy is

known to inhibit the activity of dimethylarginine dimethylaminohydrolase (DDAH2), an enzyme involved in the nitric oxide synthase pathway, causing endothelial dysfunction. Previous studies report hypermethylation of DDAH2 promoters in HHcy [177]. Also, an earlier study in our lab, showed increased DNMT1 expression in HHcy mice suggesting hypermethylation [37]. In the present study, we confirmed global hypermethylation by quantitating %5-mC in HHcy mice aorta and Hcy treated SMCs. Treatment with Aza decreased the expression of DNMT1, 3b and also reduced global methylation levels. Although previous results suggest a positive correlation between SAM/SAH ratio and DNA methylation [178], we found DNA hypermethylation with decreased SAM/SAH ratio in Hcy mice. The decrease in SAM/SAH ratio suggests an increased Hcy production accompanied by an increased availability of methyl groups for DNA methylation.

An approach to alleviate HHcy mediated aortic pathology is to lower plasma Hcy levels. Although folic acid supplementation decreases Hcy level to prevent disease progression, it is unable to reverse the existing HHcy induced aortic pathology, termed as Hcy memory effect [178]. The present study for the first time provides evidence that epigenetic DNMT inhibitor can be a potential therapeutic agent independently or in combination with other therapies such as folic acid in aortic diseases.

In conclusion, HHcy promotes pathological aortic remodeling and endothelial dysfunction leading to arterial hypertension. Inhibition of DNMT1, using Aza, ameliorates Hcy level and reduces high blood pressure. Aza appears to mediate its action by modifying the expression of enzymes involved in Hcy metabolism and ECM proteins to reduce vascular stiffness thus improving vascular function.

CHAPTER VI

SUMMARY, CONCLUSION AND FUTURE DIRECTIONS

Cardiovascular diseases are the leading cause of death worldwide. HHcy is identified as one of the primary risk factors for cardiovascular diseases such as diseases of the aorta [5, 139]. An elevation of the plasma Hcy level correlates with blood pressure in human subjects with methionine loading [108]. Arterial stiffness and vascular dysfunction are the characteristic features that contribute to cardiovascular diseases such as atherosclerosis, which causes resistance to blood flow leading to hypertension [20]. Previous studies suggest an increased accumulation of ECM components such as collagen and MMPs in cardiovascular diseases causing ECM remodeling [61]. Epigenetic modifications such as DNA methylation and histone modifications regulate the expression of cellular proteins. Furthermore, evidence suggests a role of DNA methylation in HHcy associated pathologies. Therefore, epigenetic inhibitor therapies are currently being considered as a promising approach in various diseases.

In the first set of experiments it was found that in CBS^{+/-} mice, increased levels of plasma Hcy caused an increase in the synthesis and accumulation of ECM components. Increased collagen deposition and decreased elastin levels contributed to aortic stiffness providing resistance to the incoming blood flow. This narrowing of blood vessels contributes to the development of arterial hypertension. Epigenetic mechanisms played a major role in regulating the transcription of these ECM remodeling genes.

HHcy triggers epigenetic changes by increasing the expression of enzymes involved in epigenetic modifications such as DNA methylation (DNMT1, 3a, MBD2) and Histone methylation (H3K9). These changes contributed to increased genome-wide global DNA methylation levels thereby regulating various pathways.

In the second set of studies, to ascertain the effect of DNA methylation in aortic remodeling in HHcy, CBS $\pm$ mice were treated with an epigenetic inhibitor, Aza. Aza treatment decreased global hypermethylation by inhibiting DNMT1 and hence lowered plasma Hcy levels in CBS $\pm$ mice. Aza treatment also decreased ECM remodeling by lowering the expression of MMP9 and deposition of collagen in the aorta wall causing decreased wall to lumen ratio and resistive index thus mitigating hypertension in HHcy mice. Hence, treatment with epigenetic inhibitors is a possible therapeutic approach to prevent HHcy associated pathological remodeling.

Future Directions:

Hypertension-induced aortic remodeling is a complex process involving enzymes that synthesize ECM components and their regulators such as MMPs and TIMPs. This study mainly focused on the effect of an epigenetic inhibitor, Aza, on ECM remodeling proteins (MMP9, TIMP1, Collagen) and Hcy metabolism enzymes (SAHH, MTHFR) in the aorta. However, as the enzymes involved in Hcy metabolism are located in other organs such as kidney and liver, additional studies are required to quantitate the specific effect of Aza on individual enzymes involved in Hcy metabolism. Although this study provides evidence of impairment in vascular function in HHcy mice and its improvement with Aza, understanding the mechanism by which HHcy affects promoter methylation of

individual genes involved in pathways such as nitric oxide synthase pathway requires elucidation.

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LIST OF ABBREVIATIONS

5-mC – 5-methylcytosine

AAA - Abdominal aortic aneurysm

Ach – Acetylcholine

ANOVA – Analysis of variance

Aza – 5-Aza-2'-deoxycytidine

BHMT – Betaine homocysteine methyltransferase

BP – Blood pressure

CBS – Cystathionine beta synthase

CDC – Center for disease control and prevention

CMN – Cystic medial necrosis

Col - Collagen

Ctrl - Control

CVD – Cardiovascular disease

DDAH – Dimethylarginine dimethylaminohydrolase

DMEM – Dulbecco's modified eagle medium

DNMT – DNA methyltransferase

ECM – Extracellular matrix

EDV – End diastolic velocity

ELISA – Enzyme-linked immunosorbent assay

FDA – Food and drug administration

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

H3K9me3 - Histone 3 trimethylated at lysine 9 residue

HAT – Histone acetyl transferase

Hcy – Homocysteine

HDAC – Histone deacetylase

HDM – Histone demethylase

HHcy – Hyperhomocysteinemia

HMT – Histone methyltransferase

HPLC-UV – High performance liquid chromatography with UV detector

HRP – Horseradish peroxidase

IHC - Immunohistochemistry

MBD - Methyl binding domain protein

MCP – Monocyte chemotactic protein

MDS – Myelodysplastic syndrome

MFS – Marfan syndrome

MMP – Matrix metalloproteinase

MS – Methionine synthase

MTHFR - 5,10 methylene tetrahydrofolate reductase

NO – Nitric oxide

PBS – Phosphate buffer saline

Phe – Phenylephrine

PSV – Peak systolic velocity

RI – Resistive index

ROS – Reactive oxygen species

RT-PCR – Reverse transcription polymerase chain reaction

SAHH – S-adenosyl homocysteine hydrolase

SAM – S-adenosyl methionine

SAH – S-adenosyl homocysteine

SEM – Standard error of mean

SMC – Smooth muscle cells

SNP – Sodium nitroprusside

SULF1 – Sulfatase 1

TAA - Thoracic aortic aneurysm

THF - Tetrahydrofolate

TIMP – Tissue inhibitor of metalloproteinase

VSMC – Vascular smooth muscle cells

WT - Wild type

CURRICULUM VITAE

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EDUCATION

B.Tech. Department of Biotechnology 2005 - 2009

Anna University, Chennai, India

Percentage: 83.5% (Secured among top 10 ranks in the class)

M.S., Department of Molecular and Cell Biology 2009 - 2011

University of Texas at Dallas (UTD), Richardson, TX

GPA: 3.45

PhD, Department of Physiology and Biophysics 2011 – 2014

University of Louisville Medical School, Louisville, KY

GPA: 3.85

RESEARCH EXPERIENCE

Epigenetic regulation of aortic remodeling in hyperhomocysteinemia Jun'11 – May'14

University of Louisville, Louisville, KY

Working with Dr. Suresh C.Tyagi

Analysis of Nitric Oxide (NO) stress on Uropathogenic strains of *E.coli* Aug '10 – May '11

University of Texas at Dallas (UTD), Richardson, TX

Worked with Dr. Stephen Spiro

Learned Python and its applications in Bioinformatics May '10 – Aug '10

University of Texas at Dallas (UTD), Richardson, TX

Worked with Dr. Micheal Zhang

Research study on assaying enzymes involved in metabolic pathways in *ytfe* mutants of *E.coli*

University of Texas at Dallas (UTD), Richardson, TX

Jan '10 – May '10

Worked with Dr. Stephen Spiro

Phylogenetic analysis of cDNA of RNA polymerase in *Physarum* and *Fuligo*

University of Texas at Dallas (UTD), Richardson, TX

Aug '09 – Dec '09

Worked with Dr. Dennis Miller

Practical Summer Training Course on Molecular Biology Techniques (PCMB)

Vellore Institute of Technology (VIT), Vellore, India

May '09 – June '09

Expression and Purification of potyvirus like particles (PVLP) from *E.coli*

Mepco Schlenk Engineering College, Sivakasi (Affiliated to Anna University), India

Worked with Dr. Stephen Raj

Jan '09 – May '09

Training program in Genomics

June '08

Sai Biosciences Research Institute (SBRI), Chennai, India

TEACHING EXPERIENCE

Teaching Assistant for “Human Anatomy and Physiology II Lab (BIOL 3456)”

Worked with Dr. Wen Yu

May '10 - Aug '10

Teaching Assistant for “Classical and Molecular Genetics (BIOL 3301)”

Jan '10 – May '10

Worked with Dr. Jeff DeJong and Dr. Dennis Miller

Assisted in training students for summer research program and Louisville Regional Science Fair

2012 - 2014

MEMBERSHIPS IN PROFESSIONAL AND HONORARY SOCIETIES

- Student member, American Physiological Society
- Member, American Association for the Advancement of Science (AAAS)
- Secretary, Toastmasters International Club #7458 (2013-2014)
- Secretary, Enlighten Charity Inc.

AWARDS AND HONORS

- Received Graduate Dean's Citation award, 2014
- Received consolation award for best oral presentation at Trainees of Physiological Genomics session, Experimental Biology, 2012
- Received travel award from Department of Physiology and Biophysics, University of Louisville to attend Experimental Biology 2012-2014
- Received travel award from School of Medicine, University of Louisville to attend Experimental Biology 2012-2014
- Received GSC travel funding to attend Experimental Biology, 2012 and 2014

EXTRA CURRICULAR ACTIVITIES

- Judge, Louisville Regional Science Fair 2013-2014
- Volunteer, TEDx Louisville, 2012
- Volunteer, Career Fair, University of Texas at Dallas 2012-2013

MANUSCRIPTS PUBLISHED

- Givvimani, S, Munjal, C, **Narayanan, N**, Aqil, F, Tyagi, G, Metreveli, N, Tyagi, S. C, *Givvimani, S., Hyperhomocysteinemia decreases intestinal motility leading to constipation.* Am J Physiol Gastrointest Liver Physiol, 2012. **303**(3): p. G281-90
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- Givvimani, S, Kundu, S, **Narayanan, N**, Armaghan, F, Qipshidze, N, Pushpakumar, S, Vacek, T. P, Tyagi, S. C *TIMP-2 mutant decreases MMP-2 activity and augments pressure overload induced LV dysfunction and heart failure.* Arch Physiol Biochem, 2013. **119**(2): p. 65-74
- **Narayanan, N.**, Tyagi, N., Shah, A., Pagni, S., Tyagi, S. C. *Hyperhomocysteinemia during aortic aneurysm, a plausible role of epigenetics.* Int J Physiol Pathophysiol Pharmacol, 2013. **5**(1): p. 32-42
- Givvimani S, **Narayanan N**, Pushpakumar SB, C Tyagi S. *Anti-Parstatin Promotes Angiogenesis and Ameliorates Left Ventricular Dysfunction during Pressure Overload* Int J Biomed Sci. 2014 Mar;**10**(1):1-7
- **Nithya Narayanan**, Sathnur Basappa Pushpakumar, Srikanth Givvimani, Sourav Kundu, Naira Metreveli, Dexter James, Adrienne P. Bratcher, Suresh C. Tyagi *Epigenetic Regulation of Aortic Remodeling in Hyperhomocysteinemia* FASEB J. 2014 Apr 16

MANUSCRIPTS IN PREPARATION

- **Nithya Narayanan**, Sourav Kundu, Srikanth Givvimani, Sathnur B Pushpakumar, Ruhi Kulkarni, Suresh C Tyagi *Global DNA Methylation and Extracellular Matrix Remodeling in Aortic Smooth Muscle versus Endothelial Cells in Hyperhomocysteinemia*
In preparation

CONFERENCE ABSTRACT PRESENTATIONS

- **Nithya Narayanan**, Neetu Tyagi, Sebastian Pagni and Suresh C. Tyagi *Epigenetic mechanism of atherosclerosis and hypertension in Hyperhomocysteinemia* FASEBJ March 2012
(selected for oral and poster presentation)
- Tyagi N, **Narayanan N**, Givvimani S, Tyagi SC *Bad to Bone: Homocysteine* FASEB J March 29, 2012 26:1143.5
- Tyagi N, **Narayanan N**, Mishra PK, Qipshidze, N, Givvimani S, Tyagi SC *Epigenetic Reprogramming of Mitochondrial Dysfunction in hyperhomocysteinemia* FASEB J March 29, 2012 26:701.17

- Givvimani S, **Narayanan N**, Armaghan F, Pushpakumar S and Tyagi SC *Attenuation of conducted vasodilation in skeletal muscle arterioles during hyperhomocysteinemia* FASEBJ March 29 2012
- Tyagi N, Qipshidze N, **Narayanan N**, Givvimani S, Tyagi SC. *Hydrogen sulfide mitigates cerebro-vascular remodeling during cerebral ischemia*. Society of Neuroscience, 2012, New Orleans
- Srikanth Givvimani, Anuradha Kalani, **Nithya Narayanan**, Sourav Kundu, Suresh Tyagi, *Micro RNA-499 Protects the Heart Against Adverse Post Myocardial Infarction Remodeling and Regenerates Damaged Myocardium by Promoting Endogenous Cardiac Stem Cell Differentiation*, HBPR 2013
- **N.Narayanan**, S.B. Pushpakumar, N. Qipshidze, A.P. Bratcher, N. Tyagi and S.C. Tyagi *Epigenetic inhibition by 5 Aza 2' deoxycytidine mitigates hypertension in hyperhomocysteinemia* FASEBJ 2013 955.9
- S. Givvimani, **N. Narayanan**, S.B. Pushpakumar and S.C. Tyagi *Anti-parstatin promotes angiogenesis and ameliorates left ventricular dysfunction during pressure overload*. FASEBJ 2013 1129.16
- A. Kalani, N. **Narayanan**, N. Qipshidze, S. Givvimani, P.K. Kamat, S.C. Tyagi and N. Tyagi. *Epigenetic regulation of cerebrovascular remodeling mediated through exosomes in hyperhomocysteinemia: role of folic acid*. 932.9
- Dexter James, **Nithya Narayanan**, Adrienne Bratcher, Christina Roberts, Suresh Tyagi, Irving Joshua *Aortic Vascular Responsiveness in Hyperhomocysteinemic Mice* Research Louisville 2013
- **Nithya Narayanan**, Sourav Kundu, Srikanth Givvimani, Sathnur B Pushpakumar, Ruhi Kulkarni, Suresh C Tyagi *Global DNA Methylation and Extracellular Matrix Remodeling in Aortic Smooth Muscle versus Endothelial Cells in Hyperhomocysteinemia* FASEBJ 2014
(selected for oral and poster presentation in Physiological Genomics Conference and Epigenetics and Epigenomics session)