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Effect Of Dimethylarginine Dimethylaminohydrolase In The Development Of Salt Sensitivity

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**EFFECT OF DIMETHYLARGININE DIMETHYLAMINOHYDROLASE IN THE
DEVELOPMENT OF SALT SENSITIVITY**

by

SAMAR ABDULLA NASSER

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

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Approved by:

Advisor

Date

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DEDICATION

I would like to thank God, for all of the blessings that he has granted me and for helping me fulfill this dream. To my best friend and my husband, Adel: Thank you for your unrelenting support, encouragement, love, and most of all, for always believing in me, for you I am eternally grateful. To my son, Jabreel: You have been a true inspiration and are the epitome of love. To my parents for their love and guidance from the beginning, I cherish and appreciate all of your enlightenment and leadership. And of course to my sister, Samra, who has always lent me a hand, an open ear, and a shoulder to lean on, and to my brothers, Karim and Karam, who have continued to be there for me, thank you all for everything!

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CHAPTER 1

INTRODUCTION

Global epidemiologic studies indicate that habitual ingestion of high levels of dietary salt is associated with elevated blood pressure (BP) (Stamler, 1997). Accordingly, one estimate suggests that approximately 58% of Americans with hypertension are salt sensitive (e.g., increased sodium intake elevates their arterial pressure and/or decreased sodium intake lowers their arterial pressure) (Weinberger, Fineberg, Fineberg, et al., 2001). Salt sensitivity is more frequently observed in African American than in Caucasian subjects, and in older than in younger subjects (Luft, Miller, Grim, et al., 1991). Salt sensitivity is so prevalent in African Americans that it is considered to be a “hallmark” of Black hypertension, as 73% of African American hypertensive patients are found to be salt sensitive (Svetkey, Chen, McKeown, et al., 1997). Salt sensitivity has also been linked to obesity (Flack, Grimm, Staffileno, et al., 2002; Rocchini, Key, Bondie, et al., 1989), increased activation of the renin-angiotensin system (RAS), and relative deficiency of nitric oxide (NO) (Hall, Brands, Henegar, 1999; Adelman, 2002), in both African Americans and Caucasians.

The mechanism or mechanisms resulting in salt-sensitive hypertension are multiple and include both activation of the RAS via increases in angiotensin II and reductions in the endogenous vasodilator, NO (Figure 1). Oxidative stress reflected by release of reactive oxygen species (ROS) such as, superoxide anion ($O_2^{\bullet-}$) has several deleterious physiological effects, most importantly a reduction of NO bioactivity. Increased dietary sodium downregulates NO, upregulates vascular angiotensin II, and raises oxidative stress via upregulation of NADPH oxidase (Zhou, Adam, Jaimes, 2003; Boddi, Poggesi, Coppo, et al., 1998). An important means of NO downregulation is

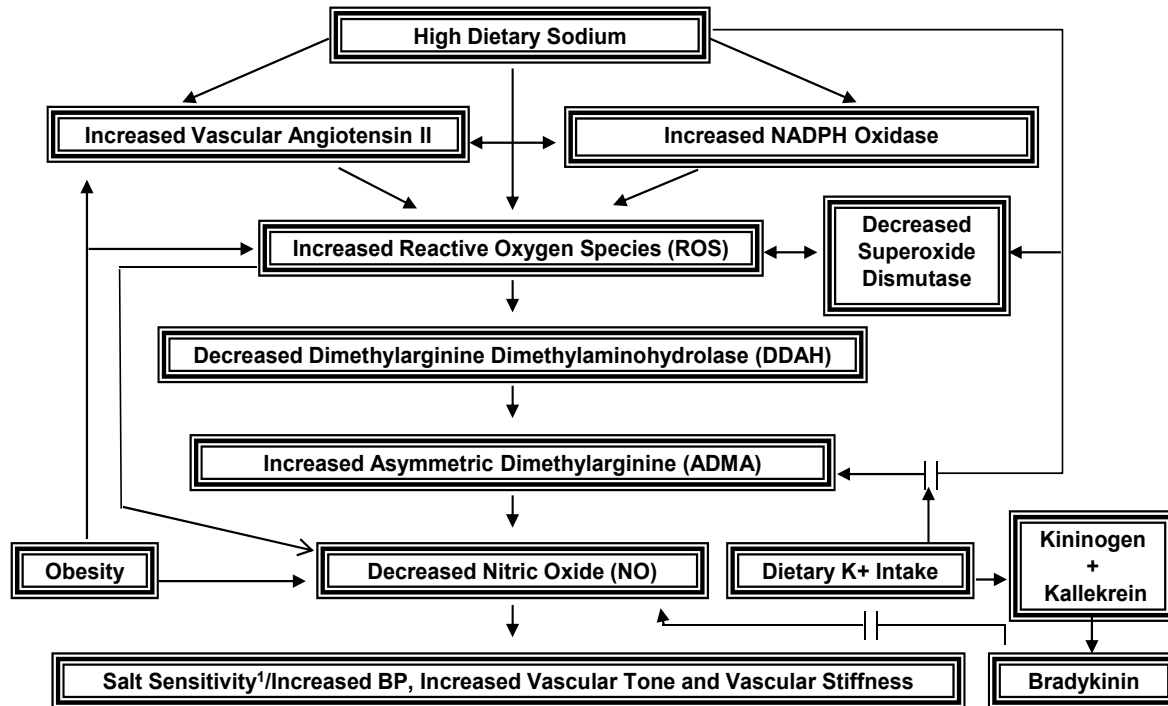


Figure 1. Proposed Model of the Sodium-Induced Reduction in Nitric Oxide and Elevation in Arterial Pressure in African Americans. Factors (i.e. obesity, high dietary sodium) associated with salt sensitivity increase vascular angiotensin II and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and reduce superoxide dismutase (SOD), an important scavenger of reactive oxygen species. The elevations of vascular angiotensin II and NADPH oxidase and reduction of SOD increase the presence of reactive oxygen species (ROS), which depress the expression and activity of dimethylarginine dimethylaminohydrolase (DDAH). The DDAH enzyme catalyzes the degradation of asymmetric dimethylarginine (ADMA), an inhibitor of nitric oxide (NO) synthase. Thus, DDAH depression by ROS results in the accumulation of ADMA, which decreases NO production. In the setting of ad lib sodium intake, the reduction in NO results in impaired natriuresis that causes a rise in systemic pressure to augment renal pressure-natriuresis, thereby maintaining steady state volume homeostasis. In the setting of ad lib potassium intake, potassium stimulates kallekrein release converting kininogen to bradykinin which increases NO release. ¹Blood pressure (BP) rises to stimulate natriuresis thereby restoring tissue perfusion homeostasis due to the NO deficiency that has shifted the pressure-natriuresis curve rightward (to higher BP). Thus, in a sodium-replete environment, tissue perfusion homeostasis is maintained at the expense of higher BP. It is plausible but not known if the sodium-induced rise in ADMA is attributable to a parallel depression in DDAH by sodium.

through asymmetric dimethylarginine (ADMA), an endogenous NO inhibitor, which is largely metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH).

The activity of DDAH is impaired by oxidative stress, thereby permitting ADMA to accumulate thus resulting in further inhibition of NO [\uparrow ROS \rightarrow \downarrow DDAH \rightarrow \uparrow ADMA \rightarrow \downarrow NO]. Increases in oxidative stress, reduction in DDAH activity, and augmented action of ADMA on depressing NO production represents a plausible mechanism in human salt sensitivity. To date, we are unaware of studies that have examined the sodium-induced effect of DDAH activity resulting in this cascade.

Therefore, the purpose of this study is to investigate and characterize the above mechanism through which salt-induced depression of NO synthesis occurs in normotensive African Americans. **The central hypothesis is that increased dietary sodium intake downregulates DDAH leading to increased ADMA which depresses NO production, resulting in a rise in BP. I further postulate that ROS will increase with sodium exposure, resulting in downregulation of DDAH.** This initial study is the first to test whether increased sodium intake is associated with a reduction in DDAH activity, leading to an increase in ADMA, and a decrease in NO production as determined by fewer NO metabolites. The study was conducted in healthy, normotensive African American men and women.

Hypertension in African Americans is a major clinical and public health problem that contributes significantly to premature morbidity and mortality as well as to the shorter lifespan in African Americans compared to Caucasians. Higher BP measurements in African Americans compared to Caucasians have been documented early in life, beginning in childhood (Muntner, He, Chen, et al., 2004). The age-adjusted prevalence of hypertension (HTN) during 2006 among individuals aged 20 years and older in the total US population was 33.3% (73,600,000). Non-Hispanic Blacks had the highest age-adjusted prevalence (44.4% men, 43.9% women), non-Hispanic Whites an

intermediate prevalence (34.1% men, 30.3% women), and Mexican Americans the lowest prevalence (23.1% men, 30.4% women) (Lloyd-Jones, Adams, Carnethon, et al., 2009).

Diet and lifestyle likely play important roles in the pathogenesis of HTN in African Americans and in the greater incidence of HTN relative to Caucasians. African Americans, especially women, are less physically active, consume more calories and are more obese beginning in the pre-adult years than Caucasians (Burke, Savage, Manolio, et al., 1992; Sharp, Bell, Grunwald, et al., 2002). Obesity, especially among women, is more common in African Americans than Caucasians (Ogden, Carroll, Curtin, et al., 2006).

Salt sensitivity is associated with a rise in BP occurring during sodium loading and/or a fall in BP during sodium restriction that exceeds directionally appropriate random fluctuations in BP. Salt sensitivity is more common in African American than Caucasian hypertensives and is also present, albeit to a lesser degree, in normotensive African Americans (Wright, Rahman, Scarpa, et al., 2003). Nevertheless, more than 50% of both African Americans and Caucasians with hypertension will manifest salt sensitivity (Peters and Flack, 2000). Salt sensitivity is a very practical concern given the nearly ubiquitous intake of dietary sodium by Americans in general and African Americans in particular, that is far in excess of any known physiological need. Even those African Americans with BPs in the normal range, have typically been found to manifest more salt sensitivity than Caucasians (Flack, Ensrud, Mascioli, et al., 1991).

The acute phase of salt sensitivity is associated with transient plasma volume expansion and increased cardiac output leading to increased tissue perfusion. The chronic phase of salt sensitivity is, however, characterized by a return of cardiac output

to normal and only those manifesting an increase in peripheral vascular resistance actually manifest a pressor response to sodium. Arguably, salt sensitivity has been conceptualized as maintenance of tissue perfusion homeostasis at the expense of higher blood pressure levels that augment pressure-induced renal natriuresis. Salt sensitivity increases at higher levels of BP, while plasma volume is simultaneously lower (frankly depressed) at higher BP levels. Thus, it is unlikely that salt sensitivity is a physiological attempt to maintain intravascular homeostasis but rather is a physiologic means for normalizing tissue perfusion via an incompletely understood mechanism(s) of whole-body autoregulation. The level of BP required to maintain steady state in/out tissue perfusion homeostasis can be influenced by factors such as nitric oxide deficiency and/or higher levels of angiotensin II, two factors that shift the pressure-natriuresis curve to the right. The pressure natriuresis relationship becomes less steep in those with salt sensitivity as higher arterial pressures are required to excrete any given amount of sodium compared to those who do not manifest salt sensitivity; it is the excretion of urinary sodium along with the rise in peripheral resistance that restores and maintains tissue perfusion homeostasis. In individuals or groups prone to salt sensitivity (those with impairments in natriuresis), the consumption of a typical western diet that contains sodium far in excess of physiological requirements, will likely lead to sustained BP elevations.

Although the physiological and genetic factors underlying salt sensitivity are increasingly understood, the mechanisms involved are not fully elucidated. This experimental study will assess the effect of sodium loading on an important physiologic cascade that has been hypothesized to contribute to salt-sensitive hypertension. This is an initial study used as basic exploratory work to establish a conceptual and plausible

model for future research. The study will enhance our knowledge in the field of salt-sensitive hypertension by potentially delineating a mechanism which can be targeted with lifestyle modifications (e.g., raised potassium intake) and via pharmacological means (e.g., DDAH stimulators) to prevent the rise in BP during habitually high dietary sodium intake.

CHAPTER 2

BACKGROUND AND SIGNIFICANCE

There are a number of obesity-related physiological effects that contribute to the intermediate BP phenotype, salt sensitivity (Rocchini, 2000; Hall, Henegar, Dwyer, et al., 2004) as well as resistance to hypertensive drug therapy (Taler, 2005; Cushman, Ford, Cutler, et al., 2002) which is, in turn, at least partially mediated by salt sensitivity. Physical activity improves endothelial function, primarily via the augmented release of nitric oxide (NO) and reduced NO destruction attributable to lower levels of oxidative stress (Franzoni, ghiadoni, Galetta, et al., 2005; Rush, Denniss, Graham, 2005) and greater NO release from the vascular endothelium. Long-term dietary patterns plausibly impact long-term trends in BP. Increased dietary sodium intake raises BP, at least in susceptible people. There is also evidence that increased dietary sodium intake augments vascular angiotensin generation (Boddi, Poggesi, Coppo et al., 1998) while, in salt-sensitive persons, reducing urinary NO metabolites (Cubeddu, Alfieri, Hoffmann, et al., 2000).

Endothelium-dependent and endothelium-independent vascular responses have consistently been observed to be abnormal in African Americans compared to Caucasians (Stein, Lang, Nelson et al., 1997; Houghton, Philbin, Strogatz, et al., 2002). There are data suggesting that the bioavailable NO, the main determinant of endothelium-dependent vascular function, is lower in African Americans than Caucasians despite much higher levels of endothelial nitric oxide synthase (NOS) activity in the former (Malinski, 2005). It appears that the synthesis of oxygen radicals, mostly via uncoupled endothelial nitric oxide synthase (eNOS) and to a smaller degree via NADPH oxidase, raises levels of oxidative stress, accelerates NO destruction, and

therefore leads to reduced NO bioavailability in African Americans (Malinski, 2005), and thus a rise in blood pressure. The section below describes the pathophysiology of each mediator in the proposed mechanism involving the sodium-induced effect of DDAH activity in the cascade of sodium-induced reduction in nitric oxide and elevation in arterial pressure in African Americans.

Nitric Oxide Synthesis

The major endothelium-derived vasoactive mediator is nitric oxide, an endogenous messenger molecule formed in healthy vascular endothelium from the amino acid precursor L-arginine. Endothelium-derived nitric oxide (EDNO) regulates arterial tone through a dilator action on vascular smooth muscle cells that depends on soluble guanylyl cyclase activation and consequent increase in intracellular cyclic 3'5'-guanosine monophosphate (cGMP). Reduced NO synthesis or increased inactivation likely plays an important role in increasing vascular tone, contributing to increased arterial resistance. The uncoupling of eNOS from its cofactors through relative L-arginine deficiency as well as by reduced tetrahydropterin, causes eNOS to produce reactive oxygen species (ROS) rather than NO, thereby reducing NO synthesis (Bervers, Braam, Post, et al., 2006) and increasing oxidative stress. Nitric oxide inactivation owing to excess generation of reactive oxygen species, increased production of endogenous vasoconstrictors such as angiotensin-II and endothelin, decreased bioavailability of L-arginine, as well as defects in intracellular transduction pathways are several proposed mechanisms implicated in the pathophysiology of hypertension (Gokce, Keaney, Vita, 1998). Lerman and colleagues demonstrated that after 6 months of oral L-arginine supplementation, endothelium-dependent coronary blood flow reserve increased by 149% in response to acetylcholine when compared to placebo

group (Lerman, Burnett, Higano, et al., 1998). Importantly, this improvement establishes a link between L-arginine at high doses and improved endothelial function that is almost entirely dependent on NO (even though it was not measured). Relative L-arginine deficiency is a known contributor to eNOS uncoupling.

Nitric Oxide Metabolism

Asymmetric Dimethylarginine (ADMA)

A growing body of evidence indicates that *in vivo* accumulation of endogenous competitive nitric oxide synthase (NOS) inhibitors may reach sufficiently high concentrations under pathological disease conditions to shift the enzymatic milieu to disrupt synthesis of NO from L-arginine. An important mechanism of NO antagonism is through asymmetric dimethylarginine (ADMA)—generated from posttranslational modification of arginine residues, which inhibits NO formation. Asymmetric dimethylarginine is one of three circulating endogenous analogues of L-arginine synthesized by methylation of arginine residues catalyzed by a group of enzymes termed protein arginine *N*-methyltransferases (PRMTs). The other two methylarginines include N(G)-monomethyl-L-arginine (L-NMMA), which has low *in vivo* circulating levels, and symmetric dimethylarginine (SDMA) which does not inhibit NOS and is not hydrolyzed by dimethylarginine dimethylaminohydrolase (DDAH) enzyme (Figure 2).

As the principal endogenous inhibitor of nitric oxide synthase, ADMA is an important regulator of NO formation. Most NO deficient states are not characterized by low NO production but rather by high oxidative stress and high NO destruction rates. Increased ADMA levels correlate with disease severity in patients with peripheral arterial disease, and are linked to increased cardiovascular risk (Boger, Bode-Boger, Szuba, et al., 1998; Cooke, 2000). ADMA levels are elevated in hypercholesterolemia,

hyperhomocysteinemia, diabetes mellitus, peripheral arterial occlusive disease, hypertension, chronic heart failure, coronary artery disease, pregnancy-induced hypertension and preeclampsia, erectile dysfunction, renal disease, as well as other clinical conditions (Lundman, Eriksson, Stuhlinger, et al., 2001; Boger, Bode-Boger, Thiele, et al., 1997; Kielstein, Boger, Bode-Boger, et al., 1999; Abbasi, Asagmi, Cooke, et al., 2001; Usui, Matsuoka, Miyazaki, et al., 1998; Surdacki, Nowicki, Sandmann, et al., 1999; Pettersson, Hedner, Milsom, et al., 1998). ADMA also causes local vasoconstriction when infused intra-arterially, and increases systemic vascular resistance and impairs renal function when infused systemically (Figure 3). More recently, Melikian and colleagues found that ADMA was the only independent correlate (inverse) of endothelial function and in a multivariable regression analysis race was the only independent determinant/correlate of plasma ADMA levels. Healthy Black African men had approximately 33% higher ADMA levels in comparison with White European men (Melikian, Wheatcroft, Ogah, et al., 2007). Also in healthy, young, normotensive Black African men, flow-mediated dilatation was significantly depressed when compared to White European men (5.2 ± 0.3 and 6.3 ± 0.4 ; $p=0.02$, respectively).

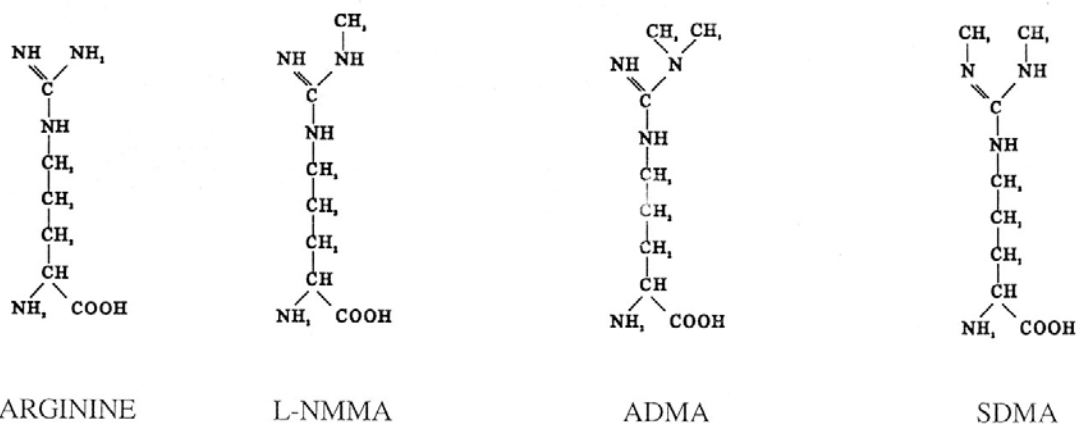


Figure 2. Structure of L-arginine and endogenous methylarginines, N(G)-monomethyl-L-arginine (L-NMMA), asymmetric dimethylarginine (ADMA) and symmetric

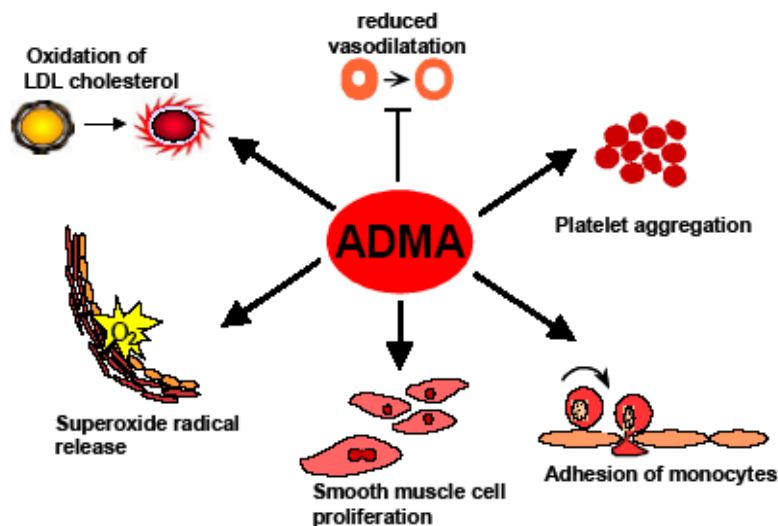


Figure 3. Schematic representation of the pathophysiological sequelae of elevated ADMA levels. Reference: Rainer H. Boger M.D., Professor and Head, Clinical Pharmacology Unit, Institute of Experimental and Clinical Pharmacology.

In salt-sensitive animal and human studies of hypertension, ADMA level correlates closely with elevation in arterial pressure (Matsuoka, Itoh, Kimoto, et al., 1997). More direct evidence that ADMA has a role in modulating cardiovascular hemodynamics was provided by Kielstein and colleagues (Kielstein, Impraim, Simmel, 2004), who conducted a series of controlled experiments with graded intravenous infusions of ADMA in healthy individuals. Evidence indicated that acute increases in plasma ADMA within a physiologically relevant range (2-10 mol/L) affected the cardiovascular system in healthy human subjects *in vivo* resulting in a sustained reduction in cardiac output, marked increase in mean arterial pressure, and systemic- and reno-vascular resistance, as well as a decrease in effective renal plasma flow. During ADMA infusion, Kielstein and colleagues also noted a significant reduction in concentrations of plasma cGMP, the main second messenger of NO (Kielstein, Impraim, Simmel, 2004). Moreover, ADMA infusion caused significant sodium retention and an increase in blood pressure (BP) via a decrease in renal sodium excretion. Recently, Fang and colleagues (2006) demonstrated that high-salt significantly raises

plasma ADMA and BP while decreasing plasma NO synthesis and urinary NO excretion in normotensive salt-sensitive Asians after salt loading but not in the salt-resistant subjects. (Fang, Mu, He, et al., 2006). This finding suggests that salt loading inhibits NO synthesis by increasing the production of ADMA in salt-sensitive subjects. Furthermore, potassium supplementation attenuated the effects of the high-salt diet on plasma ADMA, NO level, urinary NO excretion, and BP in normotensive salt-sensitive Asians; supplementing salt sensitive normotensive African Americans with potassium bicarbonate also has been shown to ameliorate salt sensitivity in a dose-dependent manner (Morris, Sebastian, Forman, et al., 1999). The data in Asians suggest that potassium supplementation in normotensive salt-sensitive subjects prevents sodium-induced depression in NO by preventing the salt-induced rise in ADMA. The data in both Asians and African Americans demonstrate that potassium supplementation ameliorates the salt-induced rise in BP.

In a nested, case-control study involving 150 middle-aged, non-smoking men, high ADMA levels were associated with a 3.9-fold elevated risk for acute coronary events (Valkonen, Paiva, Salonen, et al., 2001). ADMA is a competitive inhibitor of eNOS and its inhibitory action can be overcome by increasing the concentration of the enzyme's substrate, L-arginine. Circulating L-arginine concentrations have been found to be within the normal range in most clinical conditions associated with endothelial dysfunction. Few patients experience pathologically low L-arginine concentrations. However, clinical and experimental evidence suggests that chronic elevation of ADMA causes relative L-arginine deficiency, even in the presence of "normal" L-arginine levels. These findings collectively suggest that chronic elevation of plasma ADMA acts as a modulator of vascular physiology under certain conditions.

Dimethylarginine Dimethylaminohydrolase (DDAH)

Dimethylarginine dimethylaminohydrolase is an enzyme which converts ADMA into citrulline and dimethylamine (Ogawa, Kimoto, Sasaoka, 1987) [DDAH→ADMA→Citrulline+Dimethylamine] (Figure 4). Although, plasma asymmetric dimethylarginine is partially cleared by the kidneys, the vast majority of ADMA is degraded by DDAH. Of the total daily production of ADMA in humans, only 10% is excreted unchanged by the kidneys and the remaining 90% is metabolized by DDAH (Wilcken, Sim, Wang, et al., 2007). The activity of DDAH is impaired by oxidative stress, which permits ADMA to accumulate. A wide range of pathological stimuli induces endothelial oxidative stress such as oxidized low-density lipoprotein cholesterol, high dietary salt intake, inflammatory cytokines, hyperglycemia, and hyperhomocystinemia. The attenuated activity of DDAH allows ADMA levels to rise thereby blocking NO production.

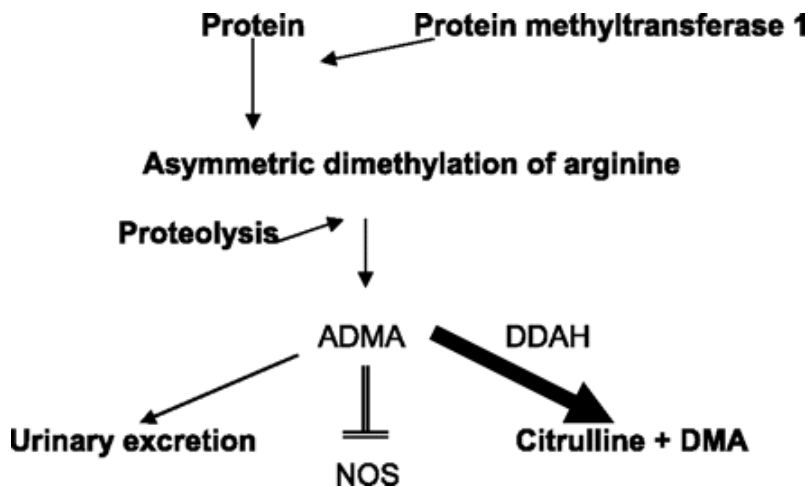


Figure 4. Simplified scheme showing the main metabolic pathways for asymmetric dimethylarginine (ADMA). DDAH, dimethylarginine dimethylaminohydrolase; DMA, dimethylamine. (Baylis, 2008)

Collectively, the ADMA-DDAH system appears to be involved in regulation of endogenous NO synthesis. Firstly, Tojo and colleagues demonstrated that male

Sprague-Dawley rats on a low sodium diet had a significantly enhanced immunohistochemical expression of eNOS in renal vascular endothelium as well as enhanced DDAH renal expression as compared to high sodium rats. Secondly, in erythropoietin (EPO)-induced hypertension, the postulated mechanism of elevated BP involves enhanced vascular reactivity and vasoconstriction. Scalera and colleagues incubated endothelial cells for 24 hours in the presence of EPO, and analyzed measures of reactive oxygen species (ROS), ADMA, and activity of DDAH. They found that after endothelial cells were exposed to EPO, ADMA concentration significantly increased in a dose-dependent manner versus control and was accompanied by a significant reduction in NO synthesis and an increase in oxidative stress, as measured by intracellular ROS. Furthermore, addition of antioxidant (pyrrolidine dithiocarbamate) preserved DDAH activity and reduced ADMA accumulation. In summary, these studies support the idea that sodium influences NO metabolism in a manner consistent with raising levels of oxidative stress and DDAH activity appears to be sensitive to oxidative stress. However, it remains unclear if **sodium**-induced changes in oxidative stress can actually be linked to downregulation of DDAH in humans.

Nitric Oxide Homeostasis

Reactive Oxygen Species, Superoxide Dismutase, and NADPH Oxidase

Superoxide anion ($O_2^{\bullet-}$) and other ROS are constant products of cellular metabolism. However, the development of oxidative stress is determined by the balance between the production of ROS such as $O_2^{\bullet-}$ mainly by NADPH oxidase (Taylor, Glocka, Liang, et al., 2006) and degradation by the antioxidant defense system involving superoxide dismutase (SOD). Superoxide is usually instantly reduced by the enzyme SOD normally present in living tissues (Kityakara, Chabrashvili, Chen, et al.,

2003). Scavenging of $O_2^{\bullet-}$ significantly reduces BP in several experimental models of hypertension (Schnackenberg, Welch, Wilcox, 1998; Welch, Blau, Xie, 2005), especially those associated with salt sensitivity (Manning, Meng, Tian, 2003; Howard, Patterson, Mullins, et al., 2005). Additionally, superoxide dismutase helps preserve the activity of NO by scavenging $O_2^{\bullet-}$, thus increasing the half-life of NO. The mechanism for NO degradation in blood vessels is its interaction with $O_2^{\bullet-}$, leading to the formation of peroxynitrite (Jung, Marklund, Xia, et al., 2007). Previous studies have indicated that inhibition of NO generation during high-salt intake leads to the development of salt-sensitive hypertension and the impairment of kidney function (Tolins, Shultz, 1994; Yamada, Sasaki, Fujihara, 1996; Nakanishi, Hara, Nagai, 2002). Thus, increases in oxidative stress as evidence by increased $O_2^{\bullet-}$ level resulting in NO deficiency appear to contribute to the development of salt sensitivity.

The exact mechanism whereby the endogenous level of $O_2^{\bullet-}$ is increased during NOS inhibition is not yet clear. The uncoupling of eNOS from its cofactors causes eNOS to produce $O_2^{\bullet-}$ instead of NO. A recent study by Taylor and colleagues (2006) found that excess renal medullary interstitial superoxide production in salt-sensitive rats contributed to salt-induced hypertension, and that NADPH oxidase was the major source of the excess superoxide. By administering a NADPH oxidase inhibitor, apocynin, there was a reduction in medullary interstitial superoxide as well as mean arterial pressure in salt-sensitive rats. Both NO and $O_2^{\bullet-}$ are constant products of cellular metabolism, and both of these molecules are constantly interacting with each other in biological tissues (Modlinger, Wilcox, Aslam, 2004). Normally, $O_2^{\bullet-}$ in the tissue is kept to a minimal level by the antioxidative function of SOD. However, when NO

production is diminished in the tissue or when eNOS uncouples generating ROS instead of NO, it is expected that this balance may be altered allowing $O_2^{\bullet-}$ accumulation in the tissue (Majid, Nishiyama, Jackson, 2004).

In a study by Meng and colleagues, a 3-week intravenous infusion of Tempol, a membrane-permeable SOD mimetic, markedly blunted the salt-induced increase in arterial pressure in Dahl salt-sensitive rats by approximately 22 mm Hg (Meng, Cason Gannon, et al., 2003). Superoxide anion release was significantly higher in salt sensitive high-sodium rats compared with salt sensitive low-sodium rats, suggesting that a high sodium intake in salt-sensitive rats causes increased oxidative stress. Tempol markedly decreased $O_2^{\bullet-}$ release in the renal cortex and medulla, and the magnitude of the reduction of $O_2^{\bullet-}$ release by Tempol was significantly greater in salt sensitive high-sodium rats. Moreover, Tempol infusion in the Dahl salt-sensitive rats on a high salt diet delayed the onset and reduced the magnitude of HTN. However, the mechanism of the amelioration of hypertension in the high-sodium/Tempol salt-sensitive rats was not clear.

Schnackenberg and colleagues previously showed that an acute bolus injection of Tempol reduced arterial pressure and renal vascular resistance in the spontaneous hypertensive rat (SHR) but not the Wistar-Kyoto rat and that this response was blocked by nitro-L-arginine methyl ester, an inhibitor of NO synthases, but not by norepinephrine. This implies that Tempol might prevent inactivation of NO by $O_2^{\bullet-}$, thus increasing the bioavailability of NO (Schnackenberg, Welch, Wilcox, 1998). Furthermore, salt loading induces oxidative stress in the kidneys of normal rats; Kitiyakara and colleagues reported that the increased oxidative stress during high salt

was accompanied by increased NADPH oxidase activity and decreased renal expression of the mRNA for intracellular and mitochondrial SOD. Overall, there seems to be a delicate balance between the production and destruction of $O_2^{\bullet-}$ with NO bioactivity.

All mammalian tissue contains three forms of superoxide dismutase (SOD), differing primarily in their locations (Beyer, Imlay, and Fridovich, 1991). SOD-1 is localized in the cytosol, SOD-2 in the mitochondria, and SOD-3 in the extracellular space. Importantly, extracellular SOD-3 activity is ~100-fold higher in the vessel wall than in other tissues. Thus, in the vessel wall, SOD-3 plays a critical role in regulating the vascular re-dox state in the extracellular space. Strålin and colleagues, demonstrated that SOD-3 is a very important component of the total superoxide dismutase in the vessel wall, comprising one-third to one-half of the total vascular SOD activity (Strålin, Karlsson, Johansson, et al., 1995). Additionally, the predominant site of production of SOD-3 is the smooth muscle cell in healthy vessels. Thus, an important function of extracellular SOD-3 in the arterial wall may be the preservation of bioactivity of NO with its antiatherogenic and vasodilating effects. In support of this, Jung and colleagues revealed that a shortage of endogenous vascular extracellular SOD activity occurs in situations of combined oxidative and nitrosative stress (Jung, Marklund, Xia, et al., 2007).

Reactive Oxygen Species and Dimethylarginine Dimethylaminohydrolase

Oxidative stress by S-nitrosylation (presence of a reactive cysteine residue in the active site of DDAH) inactivates DDAH (Leiper, Murray-Rust, McDonald, et al., 2002), which provides an important mechanism leading to upregulation in the levels of ADMA, thereby limiting further NO generation. Isoprostanes are prostaglandin-like substances

that are produced *in vivo* independently of cyclooxygenase enzymes, primarily by free radical-induced peroxidation of arachidonic acid. Isoprostanes have been shown to be extremely accurate markers of oxidative stress *in vivo*, as isoprostane levels increase dramatically in experimental animal models of oxidant injury and these levels can be suppressed by administration of antioxidants (Morrow, Roberts, 1996). Environments which elevate intracellular oxidative stress including hyperglycemia and hyperhomocysteinemia have also demonstrated impaired activity of DDAH (Lin, Ito, Asagami, et al., 2002; Stuhlinger, Tsao, Her, et al., 2001). On the other hand, several antioxidants (i.e., all-*trans*-Retinoic acid, polyethylene glycol-conjugated SOD [PEG-SOD]) increased expression of DDAH, thus DDAH is considered an oxidant-sensitive enzyme (Achan, Tran, Arrigoni et al., 2002; Lin, Ito, Asagami, et al., 2002).

Nitric Oxide and Natriuresis

In Dahl salt-sensitive rats, sodium chloride loading induces endothelial dysfunction and hypertension, whereas BP and vasodilator responses remain normal when these animals consume a low-salt diet. In this animal model of hypertension, L-arginine prevents the development of hypertension, and this protection can be overcome by an inhibitor of NOS (Chen and Sanders, 1991). It is now understood that renovascular NO production modulates salt and water excretion, and that salt-sensitive hypertension may reflect an impairment of NO action (Shultz and Tolins, 1993). In humans, the fall in pressure with L-arginine administration is more pronounced in salt-sensitive subjects (Campese, Amar, Anjali, et al., 1997). From a clinical perspective, these findings strongly suggest that L-arginine exerts a significant hypotensive effect in patients who manifest salt sensitivity, presumably via augmentation of NO production.

Obesity

In all likelihood, obesity contributes significantly to HTN risk in all racial/ethnic populations, and many racial and ethnic minorities manifest obesity disproportionately. Approximately 80% of people with HTN in the United States are overweight or obese (BMI ≥ 25 kg/m²). In Black women the prevalence of extreme obesity (BMI > 40 kg/m²) is almost 1 in 6, a prevalence that is ~3 – 4-fold higher than that of White and Hispanic women (Hensrud, Klein, 2006). There are marked ethnic and age-based differences in the rates of weight accumulation. Relative to White women the onset of obesity occurred sooner for Black and Hispanic women. After 28 years of age, Black men develop obesity more rapidly than White men. Anthropometric measures, such as obesity, especially in women, can also influence biologic systems involved in BP regulation and the expression of pressure-related target-organ damage (i.e. chronic renal injury). For example, obesity is a major anthropometric correlate of salt sensitivity in Blacks and Whites (Flack, Ensrud, Mascioli, et al., 1991).

Vascular Stiffness

Blood pressure represents the confluence of vascular properties such as arterial stiffness, endothelial dysfunction, cardiac output, peripheral vascular resistance and extracellular/intravascular volume. Blood pressure is a function of blood flow and vascular resistance (i.e., BP= cardiac output x total peripheral resistance). In clinical practice, pressure is defined in terms of systolic (SBP) and diastolic (DBP) blood pressure, which refers to a pulsatile phenomenon, with SBP and DBP representing the extremes of the blood pressure oscillation around a mean BP value. These are quantitative measures of blood pressure, however, blood pressure and flow fluctuate during the cardiac cycle. The elastic and geometric properties of the arteries cause the

arterial pressure pulse to change its shape as it travels along the arterial tree. Thus, hemodynamic information contained in the shape of the arterial pressure pulse reflects the type of interaction between the heart as a pump and the arterial system as the load, and can complement the conventional measurement of BP.

Arterial stiffness is an important determinant of cardiovascular risk, and the augmentation index is a measure of systemic arterial stiffness derived from the ascending aortic pressure waveform. In normal individuals the reflected wave returns to the central aorta late in diastole (augmenting coronary flow which occurs solely in diastole) and thus there is relatively little amplification of the aortic pressure. As blood vessels become stiff due to age-related processes, and/or other co-morbidities, such as hypertension, hyperlipidemia, diabetes mellitus, and peripheral vascular diseases, the pulse wave is transmitted more rapidly and returns to the heart during systole, resulting in a greater augmentation of the central aortic systolic pressure (Gatzka, Cameron, Kingwell, et al., 1998; Wilkinson, Prasad, Hall, et al., 2002).

Theoretical Framework

Based on the review of literature, a plausible physiologic model is presented (Figure 1) to explain the relationship between sodium intake and nitric oxide activity resulting in salt sensitive increases in blood pressure.

Summary of Literature

Epidemiological and clinical studies demonstrated a clear relationship between salt intake and hypertension. However, the BP response to changes in dietary salt are heterogeneous among individuals. Dietary sodium raises oxidative stress and reactive oxygen species contribute to impaired endothelial function in salt-sensitive rats (Zhou, et al., 2003, Laffer et al., 2006). The activity of DDAH is impaired by oxidative stress

(Leiper, Murray-Rust, McDonald, et al., 2002), thus permitting ADMA to accumulate and resulting in the inhibition of NO. Although it is known that sodium stimulates oxidative stress and modulates the renin-angiotensin system, in both humans and rats; it is currently unknown whether dietary sodium has an effect on DDAH activity. The purpose of this study is to investigate and assess if there is a sodium-induced effect on DDAH activity which would represent a plausible mechanism in human salt sensitivity.

Purpose and Aims

The purpose of this study is to test the hypothesized physiologic model to determine the mechanism involved in sodium-induced depression of NO synthesis and elevation in blood pressure in African American individuals. Three specific aims will be tested.

Specific Aims:

Aim 1: Determine the correlation between DDAH activity, levels of ADMA, and urinary NO metabolites following dietary salt loading.

Hypothesis 1a: Dietary salt loading correlates with a reduction in DDAH activity (urine dimethylamine [DMA]:urine asymmetrical dimethylarginine [ADMA], is used as a marker of DDAH activity/expression; GC-Mass Spec).

Hypothesis 1b: A reduction in DDAH activity correlates with a rise in ADMA (urine ADMA; GC-Mass Spec) levels, and subsequent reductions in urinary levels of NO metabolites following dietary salt loading.

Aim 2: Determine the dietary sodium-induced changes in DDAH activity, circulating levels of ADMA, and NO metabolites, and their relationship to changes in arterial stiffness (AS) and blood pressure (BP) following dietary salt loading.

Hypothesis 2: Dietary salt loading correlates with a reduction in DDAH activity

and an increase in ADMA levels and a reduction in NO metabolites resulting in an increase in AS (SphygmoCor) and BP (cuff).

Aim 3: Determine the association between DDAH activity and ADMA levels and their correlation with the presence of the gene polymorphism (superoxide dismutase-3 [SOD-3]), circulating markers of oxidative stress, and free radical following dietary salt loading.

Hypothesis 3: Dietary salt loading correlates with increased measures of oxidative stress (total 8-isoprostanes) and free radical (nitrotyrosine) and a reduction in DDAH activity and increase in ADMA levels, with these sodium-induced changes being greatest in subjects without the SOD-3 genotype.

CHAPTER 3

METHODS

Design

The study used an integral cross-over experimental design with four-phases lasting 20 weeks. The current study was a sub-study associated with an ongoing NIH-trial “Obesity, Nitric Oxide, and Salt Sensitivity” (ONOSS) (NIH/NIEHS 1P50ES012395-01). Healthy, normotensive African American men and women from the ongoing NIH-trial ONOSS were used as the population study sample. The ONOSS study was a 39-week study of healthy African American men and women aged 35 years and older who were overweight or obese [body mass index (BMI) ≥ 25 and < 40 kg/m²].

Sample

Overall, 210 African American participants were recruited to the ONOSS study from 14 metro-Detroit community sites to participate in the study, and the data were collected between 2004-2008. The recruitment strategies used included local health fairs, bus advertisements, and radio spots. This study was reviewed and approved by the Wayne State University, Institutional Review Board and signed informed consent was obtained from all participants prior to their participation.

To be included in the overall study, participants needed to be normotensive, African American men and women aged 35 years and older with non-hypertensive BP levels based on average cuff BP readings at the third eligibility visit and who were overweight or obese with a BMI of ≥ 25 and < 40 kg/m², with the urinary sodium:creatinine ≤ 1.1 at the third eligibility visit. The exclusion criteria included the following: hypertension according to JNC VI co-morbidity definitions $> 140/90$ mm Hg, if there was kidney disease [estimated glomerular filtration rate (GFR) < 60 mL/min/1.73

m²], or diabetes mellitus [fasting blood sugar (BS) > 126 or take diabetes medication or random BS > 200 mg/dl], psychiatric illness or dementia, heart failure, angina pectoris, intermittent claudication, valvular heart disease, myocardial infarction, coronary artery bypass graft, coronary or peripheral angioplasty, history of life threatening cardiac arrhythmias, peripheral vascular surgery within the past 6 months, use of non-steroidal anti-inflammatory drugs > 4 days/week, liver function tests > 1.5 x upper normal, albumin < 3.5, planned to move > 50 miles or travel extensively from the area during the next 12 months, urine albumin:creatinine ratio > 500, night-shift work, use of supplemental vitamins/minerals/herbs, any difficulty swallowing, or positive pregnancy test (given to premenopausal women who have not had a hysterectomy and have not been surgically sterilized). They were also excluded if currently taking cardiovascular medications including lipid lowering drugs, medications for mental illness, > 3 alcoholic drinks per day, > 6 restaurant meals per week, oral steroids or nitrates, or if they refused to sign informed consent form, refused venipuncture, refused to comply with overnight urine collections, and refused to take study capsules.

Of the original 72 participants recruited in the sub-study, 63 participants were included at baseline visit, and only 43 participants were included in the analyses after application of exclusion criteria and participant drop outs. The population consisted of normotensive (BP < 140/90 mm Hg), overweight (body mass index = 25 – 39.9 kg/m²) African American men and women aged 35 years and older. The majority of participants were female (87%) and the mean age was 45 years.

Study Protocol

The current sub-study was conducted in four phases (Figure 5, Table 1) and incorporated a randomized, blinded cross-over design. In Phase I: Dietary Orientation,

a low-sodium plus weight maintenance dietary regimen was introduced for each participant during the initial 8 weeks of the study, representing the pre-randomization phase. A registered dietitian counseled each participant at their initial visit and follow-up clinic visits where she advised participants to maintain a food diary; and weekly follow-up telephone encounters were completed on each participant throughout the remaining time of the study. Participant vital signs (i.e., blood pressure, pulse rate, and weight) were measured every other week. In Phase II: Randomization, participants were randomized to either a 4-week placebo or a 100 mmol sodium loaded diet [1,800 grams]. During the blinded 4-week salt loading period, participants received sodium chloride capsules that contained 11.1 mmol of sodium [placebo was an inactive ingredient]. Participants took 3 capsules three times a day during sodium and placebo supplementation. Participants were encouraged to take their capsules with food. At the end of the 4th week, lab samples and measurements were obtained and represented the post-randomization phase of either placebo or sodium exposure. Participants continued the low-sodium diet during the treatment period with maintenance of body weight achieved. Intakes of potassium and calcium were as expected to be, close to the average for older African Americans in the US population (54 mmol/day potassium and 550 mg/day calcium) (Kant, Graubard, Kumanyika, 2007). In Phase III Wash-out, all participants underwent a wash-out period during the ensuing 4 weeks. Participant blood pressure, pulse rate, and weight were measured every other week, and they continued to undergo dietary counseling. In Phase IV: Cross-over, participants were randomized to either the 4-week remaining placebo or 100 mmol sodium loaded diet. At the end of the 20th week (final visit), lab samples and measurements were obtained

and represented the post-randomization phase of either the remaining placebo or sodium exposure.

Throughout the study, participants continued the low-sodium diet during the intervention period with maintenance of body weight. Compliance to the diet and treatment capsules were monitored by determining the urinary sodium:creatinine ratio in three pooled overnight urine samples, once before the sodium/placebo supplementation in Phase II and twice thereafter, following the sodium and placebo loading.

The choice of a study design that manipulates a single dietary nutrient, sodium chloride, on the background of a fixed dietary intake that is typical of free-living individuals, is purposeful. Studies like the DASH diet intervention (Milan, Mulatero, Rabbia, et al., 2002; Volmer, Sacks, Ard, et al., 2001) have demonstrated that simultaneously changing multiple dietary nutrients is an effective means of lowering BP. A DASH-like design is, however, less well suited for understanding the physiological effect of a single dietary nutrient. Thus, we have chosen to hold constant, as best we can, potential modifiers of the salt-BP response (e.g., potassium, calcium, fat) and to discern the impact of physiological modifiers and mediators of the BP response to dietary sodium. The chosen population, African Americans, have a high prevalence of nitric oxide deficiency, obesity, salt sensitivity, and endothelial dysfunction (Winkleby, Kraemer, Ahn, et al., 1998; Flack, Grimm, Staffileno, et al., 2002).

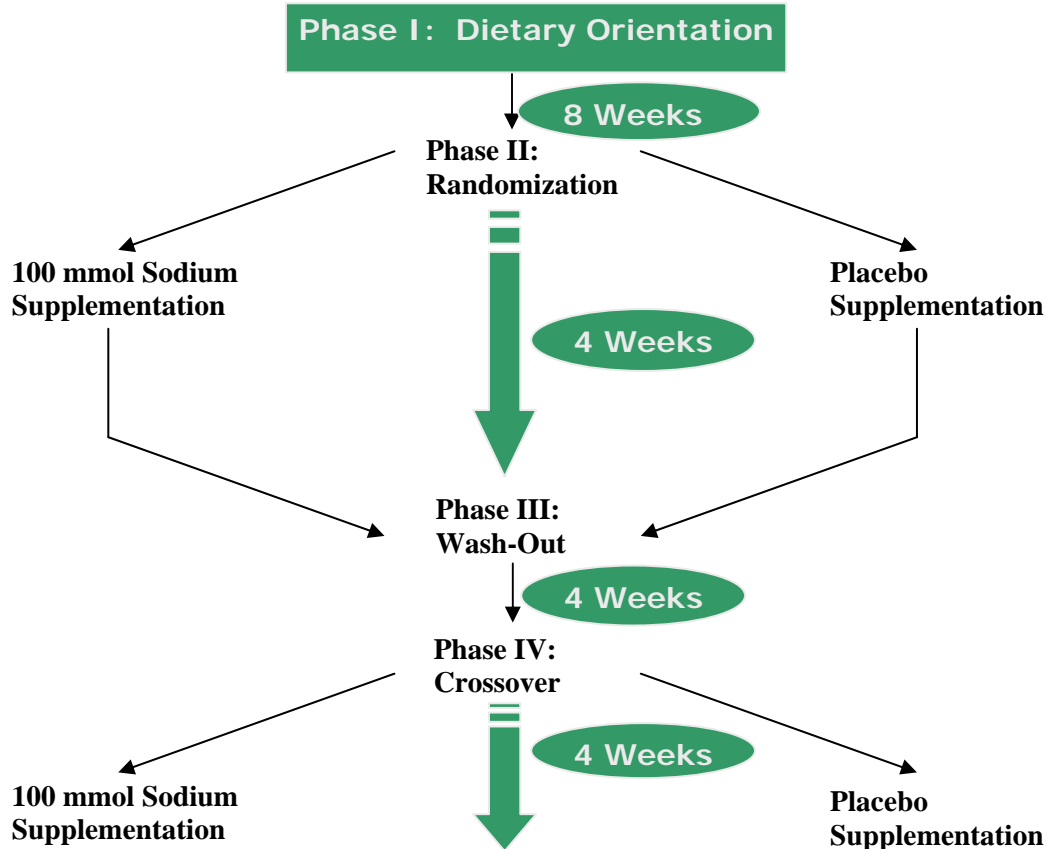


Figure 5. Four-Phase Protocol Design This flow diagram represents the cross-over design used in the current sub-study for 20 weeks. During Phase I, participants underwent 8 weeks of dietary orientation to a low-sodium plus weight maintenance diet. This dietary regimen was continued throughout all of the phases of the study. Phase II occurred after the initial 8 weeks of dietary orientation, where participants were blindly randomized to either the placebo or sodium supplementation cohort for the following 4 weeks. Next, all participants underwent a Phase III wash-out period for 4 more weeks, prior to the final phase IV cross-over phase where participants were crossed-over into the remaining cohort, either placebo or sodium supplementation. The final visit for this sub-study was at the end of the phase IV randomization placebo/sodium supplementation, representing the end of the 20th week. This protocol followed a randomized cross-over design to control for time and order effects.

Table 1. Protocol for Four-Phase Sub-Study

PERIODS FOR ANALYSIS	Phase I: Low Sodium Diet	Phase II: Placebo/Sodium					Phase III: Wash-Out				Phase IV: Placebo/Sodium			
	0--7	8	9	10	11	12	13	14	15	16	17	18	19	20
Week														
Vitals/BMI	X (every other visit)	X				X		X		X				X
Ambulatory BP		X				X								X
Dietary Assessment		X												X
Physical Activity		X				X								X
Asymmetric dimethylarginine						X								X
Dimethylarginine dimethylaminohydrolase						X								X
Plasma Isoprostanes		X				X								X
Urinary Nitric Oxide		X				X								X
Free Radicals		X				X								X
Overnight Urinary Na, K, Creatinine		X				X								X
Platelet Nitric Oxide Generation		X				X								X
Pulse Wave Velocity		X				X								X
Dispense study capsules OR broth		X								X				X
Collect study capsules						X								X
Clinic Visit	X (every other visit)	X				X		X		X				X
Phone visit /Dietary Counseling	X (every other visit)		X	X	X		X		X		X	X	X	

This chart details all visits and explicitly describes the protocol for this sub-study. Under each visit phase lists the lab/measures, or data which is to be collected for each successive visit through all four phases of the 20-week study.

Study Measures

- 1) Dietary Intake Assessment: Dietary assessments were completed by the registered dietitian once before the sodium/placebo supplementation began in Phase II and at the end of the final visit (end of 20th week). Dietary data is thought to be under-reported in obese subjects (Kretsch, Fong, Green, et al., 1999). The records were analyzed with the Nutrition Data System, research version of the software (University of Minnesota, Nutrition Coordinating Center). At the beginning and end of the study, a Food Frequency Questionnaire (Block'98 Food Frequency Questionnaire) was also administered to capture usual eating patterns over the last 12 months (Block, Woods, Potosky, et al., 1990). The intake of electrolytes was estimated (e.g. calcium, potassium, and magnesium).

2) High Dietary Sodium: During the blinded 4-week salt loading period, participants received sodium chloride capsules that contained 11.1 mmol of sodium [placebo was an inactive ingredient]. Participants took 3 capsules three times a day during sodium and placebo supplementation.

3) Reactive Oxygen Species is measured using two variables: Total 8-isoprostanes and free radicals

Total 8-Isoprostanes: 8-Isoprostane level in serum appears to be a good marker of oxidative stress in humans, and immunoassays for these oxidation products are suggested to be more specific than chromatographic methods (Proudfoot, Barden, Mori, et al., 1999). Only total 8-isoprostane was measured because free 8-isoprostane determination requires much larger sample volumes due to its low levels and there was excellent correlation between total and free 8-isoprostane in plasma. Total 8-isoprostane is expected to be a good measure of lipid peroxidation levels, based on published data from human studies (Proudfoot, Barden, Mori, et al., 1999; Morrow, Frei, Longmire, et al., 1995). Total 8-isoprostane levels in plasma were measured only once at baseline. Total 8-isoprostane plasma levels were measured using an EIA kit from Cayman Chemical Co. (Ann Arbor, MI) using a modified Sep-Pak procedure.

Free Radical: Free radical was measured once before the sodium/placebo supplementation in Phase II, considered a baseline value. The type of free radical generated in the arterial wall was estimated from quantization of nitrotyrosine. These protein degradation products are generated from nitric oxide, hydroxyl radical and hypochlorous acid, respectively. Nitrotyrosine was quantified by gas chromatography with mass spectral detection (GC/MS). It has been proposed that

assay of oxidized amino acids such as tyrosine in plasma may be a marker of oxidative stress relevant to cardiovascular diseases (Heinecke, 2002). *Free radicals are critical to the interpretation of the study results because they destroy NO. Also joint consideration of free radical and NO production will permit characterization of the impact of the relative balance between desirable NO and mostly undesirable free radical generation on BP responses to dietary sodium manipulations.*

- 4) Superoxide Dismutase (SOD): The 47C>T(A16V) polymorphism in SOD2 has been identified as functional. Accordingly, it is linked to a 30-40% decrease in enzyme activity. The 637 C>G (Arg213Gly) mutation in SOD3 has been linked to an 8-10 fold increase in enzyme activity. SOD is an antioxidant enzyme, thus increased and reduced activity is, respectively, associated with greater and lesser anti-oxidant effects. The latter would lead to higher levels of oxidative stress.
- 5) Dimethylarginine dimethylaminohydrolase (DDAH): DDAH activity was indirectly measured by the quantification of endogenous dimethylamine (DMA), product of degraded ADMA by DDAH enzyme, and ADMA (DMA/ADMA molar ratio index for creatinine, micromol/mmol creatinine) (Chobanyan, Thum, Suchy, et al., 2007). Urine DMA was measured twice during the sub-study after the dietary sodium and placebo supplementation phases, simultaneously with ADMA. Urine biomonitoring data was adjusted to the constant creatinine concentration to correct for variable dilutions among spot samples. Dimethylamine in human urine by gas chromatography-mass spectrometry (GC-MS) was determined by the Institute for Clinical Pharmacology, Hannover Medical School, Hannover, Germany.
- 6) Asymmetric dimethylarginine (ADMA): ADMA levels fall as DDAH levels rise. ADMA was measured twice during the study, after dietary sodium and placebo

supplementation (end of the placebo phase represents the “baseline” ADMA level). The ratio of urine ADMA to urine creatinine (ADMA/Cr) was used in the statistical analyses to control for urine dilution, a standard method when evaluating urine biomarkers. ADMA in human urine by GC-MS was determined by the Institute for Clinical Pharmacology, Hannover Medical School, Hannover, Germany.

- 7) Urinary Nitric Oxide (NO): Urinalysis of total nitrate and nitrite concentration was determined by the Institute for Clinical Pharmacology, Hannover Medical School, Hannover, Germany. Total nitrate and nitrite metabolites in urine, which represents crude nitric oxide production, were measured by first converting nitrate to nitrite using nitrate reductase and then treating with Griess reagents to convert nitrite into a deep purple azo compound (Tsikas, 2007). Photometric measurement of the absorbance of the azo compound was at 540 or 550 nm. The detection limit for nitrate/nitrite assay (80 μ l) and nitrite assay (100 μ l) was 2.5 μ M and 2.0 μ M, respectively, and the mean interassay CV was 3.4%. In non-contaminated urine, nitrate would be approximately 99% of the sum of nitrate and nitrite. Total nitrate and nitrite metabolites in urine were expressed as an excretion rate in units μ mol/8 hours (Tsikas, 2007). The ratio of urine NO to urine Cr (NO/Cr) was used in the statistical analyses to control for urine dilution, a standard method when evaluating urine biomarkers. NO metabolites were measured once before the sodium/placebo supplementation in Phase II and twice thereafter, following the sodium and placebo loading. Prior to these visits, participants were counseled to adhere to a low nitrate diet for the four days prior to the urine collection. Written materials were provided along with telephone counseling. Urinary nitrate measures represented nitric oxide production, a key predictor and outcome variable in this study.

- 8) Obesity – height/weight- body mass index (BMI): Height was obtained at the initial visit, while weight was measured using a standard balance beam scale. Height and weight are necessary to determine BMI.
- 9) There were two outcome measures of interest: arterial stiffness and blood pressure:
- Arterial Stiffness (AS): Measurement of AS was performed once before the sodium/placebo supplementation in Phase II and twice thereafter, following the sodium and placebo loading. AS was measured non-invasively using the SphygmoCor system (AtCor Medical, Australia). AS was measured by calculating augmentation index (AI). AS is positively correlated with AI (the difference between the first and second pressure peaks divided by pulse pressure). AS should be affected by NO metabolism, oxidative stress, and/or dietary sodium manipulations.
- Resting Blood Pressure: All BP measurements were obtained using a protocol that was adapted from the American Heart Association Recommendations for Human BP Determination (Perloff, Grim, Flack, et al., 1993). Three pressure readings were recorded within a 5-minute period taking the average of these 3 readings to record as the BP measurement for that visit. BP was measured at **every** clinic visit for all participants, however the BP at the end of sodium and placebo periods were utilized in the analysis. BP is the primary study response variable. Systolic blood pressure (SBP) was chosen as the primary response variable because it is measured more precisely than diastolic BP (DBP), and because it more closely related to target-organ injury and pressure-related mortality, even after consideration of measurement error, than DBP (He, et al., 1999). Further, preliminary studies suggest that increased dietary sodium exposure has a relatively greater impact on SBP compared to DBP, at least on a per mm Hg basis.

10) Urinary Sodium:Creatinine Ratio: Urinary sodium was quantified, as an estimate of changes in dietary intake, by determining the urinary sodium:creatinine ratio in three pooled overnight urine samples, once before the sodium/placebo supplementation in Phase II and twice thereafter, following the sodium and placebo loading.

Statistical Analysis

Descriptive analyses for means, medians, standard deviations (SD), and 95% confidence intervals (CI) were conducted for all continuous variables (i.e., age, body mass index, blood pressure) while proportional distributions were examined for all categorical variables. Continuous variables were examined for skewness/normality using Shapiro-Wilk statistic. Continuous data that deviate significantly from normality were transformed to the natural logarithm to approximate a normal distribution prior to analysis. The paired *t* test was used to compare the exposure effects at end of the sodium and placebo study periods. Subsequently, Pearson correlations were utilized to quantify associations, and were considered significant at the 5% significance level. The outcome measures of this study were the continuous variables (DDAH, ADMA, urinary NO metabolites, BP, and AS). The power analysis has been estimated for significance level of 5% and showed that 66 study patients have more than 80% power for a change in SBP. Missing data was random and participants with missing variables in regards to the main outcome measures were eliminated from the analysis, resulting in a loss of power. All analyses including appropriate statistics, p-values, and graphs were reported using Statistical Analysis Software (SAS, version 9.1).

Data Analysis

Specific Aim 1:

After both dietary sodium and placebo supplementation, the relationship of the

change in DDAH activity (urine dimethylamine [DMA]: asymmetrical dimethylarginine [ADMA], used as a marker of DDAH activity/expression) to the change in the levels of urinary ADMA and urinary NO metabolites, after dietary salt loading was examined. Paired *t* test was used to compare the differences between the end of sodium and placebo study periods. Pearson correlations were utilized to quantify associations, and were considered significant at the 5% significance level. The variables were divided by creatinine in the statistical analyses to minimize timing errors related to the collection of the urine samples, a standard method when evaluating urine biomarkers. The magnitude of timing error is much less if you take the amount of sodium in urine/creatinine in urine. Urinary biomonitoring data are typically adjusted to the constant creatinine concentration to correct for variable dilutions among spot samples.

Specific Aim 2:

The associations of sodium-induced changes in DDAH activity, circulating levels of ADMA and urinary NO metabolites after dietary salt loading in relation to the changes in BP and arterial stiffness, were quantified. Paired *t* test was used to compare the differences at the end of the sodium and placebo study periods; SBP and DBP were used separately in analyses involving BP. The variables were divided by creatinine in the statistical analyses to minimize timing errors related to the collection of the urine samples.

Specific Aim 3:

Levels of DDAH activity and ADMA were correlated with levels of circulating markers of oxidative stress (total 8-isoprostanes), and free radical (nitrotyrosine) after salt loading. Paired *t* test was used to compare the differences at the end of the sodium and placebo study periods. Independent sample *t* test was used to compare the levels

of oxidative stress and free radical at a given point and time between the two groups. The variables were divided by creatinine in the statistical analyses to minimize timing errors related to the collection of the urine samples.

Additional analyses:

There were a wide range of values in addition to a very narrowly distributed data set, thus additional analyses utilizing z-scores was done. These differences in distributions made it difficult to judge similarities or significant differences among categories in the data sets, however after standardizing with z-scores, the data results remained relatively unchanged.

Quality Control

All persons involved in handling laboratory samples were trained in the appropriate procedures of processing, labeling, and storage by Clandestine Laboratory Investigators Association (CLIA) personnel standards to assure that laboratory personnel had the appropriate training and experience to adequately perform. In order to minimize variability of urinary nitrate/nitrite levels participants were instructed to follow a low nitrate/nitrite diet for 4 days prior to each urinary nitrate/nitrite collection. The mean coefficient of variation has been shown to be significantly higher within individuals during a “free” diet ($40 \pm 9\%$) compared with a “low nitrate/nitrite” diet ($10 \pm 1\%$) ($P = 0.02$). The research staff performing BP measurements underwent periodic training on BP measurement to verify reliability and accuracy of BP measurement techniques. Weekly telephone contact was maintained with participants throughout a large portion of the study to maximize the diet intervention effect and to minimize losses to follow-up.

Actual Difficulties and Limitations

Several (N=18) patients dropped out after the first intervention period and thus did not receive the second treatment, while 11 participants withdrew from the overall study. Several patients experienced side-effects with the sodium intervention (i.e., nausea, swelling) which contributed to the dropout rate. This made within-subject comparison impossible and was particularly important as withdrawal reduced the statistical power. This further complicated the concept of intent-to-treat analysis as several patients randomized completed the first period, but not the second period.

Ethical Aspects

There were minimal risks to human subjects associated with the sub-study. Some of the interview questions could be considered sensitive. No study procedures were performed until informed consent was obtained. The Core Director ensured that the informed consent process was appropriately carried out, reviewed the consent forms for completeness, and ensured their confidentiality by locking them in a file in her office. Core personnel and study personnel from each project had access to the files for their project, but no one else could access it without the subjects' permission. All procedures were performed by appropriately trained personnel according to study protocols. Interviewers underwent training so that they were able to perceive and appropriately deal with any sensitivity participants may have to questionnaire items. We did not anticipate needing medical or emergency intervention in this minimal risk study, but there was a part-time internist and a part-time physician's assistant assigned to the Core, who were available by pager in addition to a hospital "code" team assigned to the Core. For all subjects, unique study identification numbers were utilized. Internal Review Board approved all aspects of the study.

CHAPTER 4

RESULTS

Descriptive Statistics

Overall, there were no statistically significant differences in patient characteristics at baseline in the 63 participants recruited into the sub-study; however only 43 participants were included in the analyses after application of exclusion criteria and participant drop outs. The majority of participants were female (87%) and the mean age was 45 years (Tables 2a and 2b). Average measures of BP and hemodynamic parameter did not differ by gender. Mean cuff SBP and DBP were within normal range of SBP < 140 mmHg and DBP < 90 mmHg.

Table 2a. Baseline Patient Characteristics

Patient Characteristics	Randomized Treatment Sequence	
	Placebo-Salt Mean (SD)	Salt-Placebo Mean (SD)
Age, years	45.3 (5.0)	45.9 (7.0)
Female, n(%)	28 (90%)	28 (87.5%)
Systolic blood pressure, mmHg	116.6 (9.4)	118.6 (9.7)
Diastolic blood pressure, mmHg	76.4 (4.8)	77.6 (6.3)
Body Mass Index, kg/m ²	32.7 (4.5)	32.0 (3.9)
Current smoker, n(%)	3 (9.7%)	4 (12.5%)
Cholesterol, mg/dL	196.1 (32.8)	206.9 (42.7)
Triglyceride, mg/dL	85.3 (33.4)	93.9 (41.4)

(N=63; Placebo-Salt N=31; Salt-Placebo N=32)

Table 2b. Characteristics of Patients Included in the Crossover Analysis

Patient Characteristics	Randomized Treatment Sequence	
	Placebo-Salt Mean (SD)	Salt-Placebo Mean (SD)
Age, years	48.7 (4.4)	49.6 (7.4)
Female, n(%)	16 (100%)	22 (91.7%)
Systolic blood pressure, mmHg	115.5 (9.2)	120.5 (12.6)
Diastolic blood pressure, mmHg	78.5 (7.0)	79.2 (7.7)
Body Mass Index, kg/m ²	32.3 (3.9)	32.0 (4.1)
Current smoker, n(%)	1 (6.25%)	2 (8.7%)
Cholesterol, mg/dL	188.3 (35.8)	198.8 (60.8)
Triglyceride, mg/dL	93.3 (38.0)	100.1 (40.7)

(N=43; Placebo-Salt N=17; Salt-Placebo N=26)

Specific Aim 1

Aim 1: Determine the correlation between DDAH activity, levels of ADMA, and urinary NO metabolites following dietary salt loading.

Hypothesis 1a: Dietary salt loading correlates with a reduction in DDAH activity (urine dimethylamine [DMA]:urine asymmetrical dimethylarginine [ADMA], used as a marker of DDAH activity/expression; GC-Mass Spec).

Hypothesis 1b: A reduction in DDAH activity correlates with a rise in ADMA (urine; GC-Mass Spec) levels, and subsequent reductions in urinary levels of NO metabolites following dietary salt loading.

The mean of the difference between the two treatment periods (sodium – placebo) in DDAH activity and NO metabolites was -0.83 (95% CI -2.7-1.1, SD = 4.4) and -11.65 (95% CI -38.2-14.9, SD = 61.6), respectively, indicating that post-sodium DDAH activity and NO metabolites tended to be slightly lower after sodium. As hypothesized, the DDAH activity and NO metabolite levels did decrease following exposure to sodium supplementation, however, using paired t-test the difference between DDAH activity and NO metabolite levels were not statistically significant (DDAH: $t = -0.87$, $DF = 84$, $p = 0.39$; NO: $t = -0.88$, $DF = 65.64$, $p = 0.38$). The mean DDAH:creatinine ratio following the placebo phase of the study was 13.45 (SD = 5.0); following the sodium supplementation phase of the study the mean DDAH:creatinine ratio was 12.62 (SD = 3.8), representing a 6.17% decrease (Figure 6). The mean NO:creatinine ratio following the placebo phase of the study was 101.40 (SD = 76.2); following the sodium supplementation phase of the study the mean NO:creatinine ratio was 89.71 (SD = 42.3), representing an 11.53% decrease (Figure 7). Thus, the data suggest but do not confirm the hypothesis that DDAH activity and NO metabolite levels

are significantly lower after sodium supplementation.

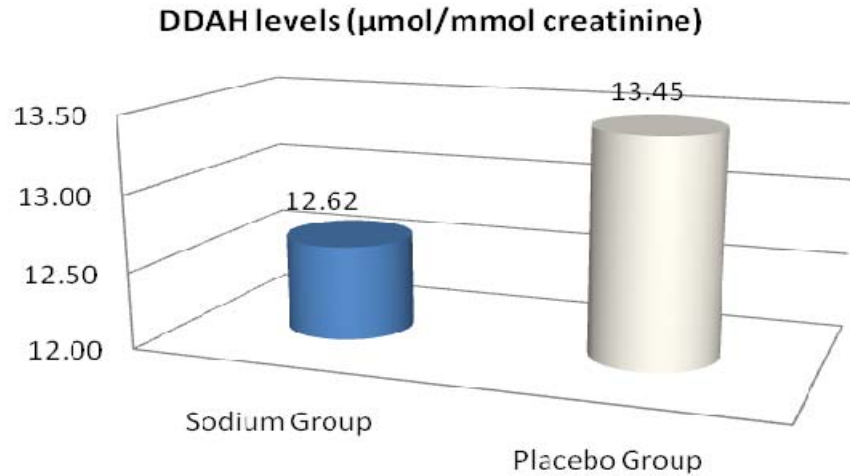


Figure 6. DDAH:creatinine levels post-sodium and pre-sodium exposure

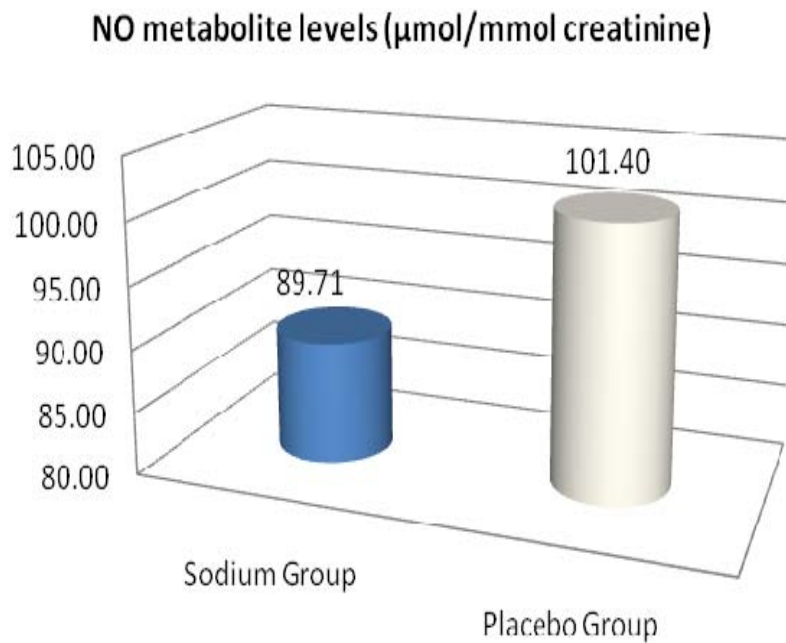


Figure 7. NO:creatinine levels post-sodium and pre-sodium exposure

The mean ADMA:creatinine ratio following the placebo phase of the study was 3.97 (SD = 1.4); following the sodium supplementation phase of the study the mean ADMA:creatinine ratio was 3.81 (SD = 0.8), representing a slight 4.03% decrease (Figure 8). The mean of the difference in ADMA:creatinine ratio was -0.164 (95% CI -

0.7-0.3, SD = 1.2), indicating that post-sodium ADMA:creatinine ratio tended to be slightly lower after sodium, however using paired t-test the difference between the two treatment periods (sodium – placebo) was not statistically significant ($t = 0.64$, $DF = 66.9$, $p = 0.52$).

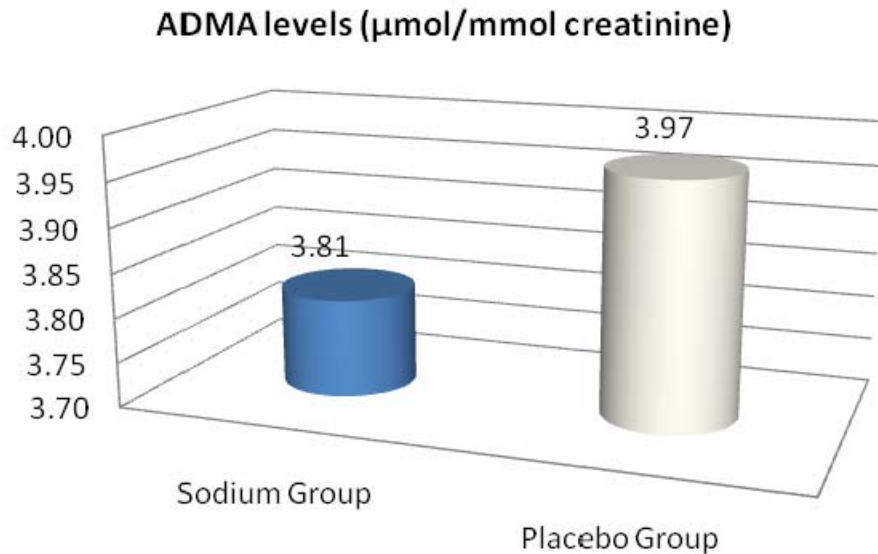


Figure 8. ADMA:creatinine levels post-sodium and pre-sodium exposure

Pearson correlation coefficient between the difference in NO:creatinine ratio and ADMA:creatinine ratio between the two treatment periods was positively correlated ($r = 0.35$; $p = 0.02$) (Table 3). Therefore, contrary to the initial hypothesis, ADMA was positively associated with NO metabolite level in the urine. ADMA inhibits NO and this inhibitory action may have stimulated NO production, however NO activity would still be reduced because of the high ADMA activity.

Furthermore, the correlation coefficient for the difference in NO:creatinine ratio and DDAH activity level between the two treatment periods demonstrated a strong, positive correlation ($r = 0.90$; $p = <0.0001$). Thus, heightened DDAH activity was significantly associated with a greater production of NO metabolites.

Additional analysis comparing the change in urine sodium:creatinine ratio after both dietary sodium and placebo supplementation:

Correlation of the change in urine sodium:creatinine ratio and ADMA:creatinine ratio between the two treatment periods was non-significant ($r = 0.07$; $p = 0.83$). Furthermore, the correlation of the change in urine sodium:creatinine and DDAH:creatinine ratio ($r = -0.41$; $p = 0.18$), and urine sodium:creatinine and NO:creatinine ratio ($r = -0.37$; $p = 0.23$) at the end of the two treatment periods were not significant.

Table 3. Pearson Correlation Coefficients

	Δ NO:Creatinine	Δ DDAH:Creatinine	Δ ADMA:Creatinine	Δ Sodium:Creatinine
Δ NO:Creatinine	1.00 43	0.90 <0.0001 43	0.35 0.02 43	-0.37 0.23 12
Δ DDAH:Creatinine	0.90 <0.0001 43	1.00 43	0.19 0.21 43	-0.41 0.18 12
Δ ADMA:Creatinine	0.35 0.02 43	0.19 0.21 43	1.00 43	0.07 0.83 12
Δ Sodium:Creatinine	-0.37 0.23 12	-0.41 0.18 12	0.07 0.83 12	1.00 12

ADMA: Asymmetric Dimethylarginine; DDAH: Dimethylarginine Dimethylaminohydrolase; NO: Nitric Oxide

Specific Aim 2

Aim 2: Determine the dietary sodium-induced changes in DDAH activity, circulating levels of ADMA, and NO metabolites, and their relationship to changes in arterial stiffness (AS) and blood pressure (BP) following dietary salt loading.

Hypothesis 2: Dietary salt loading correlates with a reduction in DDAH activity and an increase in ADMA levels and a reduction in NO metabolites resulting in an increase in AS (SphygmoCor) and BP (cuff).

The mean of the difference in SBP and DBP respectively, was +2.8 mm Hg (95%

CI -2.2-7.7, SD = 11.8) and +0.8 mm Hg (95% CI -2.6-4.1, SD = 7.9). As hypothesized, the SBP and DBP levels were increased slightly following exposure to sodium supplementation, however, using paired t-test the difference between the two SBP and DBP levels did not reach statistical significance (SBP: $t = 1.11$, $DF = 88$, $p = 0.27$; DBP: $t = 0.45$, $DF = 88$; $p = 0.66$) (Figure 9). Nevertheless, the difference in sodium:creatinine ratio was directly proportional to the difference in both SBP and DBP between the two treatment periods ($r = 0.75$, $p = 0.01$; $r = 0.49$; $p = 0.13$, respectively); however, SBP was the only significant correlation and it was positively associated with the sodium:creatinine ratio; these data suggest that the magnitude of the observed BP change was less than predicted because of a lesser between-study period change in the urinary sodium:creatinine ratio (Table 4).

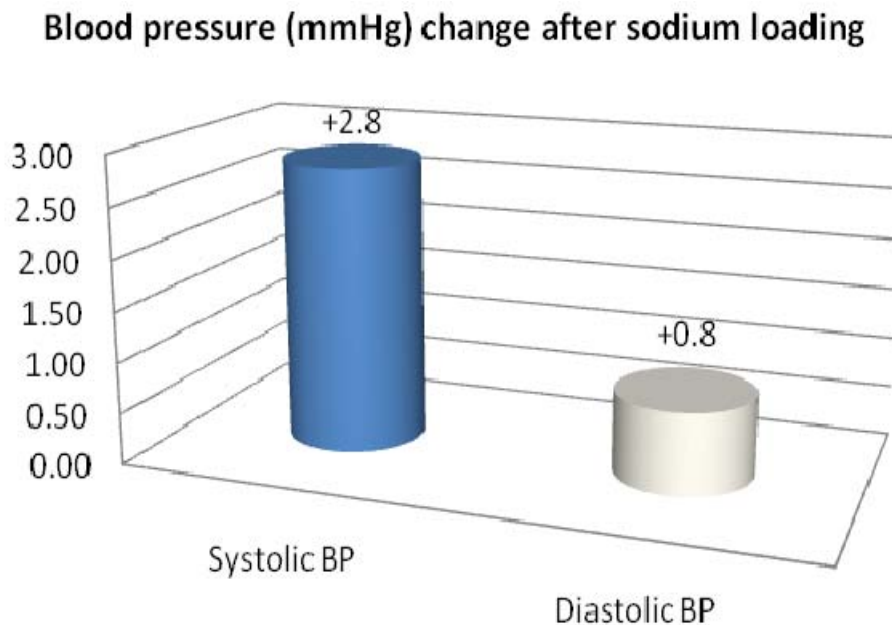


Figure 9. Blood pressure changes after sodium exposure

Table 4. Pearson Correlation Coefficients with BP

	Δ SBP	Δ DBP
Δ NO:Creatinine	0.18 0.40 24	-0.06 0.79 24
Δ DDAH:Creatinine	-0.06 0.76 24	-0.15 0.48 24
Δ ADMA:Creatinine	0.37 0.07 24	0.03 0.90 24
Δ Sodium:Creatinine	0.75 0.01 11	0.49 0.13 11

ADMA: Asymmetric Dimethylarginine; DDAH: Dimethylarginine Dimethylaminohydrolase; NO: Nitric Oxide; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure

Association of sodium-induced changes in ADMA:creatinine ratio after dietary salt loading in relation to changes in BP

The difference in ADMA:creatinine ratio was also directly proportional to the difference in SBP between the two treatment periods ($r = 0.37$, $p = 0.07$). The correlation between the difference in ADMA:creatinine ratio and SBP was borderline significant.

Association of sodium-induced changes in DDAH: creatinine ratio and NO: creatinine ratio after dietary salt loading in relation to changes in BP

The correlation between the difference in NO:creatinine ratio to the difference in SBP and DBP between the treatment periods were non-significant (SBP: $r = 0.17$; $p = 0.40$; DBP: $r = -0.06$; $p = 0.79$). Also, the correlation between the difference in DDAH:creatinine ratio to the difference in SBP and DBP were also non-significant (SBP: $r = -0.06$; $p = 0.76$; DBP: $r = -0.15$; $p = 0.48$, respectively).

Association of sodium-induced changes in arterial stiffness measures

The mean of the difference in augmentation index was +11.79 (95% CI -3.4-26.9,

SD = 19.5), a 14.9% difference, indicating that post-sodium augmentation index tended to be higher after sodium. As hypothesized, the augmentation index did increase following exposure to sodium supplementation, however, using paired t-test the difference between the two treatment periods was suggestive though not statistically significant ($t = 1.60$, $DF = 26$, $p = 0.12$).

Pearson correlation coefficient of the change in ADMA:creatinine ratio was not significantly correlated with the change in augmentation index ($r = 0.27$; $p = 0.42$) (Table 5) between the treatment periods. On the other hand, the correlation of the change in DDAH:creatinine ratio and the change in augmentation index between the treatment periods was significantly negatively correlated ($r = -0.61$; $p = 0.04$). Thus, heightened DDAH activity was strongly associated with a lower augmentation index, as the measure of arterial stiffness, as expected. Furthermore, the change in NO:creatinine ratio was negatively correlated with the change in augmentation index ($r = -0.59$; $p = 0.05$); thus, supporting the hypothesis that NO metabolite levels are inversely associated with arterial stiffness.

Table 5. Pearson Correlation Coefficients with Augmentation Index

	ΔAI
$\Delta NO:Creatinine$	-0.59 0.05 11
$\Delta DDAH:Creatinine$	-0.61 0.04 11
$\Delta ADMA:Creatinine$	0.27 0.43 11

ADMA: Asymmetric Dimethylarginine; AI: Augmentation Index; DDAH: Dimethylarginine Dimethylaminohydrolase; NO: Nitric Oxide;

Specific Aim 3

Aim 3: Determine the association between DDAH activity and ADMA levels and their correlation with the presence of the gene polymorphism (superoxide dismutase-3 [SOD-3]), circulating markers of oxidative stress, and free radical following dietary salt loading.

Hypothesis 3: Dietary salt loading correlates with increased measures of oxidative stress (total 8-isoprostanes) and free radical (nitrotyrosine) and a reduction in DDAH activity and increase in ADMA levels, with these sodium-induced changes being greatest in subjects without the SOD-3 genotype.

Nitrotyrosine and isoprostane levels were analyzed at baseline only (these oxidative markers were not measured post-sodium, only at baseline), thus cross-sectional analyses were done to determine the correlation between these oxidative stress markers and ADMA:creatinine, DDAH:creatinine, and NO:creatinine ratio levels. ADMA:creatinine, DDAH:creatinine, and NO:creatinine ratios were not significantly correlated to either baseline nitrotyrosine nor isoprostane levels.

The assay for the SOD-3 polymorphism was not completed, as the Genomics division noted that it has a very low frequency in Caucasians and even lower in African Americans. Given our small sample size and African American population, it was unlikely that I would find any heterogeneity.

CHAPTER 5

Discussion

The study population was a cohort of mostly young (mean age 45 years, SD = 6.0), female (87%), African Americans who were normotensive, and overweight. Several potentially important findings exist in this study. First of all, in a cohort of young, overweight, African American normotensive women, the data overall trended in the direction hypothesized. The DDAH level and NO metabolites came down after sodium exposure, while the blood pressure rose and the augmentation index increased by almost 12% after sodium exposure, though not statistically significant. The difference in sodium:creatinine ratio was directly proportional to the change in BP (SBP: $p = 0.01$; DBP $p = 0.13$), which likely mediated the BP effect. Additionally, nitric oxide metabolites and DDAH levels were positively correlated to each other ($r = 0.90$; $p = <0.0001$), and changes in both DDAH and NO levels were negatively correlated to changes in augmentation index (DDAH: $r = -0.61$; $p = 0.04$; NO: $r = -0.59$; $p = 0.05$). As expected, after sodium loading the DDAH and NO levels decrease while the augmentation index increases reflecting an increase in arterial stiffness.

On the other hand, the change in NO was positively correlated with the change in ADMA ($r = 0.35$; $p = 0.02$). In speculating a physiologic pathway for this discrepancy, ADMA inhibits NO and this inhibitory action may have stimulated NO production, however NO activity would still be reduced because of the high ADMA activity. Thus, the rise in NO levels would not be able to overcome the effects of ADMA inhibition. Furthermore, the expected effect upon the oxidant-sensitive enzyme DDAH was not detected, as there was no significant evidence of redox-sensitive inhibition of the enzyme DDAH activity by the free radicals. Although several studies have linked

increased oxidative stress to high salt intake in animals (Zhou, Adam, Jaimes et al., 2003; Tian, Moore, Braddy, et al., 2007; Sweit, Lacy, DeLano, et al., 1997), the human data are more limited. In a study by Laffer and colleagues, acute (one-day) sodium loading and depletion using an established protocol produced corresponding increases and decreases, respectively, in plasma F2-isoprostanes in salt sensitive hypertensive subjects.

Citizens of acculturated societies, given free access to salt, invariably consume between 100 and 200 mmol of sodium daily (INTERSALT, 1998). Several meta-analyses indicate that among hypertensive and older subjects, a 3- to 5-mm Hg systolic and approximately 1-mm Hg diastolic change in pressure is associated with a 75 to 100 mmol/24 hour difference in sodium intake (Midgley, Matthew, Greenwood, et al., 1996; Graudal, Galloe, Garred, 1998). The effect on younger and normotensive subjects is less with approximately 2 to 3 mm Hg for systolic and <1 mm Hg for diastolic. Thus, this is in line with the BP effect which occurred in our study. The major reason we likely did not see as much physiological effect on BP and other measures was because of the less than predicted difference in the urine sodium:creatinine ratio between the treatment periods, as well as the smaller sample size.

On the other hand, the inability to demonstrate a strong relationship between sodium intake and blood pressure within a population in which habitual sodium intake is relatively generous (>120 mmol/day) does not deny such a relationship as individual differences in susceptibility or temporal factors may influence the blood pressure response to such intake. In other words, individuals who are susceptible to salt-induced alterations in blood pressure may be balanced by those in whom such an effect is negligible. Alternatively, a long period of exposure to increased sodium intake may be

required for blood pressure manifestations to be apparent, while the age of the population studied may influence the observations as well. For these reasons, information from more dramatic manipulations of sodium balance in an older cohort may provide greater insight into the relationship between salt and blood pressure. Overall, substantial variation in intake (75 to 100 mmol/24 hours) can produce measurable but modest changes in blood pressure. The effect appears to be more substantial in older subjects and in those with higher pressures.

Age has been found to be significantly related to salt sensitivity of blood pressure in the majority of studies. Only a few studies using interventions to assess salt sensitivity have included large enough numbers and a sufficient age range of subjects to be able to identify such a relationship. Increasing salt sensitivity has been noted with increasing age in several other studies (Weinberger, Miller, Luft, 1986; Osanai, Kanazawa, Yokono, 1993; Overlack, Ruppert, Kolloch, et al., 1995). Additionally, this relationship appears to be stronger in hypertensive than in normotensive individuals (Weinberger, Fineberg, 1991). The mean age of participants in our study was 45 years, and thus our data may be skewed given our young cohort. Given the youth of our population, the occurrence of salt-sensitive hypertension is less prevalent, and would reflect as less significance in the study findings.

Hypertensive patients, as a group, are significantly more salt sensitive than normotensive individuals (Weinberger, Miller, Luft, 1986). Given that the participants were all normotensive, the sample size would have to be significantly greater for significance to be present. Based on the results for DDAH levels between salt loaded (mean = 12.62, SD = 3.8) versus placebo groups (mean = 13.45, SD = 5.0), and hypothesizing that people with higher salt load have lower DDAH levels compared to

lower salt loaded participants; a post-hoc power analysis was done to determine the level of power actually achieved to see if that's a factor in the non-significant findings. The power analysis revealed that 204 participants would be required for the crossover study design to achieve 0.85 statistical power ($\alpha = 0.05$) of detecting a mean difference in DDAH levels between salt loaded and placebo groups. Therefore, the selection criteria likely impacted these results, thus reflecting a major limitation to the study, as the prevalence of salt-sensitive individuals is lower in normotensive versus hypertensive patients.

By and large several limitations impacted the significance level in the study, including a smaller sample size than expected, thus the difference noted may not have allowed for significant changes to be established. Another major shortcoming was likely the inadequate difference in the urine sodium:creatinine ratio at the end of the two time periods. There may have been a lack of effect as there was not a full sodium load secondary to lack of compliance with capsule ingestion. Several participants experienced side effects (i.e., nausea) due to the size of the capsules, or they may have forgotten to take their capsules. When taking placebo, and presumably consuming a 100 mmol/day sodium diet, the urinary sodium:creatinine ratio was 0.8522. The supplementation of an additional 100 mmol/day should double the urine sodium:creatinine ratio; however, the urine sodium:creatinine ratio at the end of sodium was only 1.2651. While the expected difference between the two treatment periods was 0.8522; the observed difference that was statistically significant was 0.4130. Thus, $0.4120/0.8522 = 0.485$ or 48.5% of the expected difference between the two treatment periods was ACTUALLY observed. The conclusion is that sodium supplementation did indeed occur to a statistically significant degree but to a level that was slightly less than

50% of predicted. This is clearly related to the problem with taking capsules, perhaps of such a large size, as well as consuming high levels of sodium in concentrated amounts causing nausea. This exercise makes some assumptions that may not be entirely accurate - the linking of the urine sodium:creatinine ratio of 0.8522 to a dietary intake of 100 mmol of sodium per day - however, it is a very reasonable exercise to estimate how short the dietary intake fell short of the projected amount if everyone would have taken the sodium. In essence, the physiological changes seen occurred with ~ 50 mmol of difference in dietary sodium exposure between the two treatment periods. Additionally, the patient population was basically normotensive, and this may have impacted the change in DDAH, ADMA, and NO levels, as many patients may not have been salt sensitive.

Overall, in a cohort of young, overweight, African American normotensive women, sodium exposure was directly proportional to the difference in BP and there was a reduction in DDAH level and NO metabolites, with significant indirect correlations between DDAH, NO and augmentation index. This study attempted to characterize a novel mechanism of action through which salt-induced depression of NO synthesis may occur in normotensive African American individuals. Despite lack of significance in the differences of DDAH, ADMA, and NO between the treatment groups, the combined group results trended in the direction of the central hypothesis that increased dietary sodium intake downregulates DDAH and depresses NO production, and revealed a rise in BP and vascular stiffness. This initial study is the first demonstrating the trend that increased sodium intake was associated with a reduction in DDAH activity, and a depression in NO metabolites in healthy, normotensive African Americans. The findings provide beginning evidence of the proposed linkages, but further study is warranted

given the aforementioned limitations. This was a small study used as basic exploratory work to establish a plausible model for future research. The study advances knowledge in the field of salt-sensitive hypertension by delineating a mechanism of action which may be further studied to potentially target the prevention of elevated blood pressure.

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ABSTRACT**EFFECT OF DIMETHYLARGININE DIMETHYLAMINOHYDROLASE IN THE DEVELOPMENT OF SALT SENSITIVITY**

by

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Salt sensitivity is associated with a rise in blood pressure (BP) occurring during sodium loading and/or a fall in BP during sodium restriction that exceeds random fluctuations in BP. Salt sensitivity is more common in African American than Caucasian hypertensives and is also present, in normotensive African Americans. The mechanism or mechanisms resulting in salt-sensitive hypertension are multiple and include both activation of the renin angiotensin system via increases in angiotensin II and reductions in the endogenous vasodilator, nitric oxide (NO). An important means of NO downregulation is through asymmetric dimethylarginine (ADMA), an endogenous NO inhibitor, which is largely metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). The activity of DDAH is impaired by oxidative stress, thereby permitting ADMA to accumulate thus resulting in further inhibition of NO. Increases in oxidative stress, reduction in DDAH activity, and augmented action of ADMA on depressing NO production represents a plausible mechanism in human salt sensitivity. The study investigates and characterizes the above mechanism through which salt-induced depression of NO synthesis occurs in normotensive African

Americans. The study population was a cohort of mostly young (mean age 45 years, SD = 6.0), female (87%), African Americans who were normotensive, and overweight. The DDAH level and NO metabolites came down after sodium exposure (6.17% and 11.53%, respectively), while the BP rose (SBP: +2.8 mm Hg; DBP +0.8 mm Hg) and the augmentation index (a measure of arterial stiffness) increased by almost 12% after sodium exposure, though not statistically significant. The difference in sodium:creatinine ratio was directly proportional to the change in BP (SBP: $p = 0.01$; DBP $p = 0.13$), which likely mediated the BP effect. Additionally, NO metabolites and DDAH levels were positively correlated to each other ($r = 0.90$; $p = <0.0001$), and changes in both DDAH and NO levels were negatively correlated to changes in augmentation index (DDAH: $r = -0.61$; $p = 0.04$; NO: $r = -0.59$; $p = 0.05$). As expected, the DDAH and NO levels increased while the augmentation index decreased reflecting a reduction in arterial stiffness. Overall, in a cohort of young, overweight, African American normotensive women, sodium exposure was directly proportional to the difference in BP and there was a reduction in DDAH level and NO metabolites, with significant indirect correlations between DDAH, NO, and augmentation index. Despite lack of significance in the differences of DDAH, ADMA, and NO between the treatment groups, the combined group results trended in the direction of the central hypothesis that increased dietary sodium intake downregulates DDAH and depresses NO production, resulting in a rise in BP and vascular stiffness. This initial study is the first demonstrating the trend that increased sodium intake was associated with a reduction in DDAH activity, and a depression in NO metabolites in healthy, normotensive African Americans.

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