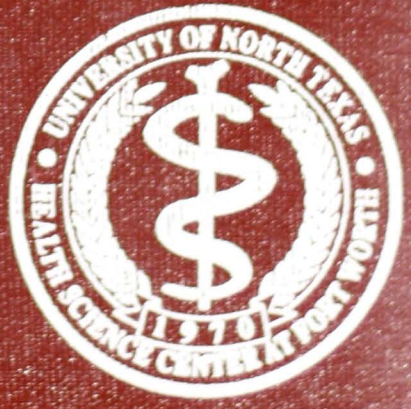
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Thakre, Tushar P., Single Nucleotide Polymorphisms of The *ATP1a2* Gene And Their Effects On Blood Pressure And Other Cardiovascular Variables In African Americans And Caucasian Americans. Doctor of Philosophy (Integrative Physiology), May 2007.

Mutations in the 3' – untranslated region (3'-UTR) of the *ATP1a2* gene, which encodes the α_2 subunit of Na^+-K^+-ATPases are reportedly associated with hypertension. This study was initiated: 1) to identify and to determine the frequency of the single nucleotide polymorphism (SNP) responsible for a 3'-UTR restriction fragment length polymorphism (RFLP) of the *ATP1a2* gene in African Americans (AAs) and Caucasian Americans (CAs), 2) to test for association of these SNPs with baseline blood pressure, 3) to determine whether pressor responses of cardiovascular variables are affected by these SNPs, and 4) to test whether endothelial dysfunction is associated with these SNPs.

RFLP analysis using the *BglII* restriction enzyme was performed on DNA obtained from 63 normotensive subjects and results were confirmed by sequencing. Responses of blood pressure, heart rate, muscle sympathetic nerve activity (MSNA) and systemic vascular resistance (SVR) to pressor stimuli of two different origins (cold pressor and hypoxic apnea) were tested in 37 individuals. Endothelial function was tested using ultrasound imaging of the brachial artery.

Six SNPs were detected in the sequenced region at mRNA positions G3756C, A3788G, T3849C, G3853A, C3913T and C3915T, of which T3849C, G3853A and C3913T are novel SNPs. Mutant allele frequencies for these SNPs were higher in AAs than in CAs. SNPs at mRNA positions G3756C, G3853A, C3913T and C3915T were associated with baseline blood pressure. The ancestral haplotype $G_{3756}G_{3853}C_{3913}C_{3915}$

constructed from these 4 SNPs associated with lower blood pressure in AAs and with higher blood pressure in CAs. Haplotypes GGTT and CATT were associated with higher mean and diastolic blood pressures, respectively in AAs. Responses of blood pressure, MSNA and SVR to the pressor stimuli were not different across haplotype ($p \geq 0.61$). Similarly, no endothelial dysfunction was associated with the SNPs ($p \geq 0.56$). Haplotype groups associated with higher baseline blood pressure tended to have higher systemic vascular resistance (SVR), suggesting increased vascular tonicity as a primary mechanism for the higher blood pressure.

These data suggest that although SNPs in the 3'-UTR of the *ATP1a2* gene and haplotypes constructed from the SNPs affect baseline blood pressure in an ethnic-specific manner at a young age, neither the responses to pressor stimuli nor endothelial function are affected. Thus, these SNPs and haplotypes are associated with an increased arterial pressure that is likely mediated by an increased vascular tone.

SINGLE NUCLEOTIDE POLYMORPHISMS OF THE *ATP1a2* GENE AND THEIR EFFECTS ON BLOOD PRESSURE AND OTHER CARDIOVASCULAR VARIABLES IN AFRICAN AMERICANS AND CAUCASIAN AMERICANS

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DISSERTATION

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DOCTOR OF PHILOSOPHY

By

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Thakre TP, Smith ML. Los; iv ıse curvilinearity of beat-to-beat heart rate in congestive heart failure. Presentea at tne annual Research Appreciation day at the University of North Texas Health Science Center in April 2004 and at the annual meeting of the Federation of American Societies of Experimental Biology (FASEB) in March 2005.

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LIST OF ABBREVIATIONS

3'-UTR	3'-Untranslated region
CO	Cardiac output
DBP	Diastolic blood pressure
MAP	Mean arterial pressure
MSNA	Muscle sympathetic nerve activity
SVR	Systemic vascular resistance
SBP	Systolic blood pressure

CHAPTER 1

INTRODUCTION

Hypertension has long been an important public health problem worldwide. According to the Centers for Disease Control (CDC), one in every three adult Americans has high blood pressure. In 2002, hypertension was listed as a primary or contributing cause of death for 277,000 Americans. In 2003, there were more than 35 million physician office visits for hypertension. About 90% of middle–aged adults will develop high blood pressure in their lifetime. The direct and indirect costs of hypertension were around $63.5 billion in the year 2006. From a public health perspective, hypertension represents a major modifiable risk factor for heart disease, stroke, congestive heart failure, and kidney disease.

Hypertension is caused by the multifactorial interplay among genetics, lifestyle and the environment. The influence of genetic factors on hypertension is supported by the observation that hypertension runs in families. Offspring and siblings of hypertensive individuals are more likely to be hypertensive themselves as compared to the relatives of non-hypertensive individuals (6, 7, 18, 51, 55). Another observation supporting the role of heredity in the causation of hypertension is the differential distribution of hypertension among ethnic groups. Hypertension is more prevalent in adult African Americans than in

Caucasian Americans (Age-adjusted prevalence in persons 20 years and older: around 41% in African Americans and around 27% in Caucasian Americans).

Early research efforts conclude that hypertension is a polygenic disease. A number of genes act in concert with environmental factors to give rise to this enigmatic disease. Multiple candidate genes have been associated with hypertension. Examples include genes coding for the adrenergic receptors, the nitiric oxide synthases and the components of the renin-aldosterone-angiotensin axis (50). Hypertension has been recently associated with the *ATP1a2* gene that codes for the alpha 2 subunit of the Na^+-K^+-ATPase (17, 57).

The Na^+-K^+-ATPases are plasma membrane enzymes found in all cells in the body. These enzymes which consume 20-30% of cellular ATP are responsible for maintaining cellular ionic gradients and thus they regulate cell volume, membrane potentials, and secondary active transport of solutes (78). Na^+-K^+-ATPases are heterodimers composed of a catalytic alpha subunit and a smaller beta subunit. There are 4 known isoforms of the α subunit (α_1- α_4) and 3 known isoforms of the β subunit (β_1-β_3). This means that there are 12 Na^+-K^+-ATPase isozymes to be expressed in a tissue specific manner. Na^+-K^+-ATPases with the alpha 2 subunit are prevalent in skeletal muscle, vascular smooth muscle, and glia of the central and peripheral nervous systems.

A number of restriction fragment length polymorphisms (RFLPs) and single nucleotide polymorphisms (SNPs) occur in both the coding and non-coding regions of the *ATP1a2* gene. Non-synonymous SNPs in the coding region of the gene are associated with some forms of migraine (29, 83, 85). In the context of hypertension, the 3'

untranslated region seems to be particularly important. RFLPs in this region are more common in hypertensives as compared with normotensives (17, 57). These RFLPs are also more common among African Americans as compared with Caucasians (17, 57). African Americans are particularly susceptible to developing hypertension which leads to the suggestion that there might be a causal relationship between the SNPs in this gene and the susceptibility to hypertension.

Considering the known and unknown aspects of the genetic susceptibility to hypertension described above, the genetic and physiological studies in this project were designed to address the following specific aims:

Specific Aim 1: To compare the prevalence of the *ATP1a2* gene's 3'-UTR SNPs in African Americans and Caucasians. The specific hypothesis tested was:

Hypothesis 1: The allele frequencies for the SNPs are differentially distributed in African Americans and Caucasians.

Specific Aim 2: To compare the responses of physiological variables (sympathetic nerve activity, heart rate and blood pressure) to graded pressor stimuli of two different types (cold pressor and hypoxic apnea) in persons with different genetic backgrounds conferred by the *ATP1a2* SNPs. In relation to specific aim 2, the following two hypotheses were tested. **Hypothesis 2a:** Persons carrying the mutations at the SNPs will have an altered sympathoexcitatory and pressor response to the cold pressor stimulus as compared to persons lacking the mutations. The differential of altered response will depend on the

number of mutated alleles in an individual, that is, mutant homozygotes will have the greatest response; heterozygotes will have an intermediate response and wild-type homozygotes will have the least response. **Hypothesis 2b:** Persons carrying the mutations at the SNPs will have an altered sympathoexcitatory and pressor response to the hypoxic apnea stimulus as compared to persons lacking the mutations. The differential of altered response will depend on the number of mutated alleles in an individual, that is, mutant homozygotes will have the greatest response; heterozygotes will have an intermediate response and wild-type homozygotes will have the least response.

Specific Aim 3: To determine whether the exaggerated pressor response in persons carrying the *ATP1a2* mutations is related to endothelial dysfunction. The following null hypothesis was tested: **Hypothesis 3:** Endothelial dysfunction is not responsible for the exaggerated response seen in persons carrying the polymorphism.

CHAPTER 2

REVIEW OF LITERATURE

Hypertension as a major public health problem

Hypertension has long been recognized as a major public health problem in the US. According to the National Heart Lung and Blood Institute, over 65 million people in the US are afflicted with this "silent" disease. Hypertension can lead to major complications including stroke, coronary artery disease, end-stage renal disease, heart failure, and many other secondary co-morbidities. Aggressive screening and treatment approaches have led to early recognition and containment of the disease; however, given the major public health impact of the disease, better approaches to identify subjects who are prone to develop hypertension are needed. Although hypertension can result from known clinical conditions (renal disease, endocrine disorders, and vascular disorders), the vast majority of patients have essential hypertension, in which the cause is unknown. A genetic predisposition may be responsible for most of the cases of essential hypertension. In support of this theory, hypertension runs in families and offspring of hypertensives and have a greater probability of becoming hypertensive themselves as compared to offspring of non-hypertensives (6, 7, 18, 51, 55). The Centers for Disease Control reports a strong racial difference in the incidence of hypertension, as hypertension is much more common in African Americans (age-adjusted prevalence 40.5%) as compared to whites (age-

adjusted prevalence 27.4%). These observations have catalyzed an intensive search for genetic substrates that are associated with hypertension. Numerous studies have identified a number of genes and their variations that are found to be associated with a greater incidence of hypertension. As reviewed by Naber and Siffert (50) some of the important genes implicated in hypertension include those encoding the renin-angiotensin system, adrenergic receptors, and nitric oxide synthases among others. Additional candidate genes include genes encoding for specific subunits of the Na^+-K^+-ATPase pump. The pump is ubiquitous and may be involved in hypertension (10, 17, 31, 39, 80). One of the genes encoding for a subunit of the Na^+-K^+-ATPase is the focus of this research.

The Na^+-K^+-ATPase and hypertension

The Na^+-K^+-ATPase is an integral membrane protein that catalyzes the active transport of Na^+ and K^+ across the cell membrane. The enzyme is composed of a catalytic α subunit and an associated β subunit that is also necessary for proper enzyme function. There are four distinct isoforms for the α subunit that have variable tissue distributions (78). The α1 subunit is ubiquitous and mutations in the gene for this subunit have been implicated in renal salt-sensitive hypertension in Dahl S rats (25). The α2 isoform is found in vascular smooth muscle, cardiac muscle sarcolemma, skeletal muscle sarcolemma and adipocytes, as well as in astrocytes, oligodendrocytes, and Schwann cells of the central and peripheral nervous systems (17, 35, 54, 75, 78, 79). Studies of the normotensive offspring of hypertensive parents indicate a possible role for Na^+-K^+-

ATPase in the susceptibility to develop hypertension later in life. A recent study has reported decreased pump activity in hypertensives (59). The authors suggest that the pump may play a role in the development of essential hypertension by affecting fatty acid metabolism. Inhibition of this pump has been shown to increase vascular smooth muscle contractility via an increase in Ca^{++} entry (19). In this regard, it should be noted that the Na^{+}-K^{+}-ATPase is linked to the Na^{+}-Ca^{++} exchanger. A decrease in the activity of the former will result in increased intracellular Ca^{++} concentration via the exchanger. Calcium activates the contractile machinery in smooth muscle cells and an increased intracellular Ca^{++} concentration will lead to greater vascular tonicity and increased peripheral vascular resistance, leading to higher blood pressure. Hypertension induced by high salt intake also seems to involve the inhibition of the Na^{+}-K^{+}-ATPase via endogenous cardiac glycosides (28).

The *ATP1a2* gene and hypertension

The *ATP1a2* gene encodes the α_2 isoform of Na^{+}-K^{+}-ATPase. This gene is present on the long arm of human chromosome 1. Restriction fragment length polymorphisms (RFLPs) in the Na^{+}-K^{+}-ATPase genes have been described (31, 39, 46, 64, 65), and these RFLPs are of interest as potential markers of susceptibility to hypertension (10, 23, 49, 74). A *Bgl* II restriction fragment polymorphism downstream of the gene (in the 3' untranslated region) is co-dominantly inherited with an allele frequency of 0.2 (17). In a middle-aged African American population, the allele frequency for the downstream mutation was higher and may be associated with

hypertension (adjusted odds ratio 7.69 in homozygotes as compared to persons not carrying the RFLP) (17). Restriction enzyme studies have shown that the mutant RFLP is 10.5 kilobase (kb) and the normal RFLP is 4.3 kb (17). Several other studies have found support for the link of the Na^+-K^+-ATPase with hypertension (21, 33, 66). Knockout of the *ATP1a2* gene in rats is lethal in homozygotes (26, 63). Partial knockout of the gene (i.e. heterozygote rats) leads to increases in force of contraction of heart muscle(41), plasma glucose levels (36), resting plasma Ca^{++} and Ca^{++}-transients (22, 63). Thus, collectively these studies in rodents provide evidence of changes in cellular function that may contribute to a risk for developing hypertension.

The 3'-UTR RFLP may be more prevalent in African Americans, a population that is particularly prone to develop hypertension, than in Caucasians (17). Also, individuals who carry the 3'-UTR RFLP are more prone to develop hypertension than those who do not (17). Thus this downstream RFLP in the *ATP1a2* gene seems to be a potential genetic locus for hypertension. This research project was directed at better defining the prevalence of SNPs in the 3'-UTR of the *ATP1a2* gene in a larger sample of African Americans (particularly those with a family history of hypertension) and in investigating whether their presence confers an increased risk for developing hypertension upon carriers. In addition, this project will be the first to relate these SNPs to a difference in physiologic function that may also be predictive of risk for hypertension.

Pressor responses of hypertensives

A number of studies have previously compared responses of physiologic parameters to pressor stimuli in hypertensives and normotensives (47, 58). The stimuli used to obtain pressor responses include cold pressor stimulus (47, 58), lower body negative pressure (47, 58), hypoxia (72, 88), among other stimuli. Hypertensives consistently have elevated baseline heart rate, blood pressure, and muscle sympathetic nerve activity as compared to normotensives. In addition, when subjected to pressor stimuli, hypertensives show an exaggerated rise in blood pressure and muscle sympathetic nerve activity as compared to normotensives, thus suggesting that the pathophysiology involves altered vascular control. In addition, this altered control involves altered neural reflex mechanisms and may also involve end-organ changes. Young normotensive hyper-responders to the stressor stimuli are reportedly at an elevated risk of developing hypertension in the future (8, 45, 48, 77, 81).

Racial differences in pressor reponses

Race is a compound construct with strong genetic undertones and is known to be an important modifier of blood pressure homeostasis (3, 42). Normotensive African Americans and Caucasians have comparable baseline heart rate, blood pressure, and muscle sympathetic nerve activity (4, 5, 11, 32, 76). However, in response to pressor stimuli, African Americans show an exaggerated pressor response compared to Caucasians (4, 5, 11, 32, 76). Genetic differences might be responsible for this differential effect between the two races.

Endothelial dysfunction and hypertension

The endothelium is an important regulator of vessel diameter and blood flow. The endothelium synthesizes both potent vasodilators and vasoconstrictors (43, 44, 87). An important vasodilator secreted by the endothelium is nitric oxide which can be released from the endothelium in response to many different stimuli including high flow states and shear stress (86). Nitric oxide acts on the vascular smooth muscle cells and causes G-protein mediated vasodilation (86). Endothelial function can be tested by occluding the forearm conduit artery (brachial artery) by inflating a cuff around the forearm to pressures above the systolic blood pressure and then releasing the cuff to produce a rapid increase in blood flow through the vessel which in turn results in shear stress-mediated nitric oxide release and subsequent vasodilation (86). If the endothelium is damaged, this flow-mediated vasodilation is attenuated (86). Change in flow-mediated dilation can be tested using ultrasound-derived measurements of the vessel lumen diameter pre- and post-occlusion. Flow-mediated dilation is expressed as a percentage of increase in vessel diameter over baseline values. Hypertensives have been shown to have endothelial dysfunction as evidenced by a reduced nitric oxide synthesis in response to post occlusion reperfusion (40, 52, 56).

Cold water as a pressor stimulus

Cold water serves as a potent pressor stimulus. The cold pressor test is usually administered by placing one hand in a cold water bath. Cold water is a painful stimulus conducted by the peripheral sensory nerves, and leads to an increase in heart rate, blood

pressure and muscle sympathetic nerve activity (12, 62, 84). The response of these physiologic variables to the cold pressor stimulus is temperature dependent – the colder the water, the greater the pressor response. Studies by Victor *et al.* (84) investigated the effects of the cold pressor test on sympathetic outflow and tested the strength of the correlation between sympathetic nerve discharge and the changes in arterial pressure, heart rate, and plasma norepinephrine. Arterial pressure rose steadily during the first and second minutes of the cold pressor stimulus. Sympathetic activity did not increase in the first 30 seconds of the test but increased significantly by the end of the first minute and during the second minute of the test. The increases in sympathetic activity during this test were correlated with the increases in both mean arterial pressure and peripheral venous norepinephrine. This stimulus is therefore a useful tool for assessing sympathoexcitatory and associated pressor responsiveness. Lafleche *et al.* (34) have found an exaggerated blood pressure response to cold pressor stimulus in borderline hypertensives as compared to normal controls. The cold pressor stimulus can be therefore be used to investigate the link between the presence of the SNPs and an exaggerated response to a cold pressor stimulus.

Hypoxia as a pressor stimulus

The chemoreflexes are important modulators of sympathetic activation. The peripheral chemoreceptors located in the carotid and aortic bodies have been shown to respond primarily to a low oxygen tension in blood (hypoxia) (15). Peripheral chemoreceptors elicit increases in MSNA, with consequent increases in blood pressure

(71, 73) and respiratory rate (16, 24). Increased blood pressure and increased minute ventilation both inhibit the sympathetic response to chemoreflex activation (71, 73). Yet, during apnea, when the inhibitory influence of lung stretch is eliminated, there is a potentiation of the sympathetic response to hypoxia. This inhibitory influence of the pulmonary afferents was more dramatic on the sympathetic response to peripheral compared with central chemoreceptor activation (30).

Several studies have shown increases in MSNA during > 5 min of hypoxia (61, 70, 73). Rowell *et al.* exposed subjects to 12%, 10%, and 8% O_2 and found that MSNA increased under all three conditions (61). Studies by Cutler *et al.* have found that short-term exposure to intermittent hypoxic apnea results in sustained elevation of MSNA and that *hypoxia* is the primary mediator of this response (9). Experimental studies in dogs (2) and rats (13, 14) support the hypothesis that intermittent hypoxia can cause persistent hypertension. Using experimental protocols that simulate obstructive sleep apnea, these studies reported that hypoxia alone can cause an increase in daytime blood pressure (2, 13, 14). This sustained increase in blood pressure has been postulated to be due to enhanced sympathetic activity (1) and has been shown to be prevented by sympathetic denervation using 6-OH dopamine (14). The link to hypertension in these studies is likely mediated in part by altered chemoreflex control of sympathetic nerve activity. Thus, hypoxic apnea serves as a potent stimulus for increased sympathetic activity and can be used to investigate the potential link between the presence of the 3'-UTR RFLP and an exaggerated pressor response.

Theoretical basis for a link between the SNPs and hypertension

The SNPs are present at the 3' end of the gene. Since they are not present in the coding region, they are not expected to affect transcription and structure of the Na^+-K^+-ATPase. However, they may affect translation or mRNA half-life. Changes in either translation efficiency or mRNA half-life could lower α2 subunit expression and hence lower Na^+-K^+ exchange. Studies have shown that there is an increased intracellular sodium concentration in hypertensives (38, 67). An increase in intracellular sodium will theoretically reduce the activity of the sodium-calcium exchanger which normally extrudes calcium out of the cell in exchange for sodium efflux. Increase in intracellular sodium reduces the concentration gradient for sodium entry into the cell, thereby reducing sodium influx and calcium efflux via the sodium-calcium exchanger. This will lead to accumulation of calcium inside the cell. Calcium activates the contractile machinery inside smooth muscle cells of the vasculature, thereby setting up a condition of increased vascular reactivity. Studies done in optic nerve astrocytes have shown increased intracellular calcium in the cells of mice with a partial knockout of the *ATP1a2* gene (22). A previous study has reported that knockout of the *ATP1a2* gene leads to malfunction in neuronal activity (26). But this should not affect the sympathetic efferent nerves as the $\alpha 2\beta_2$ isozyme is found only in astrocytes, oligodendrocytes, and Schwann cells.

CHAPTER 3

EXPERIMENTAL DESIGN AND METHODS

The studies were designed to address the following questions: (1) What are the SNPs commonly found in the 3'-UTR of the *ATP1a2* gene? (2) What is the prevalence of these SNPs in healthy African Americans and Caucasians? (3) Does the presence of these SNPs affect the baseline levels of blood pressure, muscle sympathetic nerve activity, heart rate, and other cardiovascular variables? (4) Does the presence of the SNPs affect the response of cardiovascular variables to pressor stimuli? (5) Does the presence of the SNPs affect vascular reactivity?

STUDY 1

Study 1 addressed questions 1 and 2. It was hypothesized that the allele frequencies for the mutant alleles will be different in AAs as compared to CA*s*. In addition, there will be an association with the presence of these SNPs and a family history of hypertension. Blood/buccal swab samples were obtained from healthy volunteers drawn from African American and Caucasian populations and genotyping was done to determine whether SNPs were present in the 3'-UTR of the *ATP1a2* gene.

Subjects: 63 healthy volunteers were recruited for this study. They included 38 AAs (14 males, 24 females) and 26 CAs (12 Males and 13 females). The subjects

reported no history of cardiovascular, pulmonary or neurological disease. After obtaining written informed consent, details of family history of hypertension were elicited from each subject.

Experimental Protocol: *Sample preparation* DNA samples for genotyping were obtained via one of two methods: 1) collection of 3 milliliters of blood through a venipuncture or 2) through a buccal swab. DNA was organically extracted from the WBCs from the blood samples and from the cells from the buccal swabs with Phenol-chloroform-isoamyl alcohol followed by ethanol precipitation.

PCR Amplification The ATP1A2 gene was amplified for RFLP analysis using 22.5μL of PCR Supermix (Invitrogen), 0.4μM forward primer (5'-CCAGGCATGGAAAGATGG-3'), 0.4μM reverse primer (5'-TAGCCTTGTGCCCTGAGAGT-3'), and 10-50 ng genomic DNA in a 25μL final reaction volume under the following conditions:

95°C	95°C	61°C	72°C	72°C	4°C
11:00	0:10	0:30	0:30	10:00	∞

Highly polymorphic segments of the 3' UTR of the ATP1A2 gene were PCR amplified as above with forward and reverse primers. Post-PCR clean-up was performed enzymatically using 5μL Exo-SAP-it (USB Corp.) at 37°C for 30 minutes followed by enzyme deactivation at 80°C for 15 minutes.

RFLP (Restriction Fragment Length Polymorphisms) 10μL of each PCR amplicon was digested with 1U *Bgl* II restriction enzyme with appropriate buffer for a 20μL reaction

volume. Samples were incubated at 37°C for 2 hours. Digestions were run on a 1.2% agarose gel, stained with SybrGold, and viewed on a Hitachi FMBIOII Imager.

DNA Sequencing RFLP results were confirmed by sequencing with BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems). Highly polymorphic segments of the 3' UTR of the ATP1A2 gene were sequenced using either the forward primer 5'-GCCTGAGACTGGAAAAGGTG-3' or reverse primer 5'-TGGAGCTCTGAGGACTTAGTATTACA-3'. Cycle sequencing was performed in a GeneAmp® PCR System 9700 thermal cycler using 0.8uL BigDye Terminator v3.1 Cycle Sequencing Ready Reaction mix (Applied Biosystems, USA), 1.6uL BigDye® Terminator v1.1 & v3.1 5X Sequencing Buffer (Applied Biosystems, USA), 1μL 3.3μM primer, 3μL PCR product, and 3.6μL molecular grade water. Cycling conditions consisted of 25 cycles: 96°C denature 10 sec, 50°C anneal 5 sec, 72°C extension 30 sec. DNA samples were sequenced in an ABI PRISM® 3100 sequencer (Applied Biosystems, USA). The sequence was analyzed and aligned using Sequencing Analysis Software v5.2 (Applied Biosystems, USA) and Sequencher™ v4.2 (Gene Codes Corporation, USA).

Data Analysis: Arlequin ver. 3.0 was used for locus, haplotype, and gametic phase estimation, as well as Hardy-Weinberg, linkage and sample differentiation analysis. Haplotype, and gametic phase estimation was confirmed using PHASE software. Linkage disequilibrium was tested using an extension of Fisher exact probability that uses a Markov chain to efficiently explore the space of all possible contingency tables (68). This Markov chain consists of a random walk in the space of all contingency tables. It is done in such a way that the probability to visit a particular table

corresponds to its actual probability under the null hypothesis of linkage equilibrium (20). An extended Fisher's exact test using a modified version of the Markov-chain random walk algorithm was also used to detect significant departure from Hardy-Weinberg equilibrium (20, 37). The non random association of haplotypes into individuals was tested in the same fashion using sample haplotype frequencies (60, 68, 69).

Study 2

Study 2 was designed to address questions 4 and 5 by comparing baseline values of cardiovascular variables and their values in response to pressor stimuli in people with different SNPs. Effort was made to match groups for similar mean age and Body Mass Index [BMI: calculated as (body weight in kg/(height in cm)^2].

Subjects: A subgroup of 37 subjects, of the 63 who consented to the genotyping, volunteered for this study. They included 21 AAs (12 females and 9 males) and 16 CA*s* (10 females and 6 males). The mean age of the subjects was 24 ± 5.5 years. After giving written informed consent, each subject completed a medical history questionnaire prior to participation in the study. All subjects reported no history of hypertension, diabetes, cardiovascular disease, pulmonary disease or neurological disease and were not currently using any medications other than oral contraceptives. All female subjects were administered a urine pregnancy test to ensure that they were not pregnant and female subjects were not tested during menses to eliminate potential confounding effects of menses on fluid metabolism, blood volume and cardiovascular function. Subjects were

instructed to abstain from vigorous exercise and alcohol for 24 hours and from caffeine for 12 hours prior to the start of the study. All studies were performed around the same time of day to minimize effects of diurnal variation. The studies were performed in the semi-recumbent position to minimize the diuresis that may occur in the supine position and thus help prevent increases in sympathetic nerve activity over time which can occur with severe bladder distention.

Experimental Protocol: Prior to instrumentation, all subjects were allowed to use the restroom. The subjects were then placed on a reclining chair in a semi-recumbent position in a laboratory setting with an ambient temperature of 23-24 ^{0}C. The subjects were instrumented for measurement of heart rate, blood pressure, respiratory function, arterial oxygen saturation (Sa_{O2}) and muscle sympathetic nerve activity (MSNA). After a 20-minute quiet rest, the subjects were given two kinds of pressor stimuli – 3 bouts of hypoxic apnea and 3 bouts of cold pressor test. During the hypoxic apnea stimulus, subjects breathed for 1 minute through a face mask connected to a Douglas bag containing air with different concentrations of oxygen (12%, 16%, and 21%). At the end of the minute, the subjects held their breath at end-expiration for 20 seconds (lung volume equal to FRC). The 3 bouts of hypoxic apnea were given in a completely random order. During the cold pressor stimulus, subjects immersed their one hand for 2 minutes in a cold water bath with set temperature (2°, 10°, or 18°C). The bath contained water with or without ice cubes to obtain and maintain the desired temperature. The 3 bouts of the cold pressor test were also administered in a completely random sequence. A washout window of 5 and 15 minutes was used in between the bouts of hypoxic apnea and cold

pressor stimuli, respectively. After these 6 stimuli, the subjects rested comfortably until all the recording instruments had been removed.

Data analysis: One–way ANOVA was used to discern differences in cardiovascular variable baseline values and response to stimulus across genotype. For all statistical analyses, significance was set at an α level of 0.05.

Study 3

Study 3 sought to address question 6 i.e., whether vascular reactivity is affected by the SNPs in the *ATP1a2* gene. Flow-mediated dilation in the brachial artery was tested using ultrasonographic techniques as an assessment of endothelial function.

Subjects: All the 37 subjects who consented to study 2 also volunteered to do Study 3.

Experimental Protocol: The testing was done in a supine position in a quiet laboratory setting at an ambient temperature of 23-24 ^{0}C. All studies were performed at the same of the day. The subjects were connected to an electrocardiogram and made to lie supine on an examination table. The subjects' non-dominant arm was rested on a side table on a pillow and secured in place by using a vac-pac. A small sphygmomanometer cuff was placed around the subjects' forearm. Using B-mode ultrasonography with a transducer of frequency 7.5 Hz, the subject's brachial artery was identified about 3-5 cm above the elbow and the probe was fixed in place at the point of maximum clarity using a custom designed mechanical stand with a moving mechanical arm. The images were saved to the hard disk on the ultrasound machine. After instrumentation, subjects were

given a 15 minute rest period at the end of which, the forearm cuff was inflated to a pressure 50 mm Hg above the subjects' systolic blood pressure. The cuff was left inflated for 5 minutes and then released. Images were saved every 15 seconds beginning with the release of the cuff until 2 minutes after the cuff-release. After another 15 minute baseline period, the cuff was reinflated for 5 minutes and again images were obtained at 15-second intervals for 2 minutes after the release of the cuff. All images were saved to the hard disk on the ultrasound machine for later offline analysis. Brachial artery diameter was measured at 3 locations along the length of the artery and all measurements in each subject were done at the same points in the artery for comparison.

Data analysis: Differences in increase in brachial artery diameter due to flow-mediated dilation across genotype was assessed using a one-way ANOVA. All statistical analyses were performed with a significance level (α) set at 0.05.

Measurements and Procedures

Cardiovascular measurements: Heart rate (HR) was measured using standard limb-lead electrocardiography (ECG). Continuous ECG data was fed into a data acquisition system. Beat-to-beat HR was derived from the ECG waveform using the built-in peak detection algorithm in the Windaq® software and by manually scanning the ECG waveforms.

Arterial blood pressure was measured non-invasively using photoplethysmography at the finger (Finometer model 1, Finapres Medical Systems BV, Amsterdam, Netherlands). The measurements were made continuously throughout the

duration of the experiment, with a few breaks during baseline periods. The data was fed into the WinDaq acquisition system. This method has been shown to be a reliable and valid measure of blood pressure (27, 53). Additionally, Finometer obtained blood pressure was confirmed with manual auscultation during baseline periods. The analog signal was digitally sampled at 1000 Hz for later offline analysis. Beat-to-beat systolic and diastolic blood pressure was derived from the arterial blood pressure waveform using the built-in peak-and-valley detection algorithm in the Windaq® software, a routine written in Visual Basic® and by manually scanning the blood pressure waveforms.

Respiratory measurements: Respiration was monitored using an elastic respiratory monitoring band placed around the subject's abdomen (Grass Instruments, West Warwick, RI), allowing the investigator to confirm that the apneas were at end-expiration. The breathing circuit consisted of a nasal mask, a 3-way Rudolph valve, and Douglas bags which were filled with gas containing the various concentrations of oxygen used for administering hypoxia. End-tidal carbon dioxide (ET_{CO2}) was measured using a gas mass spectrometry machine (model MGA 1100 B, Perkin-Elmer, St. Louis, MO) via a port at the side of the mouthpiece. Arterial oxygen saturation was assessed by non-invasive pulse oximetry at the finger (DS-100A Durasensor, Nellcor Puritan Bennett Inc., Pleasanton, CA).

Muscle Sympathetic Nerve Activity: Muscle sympathetic nerve activity was directly measured from the peroneal nerve either at the popliteal fossa or at the fibular head using standard microneurographic techniques (82). First, the course of the nerve in the leg was determined by stimulating through the skin with a pencil-shaped electrode.

Two sterile tungsten microelectrodes (tip diameter 5-10 μm, length 35 mm, Frederick Haer and Co., Bowdoinham, ME) were inserted with one serving as a reference and the other inserted into the peroneal nerve to measure MSNA. Due to their small size and their ability to cause only minimal discomfort, the microelectrodes were inserted without local anesthesia to avoid any effect anesthesia might have on local nerve function. Nerve signals were processed by a preamplifier and an amplifier (Nerve Traffic Analyzer Model 662C-3, Department of Bioengineering, University of Iowa, Iowa City, IA) with a total gain of 99,000. Amplified signals were band-pass filtered (700-2,000 Hz), rectified and discriminated. Finally, a resistance-capacitance (RC) circuit with a time constant of 0.1 s was used to integrate the raw nerve signals. MSNA recordings were confirmed using the following criteria: 1) pulse-synchronous bursts occurring 1.2-1.4 s after the associated QRS complex, 2) reproducible activation during phase II and III of the Valsava maneuver, and 3) no activation following a pinch, skin stroking, or startle stimuli (all of which activate skin sympathetic fibers). The MSNA signal was acquired using the Windaq® software and stored for later offline analysis. Muscle sympathetic nerve activity bursts were identified using the following criteria: 1) signal: noise ratio exceeding 2.5; 2) symmetrical shape; and 3) temporal association with a preceding (by 1.2 – 1.4 s) R wave on electrocardiogram.

Ultrasound measurements: Brachial artery diameter was measured using B-mode ultrasonography (Philips HDI 5000) with a transducer of frequency 7.5 Hz. Images were obtained during baseline periods and at 15-second intervals after release of the occluding cuff for 2-minutes. The images were stored on the machine's hard disk for later offline

analysis. Arterial diameter was measured using the in-built program in the machine at 3 points along the length of the arterial segment imaged. All repeated measurements were made on the same points for comparison. All readings were made by the same investigator to avoid inter-observer variability.

Recruitment of Subjects: Subjects were recruited from the surrounding community and area universities. The principal investigator or collaborators explained the nature of the study, the inclusion/exclusion requirements, and the right to withdraw at any time without affecting their medical care to each volunteer prior to enrollment. All subjects gave written informed consent prior to participation in the study. In addition, verbal explanation was given to each subject at the time of testing. Subjects were given financial reimbursement for their time and effort during the study.

Protection of human subjects: The risks associated with the measurements of blood pressure, heart rate, respiration, Sa_{O2}, ultrasound measurements, and buccal swabbing are minimal. Such risks are not significantly increased over that ordinarily encountered in daily life. For most people, needle sticks for blood draws do not cause any serious problems. However, they may cause dizziness or bleeding, bruising, discomfort, thrombosis or blood clotting, infections and/or pain at the needle site. The cold pressor and hypoxic apnea stimuli are standard procedures used in neurologic and physiologic testing of autonomic function. The primary risks are an excessive elevation of blood pressure and resulting possibility of stroke. Exposure to hypoxic gases may cause bradyarrhythmias, lightheadedness, and anxiety. The sympathetic nerve recording procedure occasionally (3-10%) results in transient localized soreness or numbness where

the electrode was placed. Rarely, the subject will experience some parasthesias in the foot or toes. Each of these side effects resolves within 1-7 days. The potential for physical and psychological injury from the study protocol is minimal. The laboratory was also equipped with full resuscitative equipment and drugs for any emergencies. A physician was present or on call on the Health Science Center campus during all experimental procedures. None of the study subjects experienced any adverse events during the study procedures.

Inclusion of Women, Children and Minorities: There was no exclusion based on gender, religious affiliation, or sexual orientation. Because of the very nature of the study question, the study subjects were drawn from only two ethnic groups: AAs and *CAs*. Female subjects of child-bearing age were given urine pregnancy test, and were not studied if pregnant. This exclusion is due to the potential confounding effects of pregnancy on blood volume and cardiovascular function, as well as, unnecessary risk of exposure to hypoxic gas. Female subjects were not studied during or within two days of menses to eliminate potential confounding effects of menses on fluid metabolism, blood volume and cardiovascular function. Children under the age of 18 years were excluded from these studies as the potential complications from the procedures used in the study are not well-studied in children.

Medical Exclusion Criteria: participants were excluded if they reported a personal history of any of the following conditions: 1) hypertension, 2) diabetes, 3) coronary artery disease, 4) angina pectoris, 5) myocardial infarction, 6) heart failure, 7) diabetes, 8) peripheral vascular disease, 9)chronic obstructive lung disease, 10) neuromuscular

disease, and 11) seizures. As explained above, pregnant women and children were also excluded from the study.

Risk/Benefit Assessment: A risk/benefit ratio is difficult to establish for each individual subject. Overall, the risk is low for the subjects as a group, while the potential gain in knowledge is great; the advancement of knowledge far outweighs the risks. The specific benefit to the individual subject is likely to be minimal. The overall clinical benefit has the promise of: 1) developing a better understanding of the link between genes and hypertension, 2) development of a genetic screening process for identifying people susceptible to developing hypertension, 3) the potential development of a treatment modality for the arrest of progression of the disease in susceptible individuals.

Confidentiality: All data gathering procedures were performed by trained project personnel. All medical records will remain confidential. The blood/swab samples were labeled with a number and stripped of personal identifiers. All participants were assigned code numbers. The codes will be kept confidential and locked and no unauthorized person will have access to them. Only laboratory staff, representatives of the Institutional Review Board, and federal regulatory personnel will have access to study data and medical records. Additionally, electronic transmission of data will be performed in a secure manner with no direct identifying information included.

CHAPTER 4

ETHNIC-SPECIFIC INFLUENCE OF 3'-UTR POLYMORPHISMS OF THE *ATP1a2* GENE ON ARTERIAL BLOOD PRESSURE

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ABSTRACT

Background. Na^+-K^+-ATPases of the plasma membrane play key roles in cellular homeostasis. The ATPases are heterodimers composed of a catalytic α subunit and a glycoprotein β subunit, each of which has several isoforms combining in different isozymes of the pump in a tissue-specific manner. Isozymes with the α_2 catalytic subunit are expressed in skeletal muscle, vascular smooth muscle, and glial cells. A restriction fragment length polymorphism (RFLP) in the 3'-untranslated region (3'-UTR) of the *ATP1a2* gene, encoding the α_2 isoform, associates with hypertension [Glenn *et al*, *Am J Epidemiol* 153: 537-545, 2001]. This study was initiated to identify and to determine the frequency of the single nucleotide polymorphism (SNP) responsible for the 3'-UTR RFLP of the *ATP1a2* gene in African Americans (AAs) and Caucasian Americans (CAs), as well as to confirm the previously reported association of these SNPs with baseline blood pressure.

Methods. Sixty-three normotensive subjects consented to the genotyping, of which thirty-seven volunteered to undergo physiologic testing. DNA, extracted from whole blood/buccal swab samples, was amplified using polymerase chain reaction. Agarose gel electrophoresis analysis of *BglII* digests of the amplicons were validated by sequence analysis. Arterial blood pressure was measured non-invasively using finger photoplethysmography.

Results. Six SNPs were detected in a 200 base pair (bp) region of the 3'-UTR at mRNA positions G3756C, A3788G, T3849C, G3853A, C3913T and C3915T, of which those at positions 3849, 3853 and 3913 are novel SNPs discovered in this study. All

SNPs were in Hardy-Weinberg equilibrium (HWE) and mutant allele frequencies for these SNPs were higher in AAs than in CAs. Eight haplotypes were present in the samples tested, haplotype h6 being present only in CAs and haplotypes h3, h4, h5, h7 and h8 being unique to AAs and two were shared by the two populations. All haplotypes were in HWE in both races. The mutant SNPs at mRNA positions 3756, 3853, 3913 and 3915 were associated with baseline blood pressure. The ancestral haplotype GGCC constructed from these 4 SNPs associated with lower blood pressure in AAs and with higher blood pressure in CAs. Haplotypes GGTT and CATT are associated with higher mean and diastolic blood pressures, respectively in AAs.

Conclusions. Haplotypes constructed from these SNPs appear to affect baseline blood pressure in an ethnic-specific manner. Further studies are required to investigate the mechanistic reasons for the increased baseline blood pressures seen in association with these genotypes.

INTRODUCTION

Na^+-K^+-ATPase is an integral membrane protein that transports Na^+ and K^+ across the plasma membrane against their respective concentration gradients. The enzyme is composed of two essential subunits, a catalytic α subunit and an associated glycoprotein ß subunit that is necessary for the enzyme's maturation, localization to the plasma membrane, and stabilization of the K^+-occluded intermediate for proper enzyme function (23). There are four isoforms of the α subunit with variable tissue distribution (23, 33, 34). The α1 isoform is ubiquitous and predominates in the kidney. The α2 isoform is expressed in brain, heart, vasculature, and skeletal muscle and it is encoded by the *ATP1a2* gene present on human chromosome 1. The α3 isoform is limited to neural, heart and vascular smooth muscle tissues (34), while the α4 isoform is distributed in the testes (33). The Na^+-K^+-ATPase has an evolutionarily conserved cardiac glycoside binding site that binds ouabain, digoxin, and digitoxin. This site plays an important role in adrenocorticotropic hormone (ACTH)-induced hypertension in mice. Chronic administration of ACTH causes hypertension in wild type mice but not in genetically engineered mice in which the Na^+-K^+-ATPase was made resistant to cardiac glycosides. This demonstrates that the cardiac glycoside binding site of the Na^+-K^+-ATPase plays an important role in one form of hypertension (11).

Abnormalities of myocardial Na^+-K^+-ATPase are observed in various forms of experimental hypertension (6, 8, 16, 32, 37). The induction of hypertension by low-dose ouabain augments baseline myogenic tone and myogenic reactivity in small arteries by interacting specifically with arterial myocyte Na^+-K^+-ATPase. Low-dose ouabain inhibits

α2 activity in mice, resulting in elevated myocyte Ca^{2+} and arterial constriction (38). Na^+-K^+-ATPase influences blood pressure and vascular resistance through coupling with the Na^+-Ca^{++}-exchanger (NCX). Reduced Na^+-K^+-ATPase activity promotes Ca^{2+} entry via NCX in arterial myocytes as a consequence of a reduced Na^+ concentration gradient across the plasma membrane (2, 13, 18, 19). Ouabain-induced hypertension is blocked in genetically engineered mice expressing a ouabain-insensitive α2-isoform, indicating that the *ATP1a2*$^{+/-}$ hypertension model is ouabain and salt independent (11). Therefore, ouabain-induced increase in myogenic reactivity, myogenic tone, and elevated blood pressure, are primarily due to reduction of arterial myocyte Na^+-K^+-ATPase activity. In this model, reduced Na^+-K^+-ATPase activity is both necessary and sufficient to induce hypertension, which demonstrates a central role of arterial myocyte Na^+-K^+-ATPase activity in regulating blood pressure (38).

Lower levels of Na^+-K^+-ATPase activity in erythrocytes and leukocytes are reported in hypertensive individuals (3, 35). Na^+-K^+-ATPase activity is inversely associated with systolic, diastolic, and mean blood pressure. African Americans have a significantly lower erythrocyte Na+,K+- ATPase activity than Caucasians. Provided that findings in erythrocytes also reflect the relative levels of Na^+-K^+-ATPase in vascular smooth muscle cells, decreased Na^+-K^+-ATPase activity may predispose African Americans to higher blood pressure and hypertension (28).

A *BglII* restriction fragment length polymorphism (RFLP) in the 3'-UTR of *ATP1a2* was associated with percent body fat (10), resting heart rate and rate-pressure product, post-glucose plasma insulin level with regard to resting systolic blood pressure,

resting systolic blood pressure and exercise diastolic blood pressure (10), respiratory quotient in men (20), trainability of maximal oxygen consumption in sedentary Caucasian American subjects (25), and regulation of hemodynamic phenotypes in healthy subjects (26). The variant allele was also associated with hypertension and was 1.8 times more common among African Americans than among Caucasian Americans (12).

The present study was designed to investigate whether the haplotypes derived from the single nucleotide polymorphisms (SNP) in the 3'UTR region of the *ATP1a2* gene are differentially distributed between African Americans and Caucasian Americans and whether these haplotypes are associated with blood pressure in normotensive representatives of these ethnic groups.

MATERIALS AND METHODS.

Study Subjects. Clinical information was collected on 63 consenting normotensive subjects included body mass, sleep apnea, smoking and detailed family history of hypertension, family history of diabetes mellitus; and measurement of weight (to the closest 50g), height (to the closest mm) and body mass index (BMI). Ethnicity was self-reported. Thirty seven subjects consented to the physiologic studies. All 37 were non-hypertensive, non-diabetic and reported no history of cardiovascular, pulmonary or neurologic disease. No medication use other than oral contraceptives was reported by study subjects. Urinary human chorionic gonadotropin was evaluated to rule out pregnancy in female subjects and cardiovascular function was not tested during menses to reduce the variance associated with changes in fluid volume. Subjects refrained from

vigorous exercise and alcohol for 24 hours and caffeine for 12 hours prior to the start of the study. The study was approved by the Institutional Review Board of the University of North Texas Health Science Center, Fort Worth, Texas, USA.

Sample Preparation. Whole blood samples or buccal swabs were collected from 37 African Americans and 25 Caucasian Americans. DNA was extracted with Phenol-chloroform-isoamyl alcohol followed by ethanol precipitation.

PCR Amplification. The *ATP1a2* gene was amplified for RFLP analysis using 22.5μL of PCR Supermix (Invitrogen), 0.4μM forward primer (5'-CCAGGCATGGAAAGATGG-3'), 0.4μM reverse primer (5'-TAGCCTTGTGCCCTGAGAGT-3'), and 10-50 ng genomic DNA in a 25μL final reaction volume under the following conditions:

95°C	95°C	61°C	72°C	72°C	4°C
11:00	0:10	0:30	0:30	10:00	∞

32 Cycles

Highly polymorphic segments of the 3' UTR of the *ATP1a2* gene were PCR amplified as above with forward and reverse primers (5'-GCCTGAGACTGGAAAAGGTG-3'; 5'-

TGGAGCTCTGAGGACTTAGTATTACA-3', respectively). Post-PCR clean-up was performed enzymatically using 5μL Exo-SAP-it (USB Corp.) at 37°C for 30 minutes followed by enzyme deactivation at 80°C for 15 minutes.

RFLP (Restriction Fragment Length Polymorphisms). A sample (10μL) of the PCR amplicon from each subject was digested with 1U *BglII* restriction enzyme with appropriate buffer for a 20μL reaction volume. Samples were incubated at 37°C for 2 hours. Digests were separated on a 1.2% agarose gel, stained with SybrGold, and viewed on a Hitachi FMBIOII Imager.

DNA Sequencing. RFLP sequences were confirmed by analysis with BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems). Highly polymorphic segments of the 3' UTR of the *ATP1a2* gene were sequenced using either the forward primer 5'-GCCTGAGACTGGAAAAGGTG-3' or reverse primer 5'-TGGAGCTCTGAGGACTTAGTATTACA-3'. Cycle sequencing was performed in a GeneAmp® PCR System 9700 thermal cycler using 0.8uL BigDye Terminator v3.1 Cycle Sequencing Ready Reaction mix (Applied Biosystems, USA), 1.6uL BigDye® Terminator v1.1 & v3.1 5X Sequencing Buffer (Applied Biosystems, USA), 1μL 3.3μM primer, 3μL PCR product, and 3.6μL molecular grade water. Cycling conditions consisted of 25 cycles: 96°C denature 10 sec, 50°C anneal 5 sec, 72°C extension 30 sec. DNA samples were sequenced in an ABI PRISM® 3100 sequencer (Applied Biosystems,

USA). The sequence was analyzed and aligned using Sequencing Analysis Software v5.2 (Applied Biosystems, USA) and Sequencher™ v4.2 (Gene Codes Corporation, USA).

Measurement of arterial blood pressure signal. Arterial blood pressure was measured continuously by finger photoplethysmography (Finometer model 1, Finapres Medical Systems BV, Amsterdam, Netherlands). During the testing protocol, finometer blood pressures were standardized and confirmed with manual auscultation. The analog signal was digitally sampled at 1000 Hz for offline analysis. Beat-to-beat systolic and diastolic blood pressure was derived from the arterial blood pressure waveform using the built-in peak-and-valley detection algorithm in the Windaq® software, a routine written in Visual Basic® and by manually scanning the blood pressure waveforms.

Statistical Analysis. Arlequin ver. 3.0 was used for locus, haplotype, and gametic phase estimation, as well as Hardy-Weinberg, linkage and sample differentiation analysis. Haplotype and gametic phase estimation were confirmed using PHASE software. ANOVA, Student's t test, and Wilcoxon/Kruskal-Wallis Tests were performed using JMP® 6.0.0 Statistical Discovery™ from SAS.

RESULTS.

BglII RFLP analysis of C3913T SNP (mRNA position) revealed 36 +/+ homozygotes, 19 +/- heterozygotes, and 7 -/- homozygotes. Results were confirmed by sequencing (**Figure 1**). Sequencing revealed a polymorphic region within a 250 bp range

with 5 additional SNPs not assayed by RFLP analysis (mRNA positions 3756, 3788, 3849, 3853, and 3915) (**Figure 2**). All samples were genotyped for all 6 SNPs. Allele frequencies are reported in **Table 1**. Population diversity for A3788G (Hapmap-CEU) and C3915T (Hapmap-CEU, -HCB, -JPT, and -YRI) was previously reported in NCBI's dbSNP (29). No prior population diversity was reported for G3756C, and T3849C, G3853A, and C3913T are newly identified SNPs.

Linkage disequilibrium was tested using an extension of Fisher exact probability that uses a Markov chain (chain length = 10000) for polymorphic pairs of loci in our African American (**Table 2**) and Caucasian American (**Table 3**) populations. The Markov chain consists of a random walk to efficiently explore the space of all possible contingency tables (30). In this analysis, the probability of visiting a particular table corresponds to its actual probability under the null hypothesis of linkage equilibrium (15). An extended Fisher's exact test using a modified version of the Markov-chain random walk algorithm was also used to detect significant departure from Hardy-Weinberg equilibrium (HWE) (15, 22). All SNPs for African and Caucasian American populations (**Tables 4 and 5**, respectively) were in HWE. The non random association of haplotypes was also tested in the same fashion using sample haplotype frequencies and revealed no significant departures from HWE ($p = 0.88$ and $p = 0.34$) (27, 30, 31). Maximum-likelihood haplotype frequencies were estimated using an Expectation-Maximization (EM) algorithm (9, 21, 31, 36). EM is an iterative process aiming at obtaining maximum-likelihood estimates of haplotype frequencies from multi-locus genotype data when the gametic phase is unknown. ELB algorithm is then used to

reconstruct the unknown gametic phase of multi-locus genotypes based on a Gibbs sampling strategy. Phase updates are made on the basis of a window of neighboring loci based on the local level of linkage disequilibrium (31). A total of 8 estimated gametic phase haplotypes are present in the samples tested. The most common haplotype (h1) has the highest frequencies in both populations. Haplotype h6 is unique to the Caucasian American population, while haplotypes h3, h4, h5, h7, and h8 are only present in the African American samples. Haplotype frequency distributions are reported in **Table 6**.

Genetic structure analysis was performed using the exact test of sample differentiation based on haplotype frequencies as described by Raymond and Rousset (1995). This test is analogous to Fisher's exact test of the contingency tables explored with a Markov chain similar to that described for the case of the linkage disequilibrium test. The probability of observing a table less or equally likely than the observed sample configuration is estimated under the null hypothesis of panmixia, the random mating within a breeding population (14, 27). Global test of differentiation among African Americans and Caucasian Americans (30000 Markov steps done) and differentiation test between all pairs of samples (Markov chain length: 100000 steps) yielded significant differences between both populations ($p < 0.001$).

One-way analysis of variance (ANOVA) and Student's t-test were performed to calculate the mean systolic, diastolic, and mean arterial blood pressures (SBP, DBP, and MAP) for African American and Caucasian American populations, and to test for equal means between homozygous normal (+/+), homozygous mutant (-/-) and heterozygous (+/-) genotypes for all polymorphic SNPs (1). Significant findings are summarized in

Table 7. There are significant differences in mean blood pressures in the various genotypes in African Americans. One-way ANOVA detects unequal means in G3756C, C3913T, and C3915T. DBP and MAP for African Americans with the homozygous (-/-) genotype at mRNA position 3756 was significantly higher than heterozygotes (+/-) (Student t-test, $p = 0.0058$, $p = 0.0211$). Homozygous (-/-) at mRNA positions 3913 and 3915 have significantly higher SBP, DBP, and MAP than homozygous (+/+) and heterozygous (+/-) African Americans. Student's t-test reveals significantly higher DBP in African Americans with (-/-) genotype than (+/+) at mRNA position 3853. G3756C, G3853A, C3913T, and C3915T are in linkage disequilibrium, as detailed in **Table 2**. The combination of these 4 SNPs exhibits a non-random association with each other. Therefore, mean blood pressures were compared for each individual's haplotype consisting of these SNPs. One-way analysis of variance (ANOVA) and Student's t-test were performed to compare the mean systolic, diastolic, and mean arterial blood pressures (SBP, DBP, and MAP) for African and Caucasian American populations, and to test for equal means in groups. Groups were based on the number of haplotype copies comprised in individuals: 0 = does not exhibit haplotype; 1 = 1 copy (heterozygote); 2 = 2 copies (homozygote). One-way ANOVA revealed significant differences in means of SBP ($p = 0.011$), DBP ($p = 0.001$), and MAP ($p = 0.002$) for the GGCC haplotype and SBP ($p = 0.007$), DBP ($p = 0.043$), and MAP ($p = 0.013$) for the GGTT haplotype **(Figure 3)**.

African Americans with heterozygous or homozygous GGCC haplotypes have significantly lower SBP, DBP, and MAP than the wild type group ($p < 0.05$). Significant

increases in SBP, DBP, and MAP are seen in carriers of the GGTT haplotype ($p < 0.05$). A significant increase in DBP was found in homozygous CATT and significant decrease in DBP in heterozygous CGCC compared to non-carriers ($p < 0.05$); however, these results are suspect as these haplotypes were only exhibited in one individual. Caucasians homozygous for the GGCC haplotype have a significant increase in SBP and MAP compared to other CA non-carriers ($p < 0.05$). An opposite effect was found in the African American population. Significant findings are summarized in **Table 8**.

DISCUSSION

Six SNPs at the 3' UTR of the *ATP1a2* gene were genotyped and variation in four linked SNPs, G3756C, G3853A, C3913T and C3915T was associated with baseline blood pressure. Certain haplotypes associated with BP in an ethnic-specific manner. The common haplotype GGCC was associated with lower blood pressure in African Americans, while the reverse is true with Caucasian Americans. Haplotypes GGTT and CATT were associated respectively with higher baseline MAP and DBP in African Americans.

The 3′-UTRs of human genes play an important role in regulating mRNA 3′ end formation, stability/degradation, nuclear export, subcellular localization and translation (4, 5, 7). They are rich in regulatory elements and are involved in regulating gene expression at the pre-mRNA level. (5). The processing of 3' ends is also important for

transcription termination downstream of cleavage sites, polyadenylation, and assembly of an export-competent mRNA (39).

The current study was based on a small, relatively young, population of normotensive, non-diabetic subjects with no reported history of cardiovascular, pulmonary or neurologic disease. The results demonstrated significant association between *ATP1a2* single SNPs and haplotypes with baseline blood pressure in the African American population. Further studies in large samples drawn from the general population will be needed to explain the possible effect of *ATP1a2* on BP variation. Although the exact mechanism remains to be understood, it is not irrational to speculate that increase of mean BP values results from decreased Na^+-K^+-ATPase activity due to haploinsufficiency. Future functional studies are necessary to test this hypothesis.

REFERENCES

1. JMP, version 6. SAS Institute Inc. Cary, NC, 1989-2005.

2. **Arnon A, Hamlyn JM, and Blaustein MP.** Ouabain augments Ca(2+) transients in arterial smooth muscle without raising cytosolic Na(+). *Am J Physiol Heart Circ Physiol* 279: H679-691, 2000.

3. **Canestrari F, Galli F, Boschi S, Albertini MC, Gheller G, De Crescentini S, and Bossu M.** Erythrocyte Na+,K(+)-ATPase properties and adenylate energy charge in normotensives and in essential hypertensives. *Clin Chim Acta* 224: 167-179, 1994.

4. **Chabanon H, Mickleburgh I, and Hesketh J.** Zipcodes and postage stamps: mRNA localisation signals and their trans-acting binding proteins. *Brief Funct Genomic Proteomic* 3: 240-256, 2004.

5. **Chen P, Guzman JP, Leong PK, Yang LE, Perianayagam A, Babilonia E, Ho JS, Youn JH, Wang WH, and McDonough AA.** Modest dietary K+ restriction provokes insulin resistance of cellular K+ uptake and phosphorylation of renal outer medulla K+ channel without fall in plasma K+ concentration. *Am J Physiol Cell Physiol* 290: C1355-1363, 2006.

6. **Clough DL, Huot SJ, Pamnani MB, and Haddy FJ.** Decreased myocardial Na+K+ATPase activity in rats with reduced renal mass-saline hypertension. *J Hypertens* 3: 583-589, 1985.

7. **Conne B, Stutz A, and Vassalli JD.** The 3' untranslated region of messenger RNA: A molecular 'hotspot' for pathology? *Nat Med* 6: 637-641, 2000.

8. **da Silva JC, Jr., Shi XJ, Johns CA, Jefferson DM, Grubman SA, Madias NE, and Perrone RD.** Experimental renal failure in the rat modulates cardiac Na,K-ATPase alpha 2 mRNA but not protein. *J Am Soc Nephrol* 5: 27-35, 1994.

9. **Dempster A, N. Laird and D. Rubin,.** Maximum likelihood estimation from incomplete data via the EM algorithm. *J Roy Statist Soc* 39: 1-38, 1977.

10. **Deriaz O, Dionne F, Perusse L, Tremblay A, Vohl MC, Cote G, and Bouchard C.** DNA variation in the genes of the Na,K-adenosine triphosphatase and its relation with resting metabolic rate, respiratory quotient, and body fat. *J Clin Invest* 93: 838-843, 1994.

11. **Dostanic-Larson I, Van Huysse JW, Lorenz JN, and Lingrel JB.** The highly conserved cardiac glycoside binding site of Na,K-ATPase plays a role in blood pressure regulation. *Proc Natl Acad Sci U S A* 102: 15845-15850, 2005.

12. **Glenn BS, Stewart WF, Schwartz BS, and Bressler J.** Relation of alleles of the sodium-potassium adenosine triphosphatase alpha 2 gene with blood pressure and lead exposure. *Am J Epidemiol* 153: 537-545, 2001.

13. **Golovina V, Song H, James P, Lingrel J, and Blaustein M.** Regulation of Ca2+ signaling by Na+ pump alpha-2 subunit expression. *Ann N Y Acad Sci* 986: 509-513, 2003.

14. **Goudet J, Raymond M, de Meeus T, and Rousset F.** Testing differentiation in diploid populations. *Genetics* 144: 1933-1940, 1996.

15. **Guo SW and Thompson EA.** Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* 48: 361-372, 1992.

16. **Haddy FJ.** Potassium, Na+-K+ pump inhibitor and low-renin hypertension. *Clin Invest Med* 10: 547-554, 1987.

17. **Imholz BP, van Montfrans GA, Settels JJ, van der Hoeven GM, Karemaker JM, and Wieling W.** Continuous non-invasive blood pressure monitoring: reliability of Finapres device during the Valsalva manoeuvre. *Cardiovasc Res* 22: 390-397, 1988.

18. **Iwamoto T, Kita S, Zhang J, Blaustein MP, Arai Y, Yoshida S, Wakimoto K, Komuro I, and Katsuragi T.** Salt-sensitive hypertension is triggered by Ca2+ entry via Na+/Ca2+ exchanger type-1 in vascular smooth muscle. *Nat Med* 10: 1193-1199, 2004.

19. **Juhaszova M and Blaustein MP.** Distinct distribution of different Na+ pump alpha subunit isoforms in plasmalemma. Physiological implications. *Ann N Y Acad Sci* 834: 524-536, 1997.

20. **Katzmarzyk PT, Rankinen T, Perusse L, Deriaz O, Tremblay A, Borecki I, Rao DC, and Bouchard C.** Linkage and association of the sodium potassium-adenosine triphosphatase alpha2 and beta1 genes with respiratory quotient and resting metabolic rate in the Quebec Family Study. *J Clin Endocrinol Metab* 84: 2093-2097, 1999.

21. **Lange K.** *Mathematical and Statistical Methods for Genetic Analysis.* New York: Springer, 1997.

22. **Levene H.** On a matching problem arising in genetics. *Annals of Mathematical Statistics* 20: 91-94, 1949.

23. **Lingrel J, Moseley A, Dostanic I, Cougnon M, He S, James P, Woo A, O'Connor K, and Neumann J.** Functional roles of the alpha isoforms of the Na,K-ATPase. *Ann N Y Acad Sci* 986: 354-359, 2003.

24. **Parati G, Casadei R, Groppelli A, Di Rienzo M, and Mancia G.** Comparison of finger and intra-arterial blood pressure monitoring at rest and during laboratory testing. *Hypertension* 13: 647-655, 1989.

25. **Rankinen T, Perusse L, Borecki I, Chagnon YC, Gagnon J, Leon AS, Skinner JS, Wilmore JH, Rao DC, and Bouchard C.** The Na(+)-K(+)-ATPase alpha2 gene and trainability of cardiorespiratory endurance: the HERITAGE family study. *J Appl Physiol* 88: 346-351, 2000.

26. **Rankinen T, Perusse L, Deriaz O, Theriault G, Chagnon M, Nadeau A, and Bouchard C.** Linkage of the Na,K-ATPase alpha 2 and beta 1 genes with resting and exercise heart rate and blood pressure: cross-sectional and longitudinal observations from the Quebec Family Study. *J Hypertens* 17: 339-349, 1999.

27. **Rousset F and Raymond M.** Testing heterozygote excess and deficiency. *Genetics* 140: 1413-1419, 1995.

28. **Rygielski D, Reddi A, Kuriyama S, Lasker N, and Aviv A.** Erythrocyte ghost Na+,K+-ATPase and blood pressure. *Hypertension* 10: 259-266, 1987.

29. **Sherry ST, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM, and Sirotkin K.** dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res* 29: 308-311, 2001.

30. **Slatkin M.** Linkage disequilibrium in growing and stable populations. *Genetics* 137: 331-336, 1994.

31. **Slatkin M and Excoffier L.** Testing for linkage disequilibrium in genotypic data using the Expectation-Maximization algorithm. *Heredity* 76 (Pt 4): 377-383, 1996.

32. **Sowers JR, Beck F, Stern N, and Raghavan SR.** Reduced sodium-potassium dependent ATPase and its possible role in the development of hypertension in spontaneously hypertensive rats. *Clin Exp Hypertens A* 5: 71-86, 1983.

33. **Stein CM, Lang CC, Singh I, He HB, and Wood AJ.** Increased vascular adrenergic vasoconstriction and decreased vasodilation in blacks. Additive mechanisms leading to enhanced vascular reactivity. *Hypertension* 36: 945-951, 2000.

34. **Sweadner KJ.** Isozymes of the Na+/K+-ATPase. *Biochim Biophys Acta* 988: 185-220, 1989.

35. **Touyz RM, Milne FJ, and Reinach SG.** Platelet and erythrocyte Mg2+, Ca2+, Na+, K+ and cell membrane adenosine triphosphatase activity in essential hypertension in blacks. *J Hypertens* 10: 571-578, 1992.

36. **Weir BS.** *Genetic Data Analysis II: Methods for Discrete Population Genetic Data.* Sunderland, MA: Sinauer Assoc., Inc., 1996.

37. **Whitmer KR, Lee JH, Martin AF, Lane LK, Lee SW, Schwartz A, Overbeck HW, and Wallick ET.** Myocardial Na, K-ATPase in one-kidney, one-clip hypertensive rats. *J Mol Cell Cardiol* 18: 1085-1095, 1986.

38. **Zhang J, Lee MY, Cavalli M, Chen L, Berra-Romani R, Balke CW, Bianchi G, Ferrari P, Hamlyn JM, Iwamoto T, Lingrel JB, Matteson DR, Wier WG, and Blaustein MP.** Sodium pump alpha2 subunits control myogenic tone and blood pressure in mice. *J Physiol* 569: 243-256, 2005.

39. **Zhao J, Hyman L, and Moore C.** Formation of mRNA 3' ends in eukaryotes: mechanism, regulation, and interrelationships with other steps in mRNA synthesis. *Microbiol Mol Biol Rev* 63: 405-445, 1999.

FIGURE LEGENDS

Figure 1. Representative results of agarose gel electrophoresis of B*glII* digests of the DNA samples are shown on the left. The panels on the right represent the base present at the location of the bands, indicated by circles and arrows from the left panel.

Figure 2. The structure of the *ATP1a2* gene is diagrammed above and the location of the six SNPs detected in the 3'-UTR of the *ATP1a2* gene in this population of subjects is explained below. The 3 SNPs in black have been reported previously while the 3 in red are SNPs being reported for the first time.

Figure 3. ANOVA results for GGCC and GGTT haplotypes. The means diamond (green) illustrates a sample mean and 95% confidence interval. The vertical span of each diamond represents the 95% confidence interval for each group, while the horizontal extent of each group along the *x*-axis is proportional to the sample size. Blue lines represent the mean of each group with error bars one standard error above and below each group mean. Dashed lines identify one standard deviation above and below the group means. (A-B) Compare SBP in GGCC and GGTT haplotypes. (C-D) Compare DBP in GGCC and GGTT haplotypes. (E-F) Compare MAP in GGCC and GGTT haplotypes.

Figure 1.

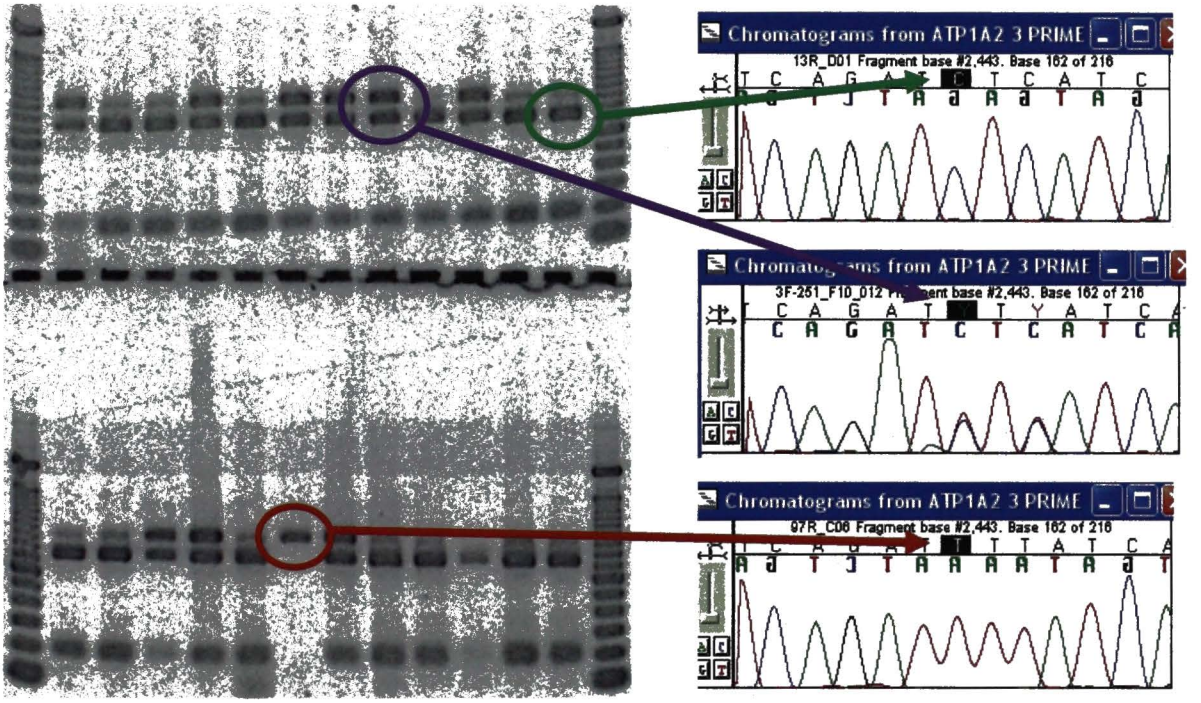
Chromatograms from ATP1A2 3 PRIME
13R_D01 Fragment base #2,443. Base 162 of 216
Chromatograms from ATP1A2 3 PRIME
Chromatograms from ATP1A2 3 PRIME
97R_C06 Fragment base #2,443. Base 162 of 216

Figure 2.

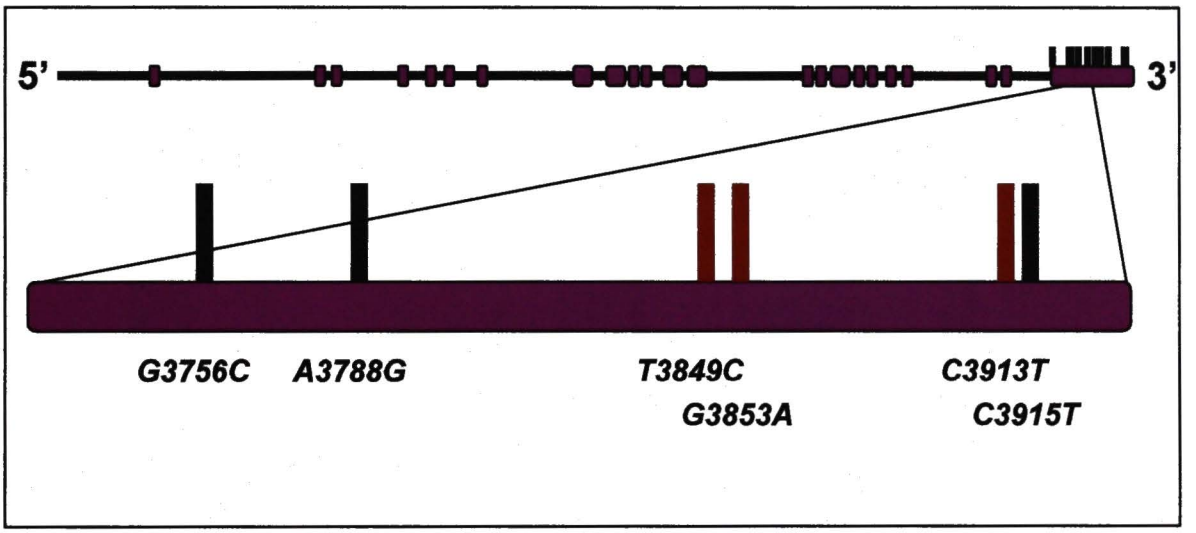

Figure 3.

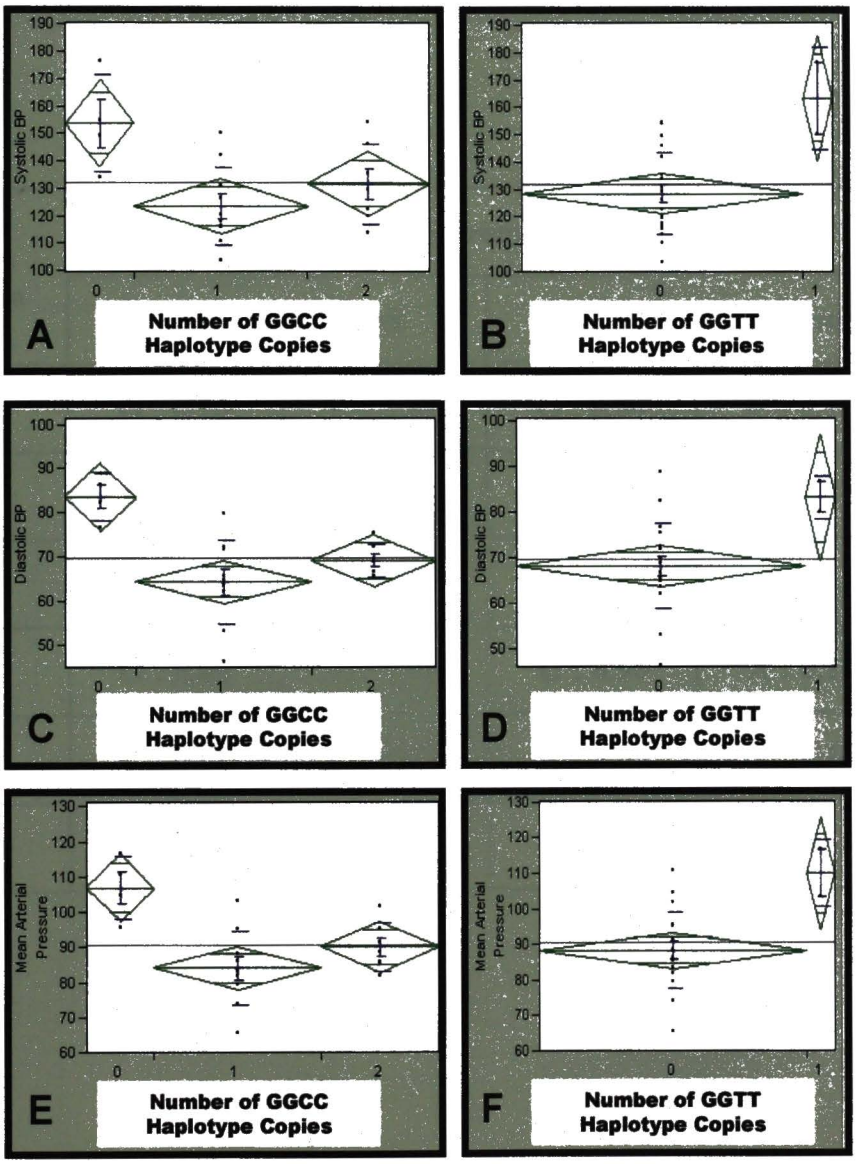
Systolic BP
Number of GGCC Haplotype Copies
A
Systolic BP
Number of GGTT Haplotype Copies
B
Diastolic BP
Number of GGCC Haplotype Copies
C
Diastolic BP
Number of GGTT Haplotype Copies
D
Mean Arterial Pressure
Number of GGCC Haplotype Copies
E
Mean Arterial Pressure
Number of GGTT Haplotype Copies
F

Table 1. Frequencies of wild type and mutant alleles are listed for the six studied SNPs present in the subject populations evaluated.

mRNA position	Allele Frequencies			
	African American		Caucasian American	
3756	G : 0.70	C : 0.30	G : 0.84	C : 0.16
3788	A : 0.82	G : 0.18	A : 1.00	
3849	T : 0.92	C : 0.08	T : 1.00	
3853	G : 0.86	A : 0.14	G : 0.90	A : 0.10
3913	C : 0.68	T : 0.32	C : 0.84	T : 0.16
3915	C : 0.68	T : 0.32	C : 0.84	T : 0.16

Table 2. The table shows significant linkage disequilibrium in African American population: Fisher exact probability (Markov chain length = 10000) for polymorphic pairs of loci.

'+' Significance level ≤ 0.05; '-' Not significant; '*' Novel SNP

	G3756C	A3788G	T3849C*	G3853A*	C3913T*	C3915T
G3756C		+	-	+	+	+
A3788G	+		-	-	+	+
T3849C*	-	-		-	-	-
G3853A*	+	-	-		+	+
C3913T*	+	+	-	+		+
C3915T	+	+	-	+	+	

Table 3. The table shows significant linkage disequilibrium in Caucasian American population: Fisher exact probability (Markov chain length = 10000) for polymorphic pairs of loci.

'+' Significance level ≤ 0.05; '-' Not significant; '*' Novel SNP

	G3756C	G3853A*	C3913T*	C3915T
G3756C		-	+	+
G3853A*	-		-	-
C3913T*	+	-		+
C3915T	+	-	+	

Table 4. Hardy-Weinberg Equilibrium for African American Population: Observed and Expected Heterozygosities were compared for each SNP. No significant departures from HWE were detected using Fisher's Exact test.

'*' Novel SNP

Locus	*Obs.Het.*	*Exp.Het.*	*P-value*	*s.d.*
G3756C	0.432	0.424	1.000	0.000
A3788G	0.297	0.294	1.000	0.000
T3849C*	0.108	0.151	0.197	0.000
G3853A*	0.216	0.237	0.503	0.000
C3913T*	0.432	0.444	1.000	0.000
C3915T	0.432	0.444	1.000	0.000

Table 5. Hardy-Weinberg Equilibrium for Caucasian American Population: Observed and expected heterozygosities were compared for each SNP. No significant departures from HWE were detected using Fisher's Exact test.

'*' Novel SNP

Locus	*Obs.Het.*	*Exp.Het.*	*P-value*	*s.d.*
G3756C	0.240	0.274	0.484	0.000
G3853A*	0.120	0.184	0.197	0.000
C3913T*	0.240	0.274	0.484	0.001
C3915T	0.240	0.274	0.484	0.001

Table 6. Haplotype frequency distribution among the samples: Relative and absolute frequencies of the 8 haplotypes for African American (AA) and Caucasian American (CA) populations.

Haplotype ID	Haplotype definition	Relative Frequencies		Absolute Frequencies		
		AA	*CA*	*AA*	*CA*	**Total**
h1	**GATGCC**	0.405	0.740	30	37	*67*
h2	**CATGTT**	0.149	0.160	11	8	*19*
h3	**GGTGCC**	0.176	---	13	0	*13*
h4	**CATATT**	0.135	---	10	0	*10*
h5	**GACGCC**	0.081	---	6	0	*6*
h6	**GATACC**	---	0.100	0	5	*5*
h7	**GATGTT**	0.041	---	3	0	*3*
h8	**CATGCC**	0.014	---	1	0	*1*
Total				**74**	**50**	**124**

Table 7. The table shows association of the SNPs with baseline systolic, diastolic and mean blood pressures. The numbers in parentheses after the p values indicate the groups being compared with 0 being wild type, 1 being heterozygotes and 2 being homozygotes for the SNP.

SNP (mRNA pos)	TEST	SBP	DBP	MAP
G3756C	**ANOVA**	**ns**	**0.0192**	**0.0381**
	Student's t	*ns*	*0.0058 (1 2)*	*0.0211(1 2)*
G3853A	**ANOVA**	**ns**	**ns**	**ns**
	Student's t	*ns*	*0.0441 (0 2)*	*ns*
C3913T	**ANOVA**	**0.0158**	**0.0029**	**0.0037**
	Student's t	*0.0049 (1 2)*	*0.0011 (1 2)*	*0.0012 (1 2)*
	Student's t	*0.0193 (0 2)*	*0.0026 (0 2)*	*0.0042 (0 2)*
C3915T	**ANOVA**	**0.0158**	**0.0029**	**0.0037**
	Student's t	*0.0049 (1 2)*	*0.0011 (1 2)*	*0.0012 (1 2)*
	Student's t	*0.0193 (0 2)*	*0.0026 (0 2)*	*0.0042 (0 2)*

Table 8. Association of haplotypes of the *ATP1a2* gene with baseline blood pressure. One-way ANOVA was used to compare means. The asterisks and daggers refer to pairs of groups compared using t-tests and the corresponding p-values.

African Americans								
Haplo-type	**Copy #**	**N**	**SBP**		**DBP**		**MAP**	
			Mean (s.d.)	**P-value**	**Mean (s.d.)**	**P-value**	**Mean (s.d.)**	**P- value**
GGCC	0	4	153.61*† (17.75)	**0.0113**	83.61*† (5.32)	**0.0014**	106.94 (8.91) *†	**0.0019**
	1	10	123.38* (14.23)	*0.0031*	64.29* (9.55)	*0.0003*	83.99 (10.48) *	*0.0005*
	2	7	131.56 † (14.58)	*†0.0306*	69.11† (3.74)	*†0.0059*	89.92 (6.76)†	*†0.0082*
CATT	0	14	129.30 (16.70)	**ns**	67.60* (9.49)	**ns**	88.16 (11.33)	**ns**
	1	6	134.07 (21.85)		70.98 (9.48)	*0.0441*	92.01 (12.95)	
	2	1	154.61		88.85*		110.77	
GGTT	0	19	128.55 * (3.51)	**0.0065**	68.14* (9.51)	**0.043**	88.28 (10.73) *	**0.0134**
	1	2	163.37* (10.83)	*0.0065*	83.16* (4.73)	*0.043*	109.90 (9.42)*	*0.0134*
CGCC	0	20	132.67 (18.33)	**ns**	70.40* (9.65)	**ns**	91.16 (11.98)	**ns**
	1	1	115.77		52.99*	*0.0471*	73.91	
Caucasian Americans								
GGCC	0	1	113.86*	**ns**	54.71	**ns**	74.42*	**ns**
	1	4	126.68 (3.72)	*0.0443*	63.23 (5.93)		84.38 (4.38)	*0.0492*
	2	11	130.02* (7.66)		65.91 (9.36)		87.28* (6.01)	

CHAPTER 5

PHYSIOLOGIC PHENOTYPE OF POLYMORPHISMS OF THE *ATP1a2* GENE IN AFRICAN AMERICANS AND CAUCASIAN AMERICANS: RELATION TO HYPERTENSION

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ABSTRACT

Background. The Na^+-K^+ ATPase is a key determinant of blood pressure homeostasis. Restriction fragment length polymorphisms (RFLPs) in the 3' untranslated (UTR) region of the *ATP1a2* gene that encodes the α2 catalytic subunit have been reported to be associated with hypertension [Glenn *et al, Am J Epidemiol 153: 537-545, 2001*]. In a study of healthy normotensive subjects, 6 single nucleotide polymorphisms (SNPs) were discovered in a 200 base pair region in the 3'-UTR of this gene. Four of these SNPs (at mRNA positions 3756, 3853, 3913 and 3915) and haplotypes constructed from these SNPs (particularly the ancestral GGCC haplotype and the haplotypes CGTT and CATT) appeared to significantly influence baseline blood pressure in an ethnic-specific manner. The follow-up study was conducted to determine whether the differences in basal blood pressure were associated with altered reflex control mechanism of blood pressure, endothelial dysfunction and/or altered basal tone in the vasculature.

Methods. Responses of cardiovascular variables (blood pressure, heart rate, muscle sympathetic nerve activity, cardiac output and systemic vascular resistance) to pressor stimuli of two different origins (cold pressor and hypoxic apnea) were studied in 37 individuals. Endothelial function was tested using ultrasound-aided measures of brachial artery flow-mediated dilation.

Results. Responses of systolic and diastolic blood pressures, muscle sympathetic nerve activity and systemic vascular resistance to the pressor stimuli were not different across haplotype ($p \geq 0.61$). Similarly, no endothelial dysfunction was present in individuals carrying the SNPs associated with higher baseline blood pressure levels ($p \geq$

0.56). Haplotype groups associated with higher baseline blood pressure tended to have higher systemic vascular resistance (SVR), suggesting increased vascular tonicity as a likely primary mechanism for the higher blood pressure levels.

Conclusions. These results suggest that although the SNPs influence baseline blood pressure levels at a young age, neither the responses to pressor stimuli nor endothelial function are affected; thus, collectively, these data suggest that these SNPs and haplotypes are associated with an increased arterial pressure that is likely mediated by an increased vascular tone.

INTRODUCTION

Hypertension is a major public health problem associated with long-term complications affecting the heart, kidneys and nervous system. Hypertension represents a major burden on the health care industry in terms of direct and indirect costs. The majority of cases of hypertension are idiopathic and genetic mechanisms are strongly suspected in many cases. Genetic influences on hypertension are supported by the observations that offspring and siblings of hypertensive individuals have greater susceptibility to hypertension than do relatives of non-hypertensive individuals (4, 7, 16, 31, 34). Hypertension is also subject to racial influences and is more prevalent in the African American (AA) population than in Caucasian Americans (CA) (1). The *ATP1a2* gene is among the genes with mutations that have been implicated in hypertension. The gene encodes the α_2 subunit of the Na^+-K^+-ATPases, a family of plasma membrane enzymes present in all cells in the body. Because the Na^+-K^+-ATPases are responsible for maintaining ionic gradients across the cell membrane, they regulate cell volume, membrane potentials, and secondary active transport of solutes. They consume 20-30% of cellular ATP for the vectoral transport of Na^+ and K^+ across the membrane. Na^+-K^+-ATPases are heterodimers composed of a catalytic alpha subunit and a smaller beta subunit. Because there are 4 isoforms of the α subunit (α_1- α_4) and 3 isoforms of the β subunit (β_1- β_3), there are 12 possible isozymes of the pump that appear to be expressed in a tissue specific manner. Na^+-K^+-ATPases with the α_2 subunit are prevalent in skeletal muscle, vascular smooth muscle, and glia (15, 24, 33, 46-48). Mutations in the *ATP1a2*

gene can potentially affect the function of the pump in these tissues and thus alter blood pressure via central or end-organ mechanisms.

A number of restriction fragment length polymorphisms (RFLPs) and single nucleotide polymorphisms (SNPs) are known to occur in both the coding and non-coding regions of the Na^+-K^+-ATPase genes (15, 36). Rankinen *et al* (36) demonstrated that an RFLP in the *ATP1a2* gene is associated with higher resting systolic blood pressure in healthy Canadians. Two years later, Glenn *et al* (15) demonstrated that the same RFLP influences the association between blood pressure and blood lead levels. They also reported that the prevalence of this polymorphism is higher in populations of African descent in whom the prevalence of hypertension is also high (15, 36). Since then, there have been several murine studies (8, 10, 19, 49, 57) that have established the cardinal role of this gene in blood pressure regulation. The genetic variation studied by Rankinen *et al* (36) and Glenn *et al* (15) was localized in the 3' untranslated region (3' UTR) of the *ATP1a2* gene. Whether this region has a functional role in blood pressure control is not currently known. No human studies comparing physiologic responses in individuals having different genetic variations in this region have been reported. However, as reported elsewhere for a cohort of Caucasian American and African American subjects, SNPs (and haplotypes constructed from the SNPs) in this region were associated with differential baseline blood pressure levels in an ethnic-specific manner (Thakre *et al*, unpublished report). As a result, the same subject population was evaluated to determine if blood pressure control mechanisms are altered in subjects with selected SNPs in the 3'-UTR of the *ATP1a2* gene by investigating whether a) the SNPs and haplotypes are

associated with differential cardiovascular responses to pressor stimuli and b) the SNPs and haplotypes are associated with endothelial dysfunction

MATERIALS AND METHODS

Study Subjects. Thirty-seven subjects volunteered for the study. These included 21 AAs (12 females and 9 males) and 16 CAs (10 females and 6 males). The mean age of the subjects was 23.95 ± 5.43 years. The clinical information collected on these subjects included history of sleep apnea, smoking and detailed family history of hypertension, family history of diabetes mellitus; and measurement of weight (to the closest 50g), height (to the closest mm) and body mass index (BMI). Ethnicity was self-reported. All of these subjects were non-hypertensive, non-diabetic and reported no history of cardiovascular, pulmonary or neurologic disease. None were currently using medications other than oral contraception. Urinary human chorionic gonadotropin was evaluated to rule out pregnancy in female subjects and cardiovascular function was not tested during menses to reduce the variance associate with changes in fluid volume. Subjects refrained from vigorous exercise and alcohol for 24 hours and caffeine for 12 hours prior to the start of the study. The study was approved by the Institutional Review Board of the University of North Texas Health Science Center, Fort Worth, Texas, USA.

***ATP1a2* genotyping.** DNA was organically extracted from the WBCs from the blood samples and from the cells from the buccal swabs with Phenol-chloroform-isoamyl alcohol followed by ethanol precipitation. Sequencing was performed on the plus and

minus strands using either the forward primer 5'-GCCTGAGACTGGAAAAGGTG-3' or reverse primer 5'-TGGAGCTCTGAGGACTTAGTATTACA-3'. Cycle sequencing was performed in a GeneAmp® PCR System 9700 thermal cycler using 0.8uL BigDye Terminator v3.1 Cycle Sequencing Ready Reaction mix (Applied Biosystems, USA), 1.6uL BigDye® Terminator v1.1 & v3.1 5X Sequencing Buffer (Applied Biosystems, USA), 1μL 3.3μM primer, 3μL PCR product, and 3.6μL molecular grade water. Cycling conditions consisted of 25 cycles: 96°C denature 10 sec, 50°C anneal 5 sec, 72°C extension 30 sec. DNA samples were sequenced in an ABI PRISM® 3100 sequencer (Applied Biosystems, USA). The sequence was analyzed and aligned using Sequencing Analysis Software v5.2 (Applied Biosystems, USA) and Sequencher™ v4.2 (Gene Codes Corporation, USA).

Experimental protocol for pressor responsiveness testing. The experimental protocol is depicted in **Figure 1A**. The experiments were performed in a semi-recumbent position. After voiding, subjects were instrumented for measurement of heart rate (HR), arterial blood pressure, respiratory function, arterial oxygen saturation (Sa_{O2}) and muscle sympathetic nerve activity (MSNA). Heart rate was measured using standard limb-lead electrocardiography (ECG). Blood pressure was measured using finger photoplethysmography (Finometer model 1, Finapres Medical Systems BV, Amsterdam, Netherlands). The measurements were made continuously throughout the duration of the experiment, with a few breaks during baseline periods. Respiration was monitored using an elastic respiratory monitoring band placed around the subject's abdomen (Grass

Instruments, West Warwick, RI), allowing the investigator to confirm that apnea was initiated at end-expiration. The breathing circuit consisted of a nasal mask, a 3-way Rudolph valve, and Douglas bags which were filled with gas containing the various concentrations of oxygen used for administering hypoxia. Arterial oxygen saturation was assessed by pulse oximetry at the finger (DS-100A Durasensor, Nellcor Puritan Bennett Inc., Pleasanton, CA). Muscle sympathetic nerve activity was directly measured from the peroneal nerve either at the popliteal fossa or at the fibular head using standard microneurographic techniques (51, 52). Two sterile tungsten microelectrodes (tip diameter 5-10 μm, length 35 mm, Frederick Haer and Co., Bowdoinham, ME) were inserted with one serving as a reference and the other inserted into the peroneal nerve to measure MSNA. Nerve signals were processed by a preamplifier and an amplifier (Nerve Traffic Analyzer Model 662C-3, Department of Bioengineering, University of Iowa, Iowa City, IA) with a total gain of 99,000. Amplified signals were band-pass filtered (700-2,000 Hz), rectified and discriminated. Finally, a resistance-capacitance (RC) circuit with a time constant of 0.1 s was used to integrate the raw nerve signals. Sympathetic nerve recordings were confirmed using the following criteria: 1) pulse-synchronous bursts occurring 1.2-1.4 s after the associated QRS complex, 2) reproducible activation during phase II and III of the Valsava maneuver, and 3) no activation following a pinch, skin stroking, or startle stimuli (all of which activate skin sympathetic fibers).

The subject responses to two different pressor stimuli were assessed: hypoxic apnea and cold pressor test. During the hypoxic apnea stimulus, subjects breathed for 1

minute through a face mask connected to Douglas bags containing air with different concentrations of oxygen. At the end of the minute, the subjects held their breath at end-expiration for 20s. During the cold pressor stimulus, subjects immersed one hand for 2 minutes in cold water baths with one of three set temperatures. Each stimulus was applied in three bouts of different intensities – Hypoxic apnea was conducted with 12%, 16%, and 21% oxygen in the inspired air and the cold pressor stimulus was applied at 2°, 10°, and 18°C. Subjects were permitted 5 and 15 minutes was used in between the bouts of hypoxic apnea and cold pressor stimuli, respectively to recover. The order of the six experimental conditions was randomized for each subject. Representative responses of Blood pressure, MSNA and HR to the two stimuli are depicted in **Figure 2**.

Experimental protocol for endothelial dysfunction. The testing protocol is depicted in **Figure 1B**. The testing was conducted in a supine position in a quiet laboratory setting at an ambient temperature of 23-24 ^{0}C. All studies were performed at the same time of the day. The subjects were connected to an electrocardiogram and made to lie supine on an examination table. The subjects' non-dominant arm was rested on a side table on a pillow and secured in place by an orthopedic pillow that was shaped around the arm (vac-pac). A small occlusion cuff was placed around the subjects' forearm. Using B-mode ultrasonography (Philips HDI 5000) with a broadband linear array transducer (L7-4), the subject's brachial artery was identified about 3-5 cm above the elbow and the probe was fixed in place at the point of maximum clarity using a custom designed mechanical stand with a moving mechanical arm. The images were

recorded digitally. After instrumentation, a standard flow-mediated dilation procedure was performed (12). After a 15 minute rest period, the forearm cuff was inflated to a pressure 50 mm Hg above the subjects' systolic blood pressure. The cuff was left inflated for 5 minutes and then released. Images were saved every 15 seconds for 2 minutes beginning with the release of the cuff. After another 15 minute baseline period, the procedure was repeated. Brachial artery diameter was measured at 3 locations along the length of the artery and all subsequent measurements in each subject were done at the same points in the artery for comparison.

Statistical Analysis. Baseline cardiovascular variables were compared in the two races using a Student's t-test. Baseline cardiovascular variables by genotype were compared using a Student's t-test and a one-way analysis of variance (ANOVA). Association of the haplotypes of the *ATP1a2* gene with responses of HR, BP and MSNA to the pressor stimuli was examined separately in AAs and CAs by using a two-way ANOVA. Association of haplotypes with change in brachial artery diameters to test for presence of endothelial dysfunction was tested using a one-way ANOVA. Slopes of the responses to the pressor stimuli were derived using linear regression. All values are expressed as means ± S.D. For all statistical analysis, significance was set at an α-error rate of 0.05.

RESULTS

Race and baseline cardiovascular variables. Table 1 shows baseline cardiovascular variables in the two races. Baseline Systolic blood pressure, Mean arterial pressure, MSNA and cardiac output were not different in the two races ($p \geq 0.16$). Baseline heart rates were significantly higher in AAs than in CAs ($p = 0.01$). Disatolic blood pressure tended to be higher in AAs than in CAs, although the difference did not reach statistical significance ($p = 0.08$).

To facilitate the following discussion of the results, individuals with 2 copies of the ancestral haplotype ($G_{3756}G_{3853}C_{3913}C_{3915}$) are wild type (WT), individuals with one copy are heterozygous (MT 1) and individuals with zero copies are homozygous (MT 2). For the $C_{3756}G_{3853}T_{3913}T_{3915}$ haplotype, individuals with 2 copies are homozygotes (MT 2), individuals with one copy are heterozygotes (MT 1) and individuals with zero copies are wild type (WT).

Baseline values and responses of cardiovascular variables by the GGCC haplotype. In AAs, baseline HR and CO were not significantly different in the WT, MT 1 or MT 2 groups. Muscle sympathetic nerve activity tended to be higher in the MT 2 group as compared to the MT 1 and WT groups ($p = 0.06$). Baseline Systemic vascular resistance (SVR) tended to be higher in the homozygous mutant group as compared to the heterozygous and wild type groups ($p = 0.08$, **Figure 3**). **Figures 4-6** and **Table 2** show the responses to pressor stimuli. In response to either pressor stimulus, SBP, DBP, HR and MSNA increased by similar amounts in the WT, MT 2 and MT 1 groups although the

absolute values reached were higher for SBP, DBP and MSNA in the homozygous group as compared to the heterozygous and wild type groups ($p > 0.17$). In CAs, baseline HR ($p = 0.45$) and MSNA ($p = 0.63$) were not different in the wild type and heterozygous groups (data not presented). Similarly changes in SBP, DBP and MSNA were not different in WT and MT 1 groups in response to the pressor stimuli in CAs ($p \geq 0.10$).

Baseline values and responses of cardiovascular variables by the CGTT haplotype. In AAs, baseline HR ($p = 0.8039$) and CO ($p = 0.5127$) were not different in WT, MT 1 and MT 2 groups. Muscle sympathetic nerve acitivity tended to be higher in the MT 2 group as compared to WT and MT 1 groups ($p = 0.0687$). **Table 3** shows the responses of SBP and DBP to pressor stimuli in the two races. In response to the pressor stimuli, SBP and DBP reached higher values in the MT 2 group as compared to the WT and MT 1 groups. However, changes from baseline were similar in the three groups ($p \geq 0.14$). In CAs, baseline HR ($p = 0.3468$) and MSNA ($p = 0.8115$) were not significantly different in the WT and MT 1 groups. Responses of HR, MSNA, SBP and DBP to either the cold pressor or the hypoxic apnea stimulus were not different in the wild type and heterozygous groups ($p \geq 0.26$).

Flow-mediated dilatation. Flow-mediated dilatation (FMD) tended to be higher in CAs as compared to AAs, although the difference was not statistically significant (8.49 ± 2.25 % in CAs vs. 7.07 ± 2.69 % in AAs, $p = 0.0874$). In AAs, WT, MT 1 and MT 2 groups for the GGCC haplotype had similar FMD (**Figure 7A**). Flow-mediated dilation

was also similar in the WT, MT 2 and MT 1 groups for the CGTT haplotype ($p = 0.2996$). In CAs, WT, MT 1 and MT 2 groups for the GGCC haplotype had similar FMD ($p = 0.565$, **Figure 7B**). FMD was also similar in the WT and MT 1 groups for the CGTT haplotype ($p = 0.779$). Thus, neither race nor genotype was associated with endothelial dysfunction in this sample of individuals.

DISCUSSION

In accord with the findings of Rankinen *et al* (36), selected 3'-UTR SNPs of the *ATP1a2* gene are associated with differences in baseline blood pressure. However, Rankinen *et al* found (36) this effect for systolic blood pressure only, whereas the current findings suggest that diastolic blood pressure is also elevated. The current study was designed to test whether these differences in basal blood pressure are associated with impairment of neural reflex control or endothelial function. Although the absolute BP achieved during the pressor stimuli was greater in the groups associated with higher baseline BP, the physiologic response was the same, suggesting that reflex control of blood pressure was normal. In addition, neither the haplotypes of the *ATP1a2* gene nor race was associated with endothelial dysfunction. Therefore, people with these particular haplotypes have higher blood pressure that is likely due to increased basal vascular tone and/or cardiac contractility independent of neural, humoral or endothelial modulation. In this context, the connection between the Na^+-K^+-ATPase and the Na^+-Ca^{++}-exchanger is important. Depression in activity of the former pump decreases the capability of the latter to extrude Ca^{++} out of the cell and leads to an increased intracellular Ca^{++} concentration.

Higher intracellular Ca^{++}may lead to an increased baseline vascular tone and consequent increase in baseline blood pressures. Indeed, the MT 2 group for the GGCC haplotype was associated with a higher basal SVR, suggesting an increased vascular tone. Future studies with a larger number of subjects are needed to address whether this is the operating mechanism for the association of these SNPs with increased baseline blood pressure.

The reflex pathway. Blood pressure is determined and regulated by vascular tone, cardiac output and by neurohumoral control pathways. Changes in sympathetic nerve activity affect blood pressure by affecting cardiac function and systemic vascular resistance. The reflex pathway can be activated by pressor stimuli and leads to a subsequent increase in blood pressure. The cold pressor test is a potent pressor stimulus leading to an increase in blood pressure and MSNA (11, 39, 53). Victor *et al* (53) reported a positive correlation between increases in MSNA and blood pressure and between increase in MSNA and plasma venous norepinepinephrine levels during the cold pressor test, suggesting an elevated neurohumoral discharge as the primary mechanism for elevation of blood pressure. They used ice-cold water in their studies (equivalent to the 2^0 C level cold pressor in the current study). In the current study, we used 3 levels of cold pressor and found a graded response of MSNA and blood pressure to the stimulus. Kregel *et al* (20) reported that increases in MSNA were correlated with the level of pain sensation being perceived by the subjects and suggest that the stimulation of MSNA is primarily due to stimulation of high-threshold, cold-pain (primarily C) fibers. Lafleche *et*

al (21) have found an exaggerated blood pressure response to cold pressor stimulus in borderline hypertensives when compared to normal controls. Miyajima *et al* reported exaggerated MSNA and blood pressure responses to the cold pressor stimuli in borderline hypertensives as compared to normal controls (29). Borderline hypertensives also have greater increases in SVR than normotensive controls during the cold pressor test (40), suggesting an increased vascular reactivity as the mechanism for exaggerated blood pressure responses. Hypoxia serves as a potent pressor stimulus acting via the peripheral chemoreceptors located in the carotid and aortic bodies responding primarily to hypoxia (14) and eliciting increases in MSNA and SVR (6), with consequent increases in BP (42, 45). Morgan *et al* (30) and Xie *et al* (55) reported that hypoxia significantly raises MSNA leading to elevation in blood pressure. Apnea superimposed on top of hypoxia removes the pulmonary-afferent mediated inhibition of sympathetic activity and augments the MSNA and BP response to hypoxia (9, 43). Several studies have shown exaggerated increases in MSNA and BP in response to hypoxic apnea in sleep apnea patients (who have a high propensity for developing hypertension) as compared to non-sleep apneics (37, 41, 45). The chemoreceptor reflex is exaggerated in spontaneously hypertensive rats (13) and in hypertensive humans (50), suggesting that this reflex may be involved in the pathophysiology of essential hypertension. Borderline hypertensives also have been reported to have exaggerated MSNA responses to hypoxia (43, 44), thus suggesting an abnormality in the reflex control pathway. Thus, responses to cold pressor test and hypoxic apnea serve as valid measures of testing the reflex control of blood pressure.

Exaggerated responses to either or both of these stimuli point toward an abnormality in this reflex control mechanism of blood pressure.

In this study, although the absolute values of BP in response to pressor stimuli reached in those with higher baseline blood pressure were higher than those reached in other groups, the change from baseline was not higher. This implies that the responses to pressor stimuli were not exaggerated in these individuals. Thus, although the baseline blood pressure was higher in these individuals, the reflex pathway for blood pressure control was not affected. The cold pressor test and hypoxic apnea are pressor stimuli having different afferent pathways leading to the brain stem. Since both stimuli failed to elicit exaggerated responses, it is likely that the afferent arms are not affected and that the brainstem or the efferent arm of the pathway (including central nervous discharge and end-organ reactivity) are reacting in similar manner to the two stimuli.

Endothelial dysfunction. The endothelium is an important controller of vessel diameter and blood flow. The endothelium synthesizes both potent vasodilator and vasoconstrictor substances (27, 28, 56). Nitric oxide is an important vasodilator secreted by the endothelium in response to many different stimuli including high flow states and shear stress (54). Nitric oxide acts on the vascular smooth muscle cells and causes G-protein mediated vasodilation (54). Endothelial health is usually evaluated by occluding the forearm conduit artery (brachial artery) by inflating a cuff around the forearm to pressures above the systolic blood pressure and then releasing the cuff to produce a rapid increase in blood flow through the vessel which in turn results in shear stress-mediated

nitric oxide release and subsequent vasodilation (54). Endothelial dysfunction can be tested using ultrasound-derived measurements of the vessel lumen diameter pre- and post-occlusion. This ultrasound-aided evaluation of flow-mediated vasodilation via nitric oxide pathway is an important measure of endothelial function. Flow-mediated dilation is expressed as a percentage of increase in vessel diameter over baseline values. Endothelial dysfunction is an important marker of hypertension. Failure to vasodilate appropriately in normotensive individuals may point toward a propensity to develop hypertension later in life (5). Hypertensives have a reduced nitric oxide synthesis and flow-mediated dilation in response to post occlusion reperfusion (25, 32, 35). There is evidence that the endothelial dysfunction associated with hypertension may represent the earliest stage of target organ damage (3, 18, 23). Endothelial function evaluation in this cohort of subjects was conducted to test whether higher basal BP in young individuals is associated with an early manifestation of endothelial dysfunction. If present, endothelial dysfunction may be the harbinger of abnormalities in the control of blood pressure later in life. None of the haplotypes of the *ATP1a2* gene investigated in this study or race was associated with endothelial dysfunction. This implies that the blood pressure differences in these individuals are not explained by a decline in flow-mediated vasodilation.

Limitations. The following limitations restrict the scope of conclusions from these data. First, the number of subjects studied is small. For genotyping individuals and comparing physiologic responses in various groups, a larger number of subjects need to be studied. Nevertheless, even with a relatively limited number of subjects, the results

suggest that these genotypes affect baseline blood pressures. Second, only young individuals were studies. The youth and general good health of the subjects might explain the similar responses to pressor stimuli that were seen across various genotype groups. Also, the absence of endothelial dysfunction in these individuals could be related to their young age. Importantly, these data demonstrate that these genotypes are not associated with a primary dysfunction of reflex control of blood pressure or endothelial mechanisms. Therefore, studies need to be conducted in older subjects to test whether responses to pressor stimuli are different across genotype groups and whether endothelial dysfunction is associated with particular genotypes. Third, normotensive individuals were studied. Studies comparing prevalences of these SNPs in hypertensives and normotensives need to be pursued. Also, studies are needed to test whether pressor responsiveness is different within hypertensives depending upon their genotype. However, these findings are important in identifying a basal change in blood pressure prior to the anticipated development of frank hypertension in these young subjects. Fourth, the reason to examine the putative association between these *ATP1a2* haplotypes and response to stressor stimuli was to assess for clues to an altered risk of future hypertension. Lack of a longitudinal follow-up in our study does not permit us to directly address this objective and, based on our results, the question of future risk of hypertension remains open. However, considering the knowledge that subjects with high baseline systolic blood pressure are also likely to show higher age-related increase in blood pressure (26, 36), it appears plausible that *ATP1a2* haplotypes may play a causal role in the development of hypertension by changing the baseline blood pressure.

Future directions. As mentioned above, studies are needed to test older subjects and hypertensives. Also, the prevalence of these SNPs in families and association with higher incidence of hypertension in family members needs to be studied. Also, a larger number of subjects need to be studied to test the association of genotype with CO and peripheral vascular resistance. Also future studies need to better characterize the cellular and physiological mechanisms that can contribute to the effects of the SNPs of the *ATP1a2* gene on expression of the pump and thereby baseline blood pressure.

Conclusion. That the baseline blood pressures are altered by genotype but the reflex pathway to pressor stimuli is not, points toward an alteration in vascular (end-organ) function or tone. This may reflect a phase of developing hypertension in that an early alteration in baseline blood pressure is followed by impairment in the reflex pathway and endothelial dysfunction, which in turn facilitate the progression to essential hypertension. The most important observation in this study was the striking difference in the pattern of association of the $G_{3756}G_{3853}C_{3913}C_{3915}$ (wild type) haplotype with baseline blood pressure in the two ethnic groups studied. Race is a compound construct with strong genetic undertones and is known to be an important modifier of blood pressure homeostasis (2, 26). The seemingly perplexing interaction between ethnicity and *ATP1a2* haplotypes that we observed in our study can be partly examined by a potential interaction of the *ATP1a2* haplotypes with one or more of the race-specific polymorphisms (17) that are known to play a critical role in the control of blood pressure in humans. The post-genomics era is expected to be beneficial for management of

diseases by permitting personalized care (22, 38). The results of study point to the possibility that the management of human hypertension may, in future, need refinement based on the knowledge of an individual's *ATP1a2* genotype and ethnic background. Future studies are required to better delineate the effects of polymorphism of this gene on blood pressure control mechanisms.

REFERENCES

1. http://www.cdc.gov.

2. **Brownley KA, Hurwitz BE, and Schneiderman N.** Ethnic variations in the pharmacological and nonpharmacological treatment of hypertension: biopsychosocial perspective. *Hum Biol* 71: 607-639, 1999.

3. **Cardillo C, Kilcoyne CM, Cannon RO, 3rd, and Panza JA.** Impairment of the nitric oxide-mediated vasodilator response to mental stress in hypertensive but not in hypercholesterolemic patients. *J Am Coll Cardiol* 32: 1207-1213, 1998.

4. **Celentano A, Galderisi M, Garofalo M, Mureddu GF, Tammaro P, Petitto M, Di Somma S, and de Divitiis O.** Blood pressure and cardiac morphology in young children of hypertensive subjects. *J Hypertens Suppl* 6: S107-109, 1988.

5. **Clarkson P, Celermajer DS, Powe AJ, Donald AE, Henry RM, and Deanfield JE.** Endothelium-dependent dilatation is impaired in young healthy subjects with a family history of premature coronary disease. *Circulation* 96: 3378-3383, 1997.

6. **Cooper VL, Pearson SB, Bowker CM, Elliott MW, and Hainsworth R.** Interaction of chemoreceptor and baroreceptor reflexes by hypoxia and hypercapnia - a mechanism for promoting hypertension in obstructive sleep apnoea. *J Physiol* 568: 677-687, 2005.

7. **Corvol P, Jeunemaitre X, Charru A, and Soubrier F.** Can the genetic factors influence the treatment of systemic hypertension? The case of the renin-angiotensin-aldosterone system. *Am J Cardiol* 70: 14D-20D, 1992.

8. **Dostanic-Larson I, Van Huysse JW, Lorenz JN, and Lingrel JB.** The highly conserved cardiac glycoside binding site of Na,K-ATPase plays a role in blood pressure regulation. *Proc Natl Acad Sci U S A* 102: 15845-15850, 2005.

9. **Eckberg DL, Nerhed C, and Wallin BG.** Respiratory modulation of muscle sympathetic and vagal cardiac outflow in man. *J Physiol* 365: 181-196, 1985.

10. **Efendiev R, Krmar RT, Ogimoto G, Zwiller J, Tripodi G, Katz AI, Bianchi G, Pedemonte CH, and Bertorello AM.** Hypertension-linked mutation in the adducin alpha-subunit leads to higher AP2-mu2 phosphorylation and impaired Na+,K+-ATPase trafficking in response to GPCR signals and intracellular sodium. *Circ Res* 95: 1100-1108, 2004.

11. **Fagius J, Karhuvaara S, and Sundlof G.** The cold pressor test: effects on sympathetic nerve activity in human muscle and skin nerve fascicles. *Acta Physiol Scand* 137: 325-334, 1989.

12. **Faulx MD, Wright AT, and Hoit BD.** Detection of endothelial dysfunction with brachial artery ultrasound scanning. *Am Heart J* 145: 943-951, 2003.

13. **Fukuda Y, Sato A, and Trzebski A.** Carotid chemoreceptor discharge responses to hypoxia and hypercapnia in normotensive and spontaneously hypertensive rats. *J Auton Nerv Syst* 19: 1-11, 1987.

14. **Garcia-Rio F, Racionero MA, Pino JM, Martinez I, Ortuno F, Villasante C, and Villamor J.** Sleep apnea and hypertension. *Chest* 117: 1417-1425, 2000.

15. **Glenn BS, Stewart WF, Schwartz BS, and Bressler J.** Relation of alleles of the sodium-potassium adenosine triphosphatase alpha 2 gene with blood pressure and lead exposure. *Am J Epidemiol* 153: 537-545, 2001.

16. **Grobbee DE.** Predicting hypertension in childhood: value of blood pressure measurement and family history. *J Am Coll Nutr* 11 Suppl: 55S-59S, 1992.

17. **Halushka MK, Fan JB, Bentley K, Hsie L, Shen N, Weder A, Cooper R, Lipshutz R, and Chakravarti A.** Patterns of single-nucleotide polymorphisms in candidate genes for blood-pressure homeostasis. *Nat Genet* 22: 239-247, 1999.

18. **Kelm M, Preik M, Hafner DJ, and Strauer BE.** Evidence for a multifactorial process involved in the impaired flow response to nitric oxide in hypertensive patients with endothelial dysfunction. *Hypertension* 27: 346-353, 1996.

19. **Kim JS, Choi KC, Jeong MH, Kim SW, Oh YW, and Lee JU.** Increased expression of sodium transporters in rats chronically inhibited of nitric oxide synthesis. *J Korean Med Sci* 21: 1-4, 2006.

20. **Kregel KC, Seals DR, and Callister R.** Sympathetic nervous system activity during skin cooling in humans: relationship to stimulus intensity and pain sensation. *J Physiol* 454: 359-371, 1992.

21. **Lafleche AB, Pannier BM, Laloux B, and Safar ME.** Arterial response during cold pressor test in borderline hypertension. *Am J Physiol* 275: H409-415, 1998.

22. **Landon MR.** Ethics and policy perspectives on personalized medicine in the post-genomic era. *J Biolaw Bus* 8: 28-36, 2005.

23. **Lapu-Bula R and Ofili E.** From hypertension to heart failure: role of nitric oxide-mediated endothelial dysfunction and emerging insights from myocardial contrast echocardiography. *Am J Cardiol* 99: 7D-14D, 2007.

24. **Lecuona E, Luquin S, Avila J, Garcia-Segura LM, and Martin-Vasallo P.** Expression of the beta 1 and beta 2(AMOG) subunits of the Na,K-ATPase in neural tissues: cellular and developmental distribution patterns. *Brain Res Bull* 40: 167-174, 1996.

25. **Linder L, Kiowski W, Buhler FR, and Luscher TF.** Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation in vivo. Blunted response in essential hypertension. *Circulation* 81: 1762-1767, 1990.

26. **Livshits G, Ginsburg E, and Kobyliansky E.** Heterogeneity of genetic control of blood pressure in ethnically different populations. *Hum Biol* 71: 685-708, 1999.

27. **Luscher TF.** Endothelium-derived relaxing and contracting factors: potential role in coronary artery disease. *Eur Heart J* 10: 847-857, 1989.

28. **Luscher TF, Yang ZH, Diederich D, and Buhler FR.** Endothelium-derived vasoactive substances: potential role in hypertension, atherosclerosis, and vascular occlusion. *J Cardiovasc Pharmacol* 14 Suppl 6: S63-69, 1989.

29. **Miyajima E, Yamada Y, Matsukawa T, Tochikubo O, Ishii M, and Kaneko Y.** Neurogenic abnormalities in young borderline hypertensives. *Clin Exp Hypertens A* 10 Suppl 1: 209-223, 1988.

30. **Morgan BJ, Crabtree DC, Palta M, and Skatrud JB.** Combined hypoxia and hypercapnia evokes long-lasting sympathetic activation in humans. *J Appl Physiol* 79: 205-213, 1995.

31. **Neutel JM and Smith DH.** Hypertension control: multifactorial contributions. *Am J Hypertens* 12: 164S-169S, 1999.

32. **Panza JA, Quyyumi AA, Brush JE, Jr., and Epstein SE.** Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N Engl J Med* 323: 22-27, 1990.

33. **Peng L, Martin-Vasallo P, and Sweadner KJ.** Isoforms of Na,K-ATPase alpha and beta subunits in the rat cerebellum and in granule cell cultures. *J Neurosci* 17: 3488-3502, 1997.

34. **Poulter NR.** Major risk factors for coronary heart disease in hypertension: implications for management. *J Hum Hypertens* 4 Suppl 3: 3-6, 1990.

35. **Preik M, Kelm M, Schoebel F, Schottenfeld Y, Leschke M, and Strauer BE.** Selective impairment of nitric oxide dependent vasodilation in young adults with hypercholesterolaemia. *J Cardiovasc Risk* 3: 465-471, 1996.

36. **Rankinen T, Perusse L, Deriaz O, Theriault G, Chagnon M, Nadeau A, and Bouchard C.** Linkage of the Na,K-ATPase alpha 2 and beta 1 genes with resting and exercise heart rate and blood pressure: cross-sectional and longitudinal observations from the Quebec Family Study. *J Hypertens* 17: 339-349, 1999.

37. **Rowell LB, Johnson DG, Chase PB, Comess KA, and Seals DR.** Hypoxemia raises muscle sympathetic activity but not norepinephrine in resting humans. *J Appl Physiol* 66: 1736-1743, 1989.

38. **Schunkert H and Mayer B.** In transition: from family history into the post-genomic era. *Am Heart J* 152: 204-206, 2006.

39. **Seals DR.** Sympathetic activation during the cold pressor test: influence of stimulus area. *Clin Physiol* 10: 123-129, 1990.

40. **Sherwood A, Hinderliter AL, and Light KC.** Physiological determinants of hyperreactivity to stress in borderline hypertension. *Hypertension* 25: 384-390, 1995.

41. **Smith ML, Niedermaier ON, Hardy SM, Decker MJ, and Strohl KP.** Role of hypoxemia in sleep apnea-induced sympathoexcitation. *J Auton Nerv Syst* 56: 184-190, 1996.

42. **Somers VK, Mark AL, and Abboud FM.** Interaction of baroreceptor and chemoreceptor reflex control of sympathetic nerve activity in normal humans. *J Clin Invest* 87: 1953-1957, 1991.

43. **Somers VK, Mark AL, and Abboud FM.** Potentiation of sympathetic nerve responses to hypoxia in borderline hypertensive subjects. *Hypertension* 11: 608-612, 1988.

44. **Somers VK, Mark AL, and Abboud FM.** Sympathetic activation by hypoxia and hypercapnia--implications for sleep apnea. *Clin Exp Hypertens A* 10 Suppl 1: 413-422, 1988.

45. **Somers VK, Mark AL, Zavala DC, and Abboud FM.** Contrasting effects of hypoxia and hypercapnia on ventilation and sympathetic activity in humans. *J Appl Physiol* 67: 2101-2106, 1989.

46. **Songu-Mize E, Liu X, Stones JE, and Hymel LJ.** Regulation of Na+,K+-ATPase alpha-subunit expression by mechanical strain in aortic smooth muscle cells. *Hypertension* 27: 827-832, 1996.

47. **Sweadner KJ.** Isozymes of the Na+/K+-ATPase. *Biochim Biophys Acta* 988: 185-220, 1989.

48. **Sweadner KJ, Herrera VL, Amato S, Moellmann A, Gibbons DK, and Repke KR.** Immunologic identification of Na+,K(+)-ATPase isoforms in myocardium. Isoform change in deoxycorticosterone acetate-salt hypertension. *Circ Res* 74: 669-678, 1994.

49. **Tian G, Dang C, and Lu Z.** The change and significance of the Na+-K+-ATPase alpha-subunit in ouabain-hypertensive rats. *Hypertens Res* 24: 729-734, 2001.

50. **Trzebski A, Tafil M, Zoltowski M, and Przybylski J.** Increased sensitivity of the arterial chemoreceptor drive in young men with mild hypertension. *Cardiovasc Res* 16: 163-172, 1982.

51. **Vallbo AB, Hagbarth KE, Torebjork HE, and Wallin BG.** Somatosensory, proprioceptive, and sympathetic activity in human peripheral nerves. *Physiol Rev* 59: 919-957, 1979.

52. **Vallbo AB, Hagbarth KE, and Wallin BG.** Microneurography: how the technique developed and its role in the investigation of the sympathetic nervous system. *J Appl Physiol* 96: 1262-1269, 2004.

53. **Victor RG, Leimbach WN, Jr., Seals DR, Wallin BG, and Mark AL.** Effects of the cold pressor test on muscle sympathetic nerve activity in humans. *Hypertension* 9: 429-436, 1987.

54. **Wennmalm A.** Endothelial nitric oxide and cardiovascular disease. *J Intern Med* 235: 317-327, 1994.

55. **Xie A, Skatrud JB, Puleo DS, and Morgan BJ.** Exposure to hypoxia produces long-lasting sympathetic activation in humans. *J Appl Physiol* 91: 1555-1562, 2001.

56. **Yang ZH, Diederich D, Schneider K, Siebenmann R, Stulz P, von Segesser L, Turina M, Buhler FR, and Luscher TF.** Endothelium-derived relaxing factor and protection against contractions induced by histamine and serotonin in the human internal mammary artery and in the saphenous vein. *Circulation* 80: 1041-1048, 1989.

57. **Zhang J, Lee MY, Cavalli M, Chen L, Berra-Romani R, Balke CW, Bianchi G, Ferrari P, Hamlyn JM, Iwamoto T, Lingrel JB, Matteson DR, Wier WG, and Blaustein MP.** Sodium pump alpha2 subunits control myogenic tone and blood pressure in mice. *J Physiol* 569: 243-256, 2005.

FIGURE LEGENDS

Figure 1. The experimental protocol used for pressor responsiveness testing and for testing endothelial function. **A.** Protocol for pressor responsiveness testing. 3 levels of hypoxic apnea (at 21%, 16% and 12% oxygen) and 3 levels of cold pressor test (at 2^0 C, 10^0 C and 18^0 C) were administered in a randomized order. **B.** Protocol for testing for endothelial dysfunction. Brachial artery images were obtained using ultrasound during baseline periods and after release of the occluding cuff.

Figure 2. The figure depicts responses of blood pressure, MSNA and heart rate to graded cold pressor (A) and hypoxic apnea (B) stimuli. BP, MSNA and HR show graded increases in response to the graded pressor stimuli.

Figure 3. The figure depicts baseline systemic vascular resistance in African Americans with 0 (MT 2), 1 (MT 1) or 2 (WT) copies of the ancestral (GGCC) haplotype. The red symbols with error bars are Mean ± S.D. SVR tended to be higher in the mutant homozygous group as compared to the heterozygous and wild type groups ($p = 0.079$).

Figure 4. The figure depicts responses of cardiovascular variables (as change from baseline levels) to the cold pressor test in African Americans with 0, 1 or 2 copies of the ancestral (GGCC) haplotype. **A.** Systolic Blood Pressure response. **B.** Diastolic Blood Pressure response. **C.** Heart rate response. **D.** MSNA response. 0 – mutant homozygote, 1 – mutant heterozygote, 2 – wild type. CP 2 – cold pressor test at 2^0 C, CP 10 – cold

pressor test at 10^0 C, CP 18 – cold pressor test at 18^0 C.All values are Mean ± S.D. None of the responses were significantly higher in any group ($p \geq 0.064$).

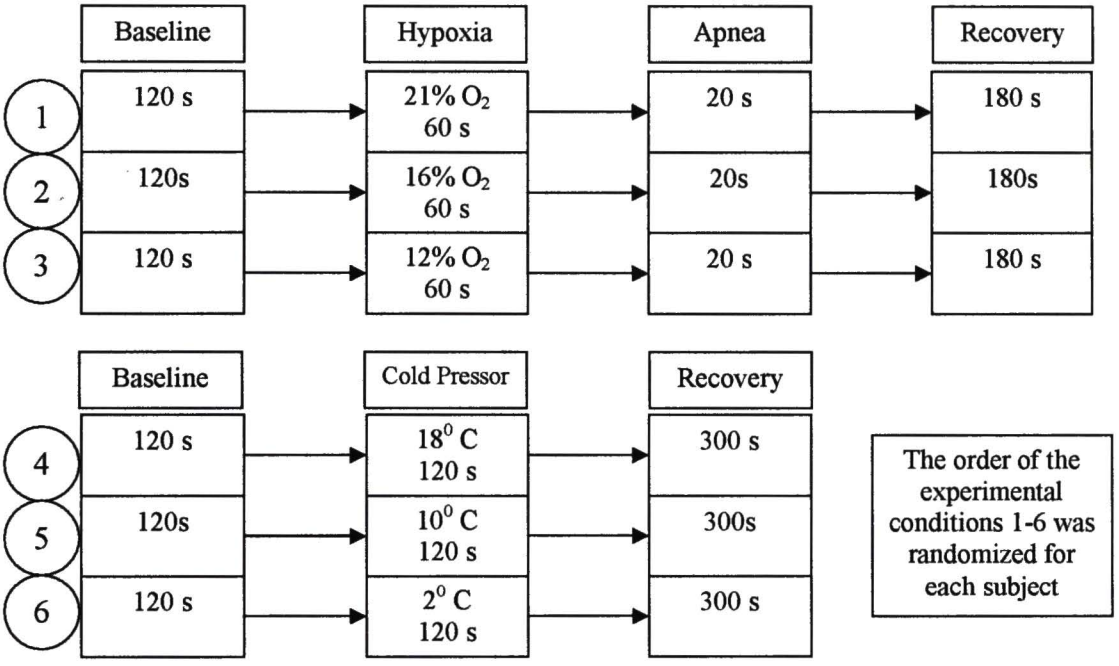
Figure 5. The figure depicts responses of Cardiovascular variables (as change from baseline levels) to hypoxic apnea in African Americans with 0 (MT 2), 1 (MT 1) or 2 (WT) copies of the ancestral (GGCC) haplotype. **A.** Systolic Blood Pressure response. **B.** Diastolic Blood Pressure response. **C.** Heart rate response. **D.** MSNA response. HY12 – hypoxic apnea at 12% inspired O_2, HY16 – hypoxic apnea at 16% inspired O_2, HY21 – hypoxic apnea at 21% inspired O_2. All values are Mean ± S.D. None of the responses were significantly higher in any group ($p \geq 0.172$).

Figure 6. The figure depicts regression curves for individual subjects for peak systolic blood pressure values reached during hypoxic apnea in African Americans with 0 (MT 2), 1 (MT 1) or 2 (WT) copies of the ancestral (GGCC) haplotype. The red lines show the mean response curves. **A.** Response curves for mutant homozygotes. **B.** Response curves for mutant heterozygotes. **C.** Response curves for wild type. Note the graded responses to the stimulus at the 3 levels of stimulus intensity. R^2 for the curves ranged from 0.693 to 0.999.

Figure 7. Flow mediated dilation is not different in the 3 groups of the GGCC haplotype. The graphs show flow-mediated dilation, as percent change in brachial artery diameter over baseline values in individual. The red symbols with error bars are Mean ± S.D. **A** –

Responses in African Americans ($p = 0.8937$). **B** – Responses in Caucasian Americans ($p = 0.565$).

Figure 1A.


Baseline
Hypoxia
Apnea
Recovery
1
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120s
120 s
21% O_2 60 s
16% O_2 60 s
12% O_2 60 s
20 s
20s
20 s
180 s
180s
180 s
Baseline
Cold Pressor
Recovery
4
5
6
120 s
120s
120 s
18^0 C 120 s
10^0 C 120 s
2^0 C 120 s
300 s
300s
300 s
The order of the experimental conditions 1-6 was randomized for each subject

Figure 1B.

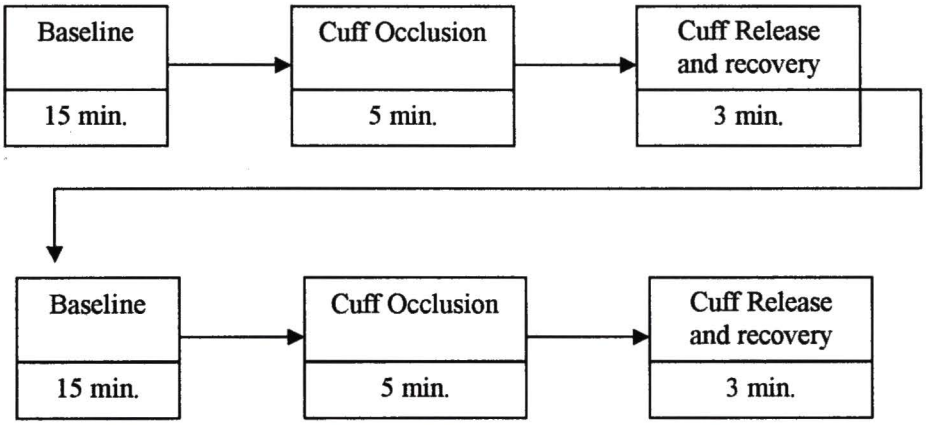

Baseline
15 min.
Cuff Occlusion
5 min.
Cuff Release and recovery
3 min.
Baseline
15 min.
Cuff Occlusion
5 min.
Cuff Release and recovery
3 min.

Figure 2 A.

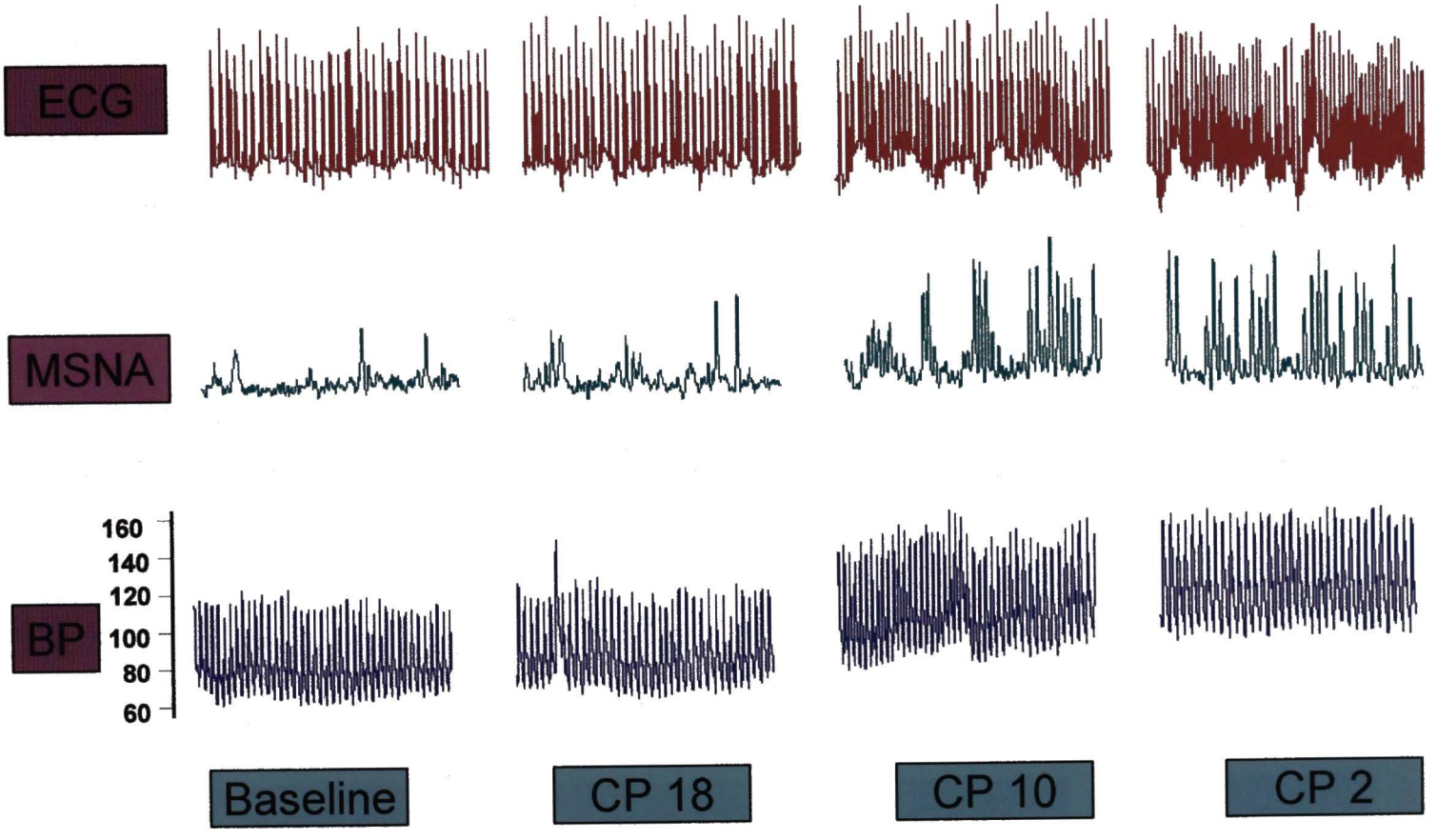


Figure 2 B.

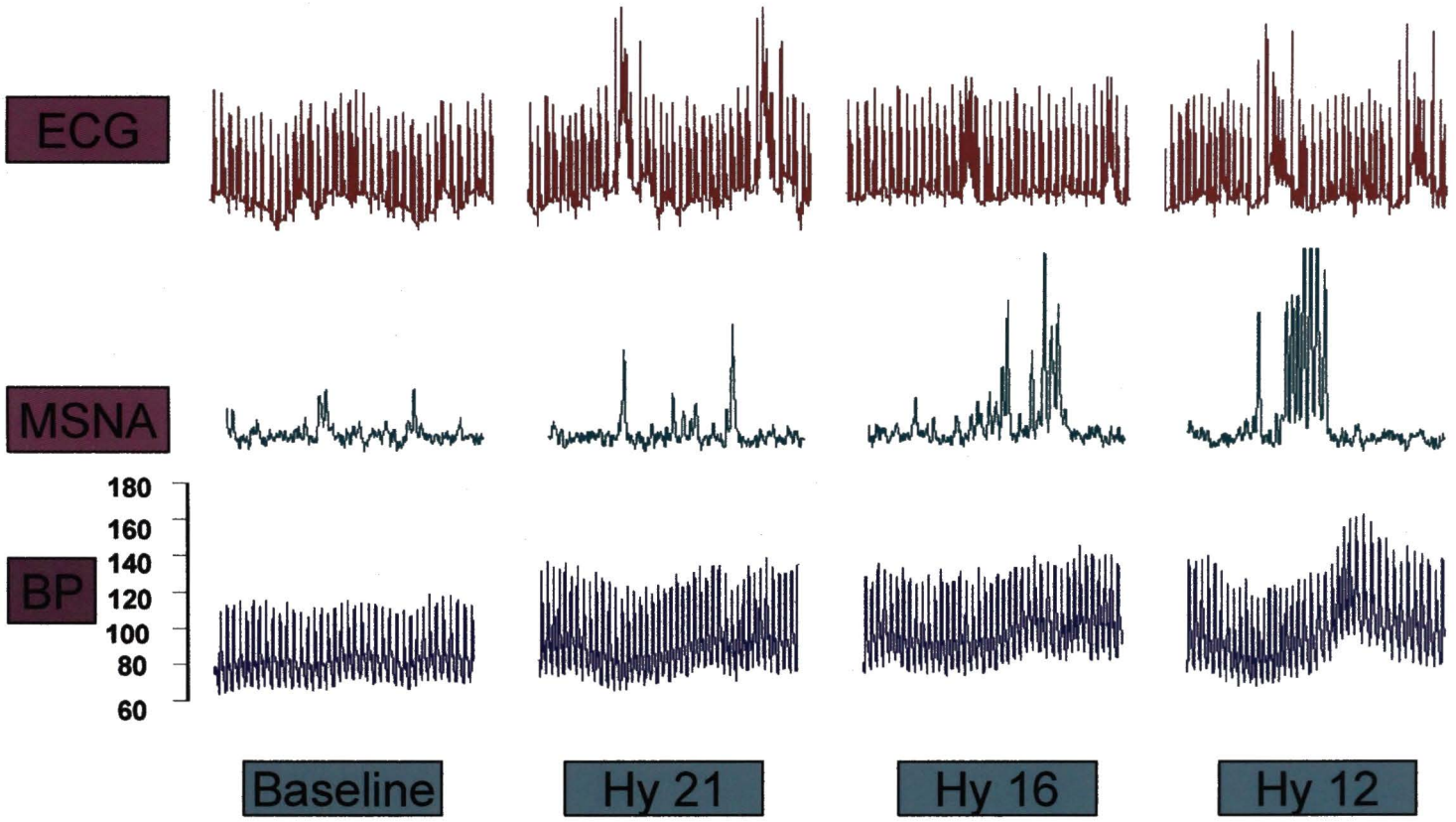


Figure 3.

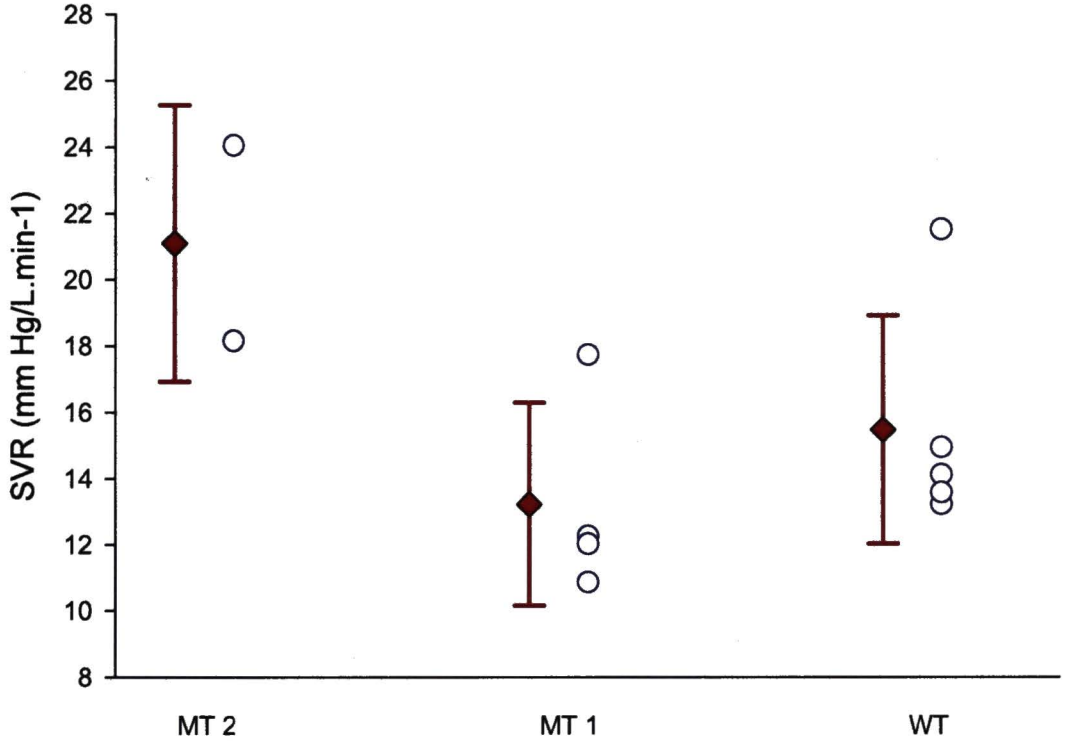


Figure 4.

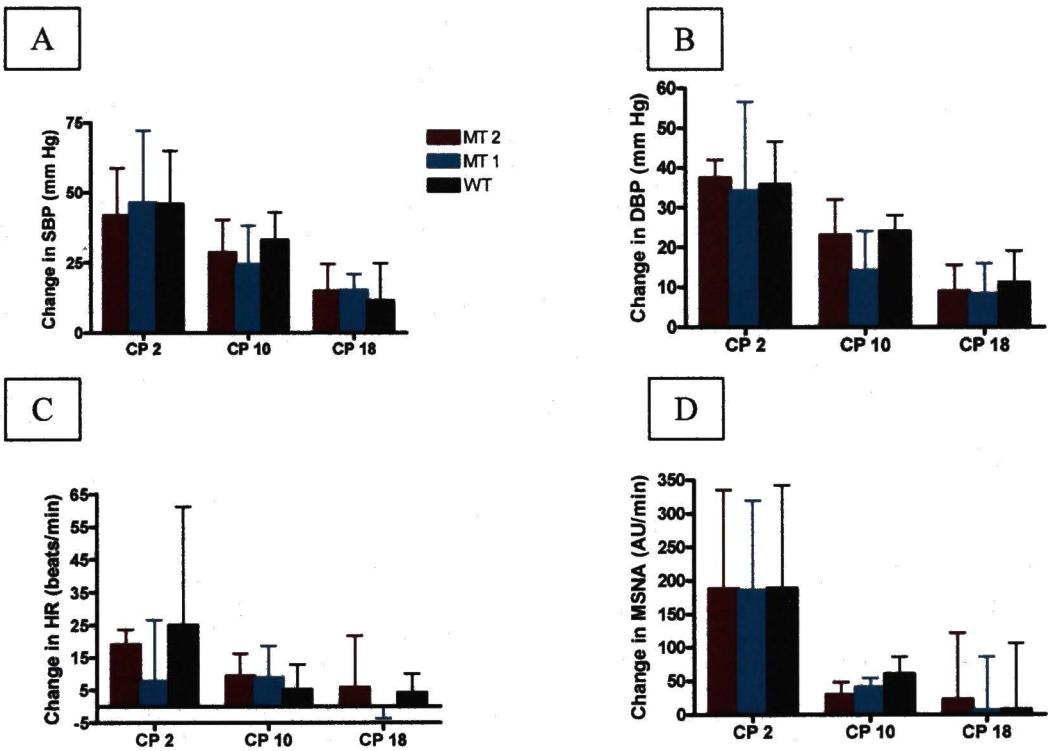

A
Change in SBP (mm Hg)
75
50
25
0
CP 2
CP 10
CP 18
MT 2
MT 1
WT
B
Change in DBP (mm Hg)
60
50
40
30
20
10
0
CP 2
CP 10
CP 18
C
Change in HR (beats/min)
65
55
45
35
25
15
5
-5
CP 2
CP 10
CP 18
D
Change in MSNA (AU/min)
350
300
250
200
150
100
50
0
CP 2
CP 10
CP 18

Figure 5.

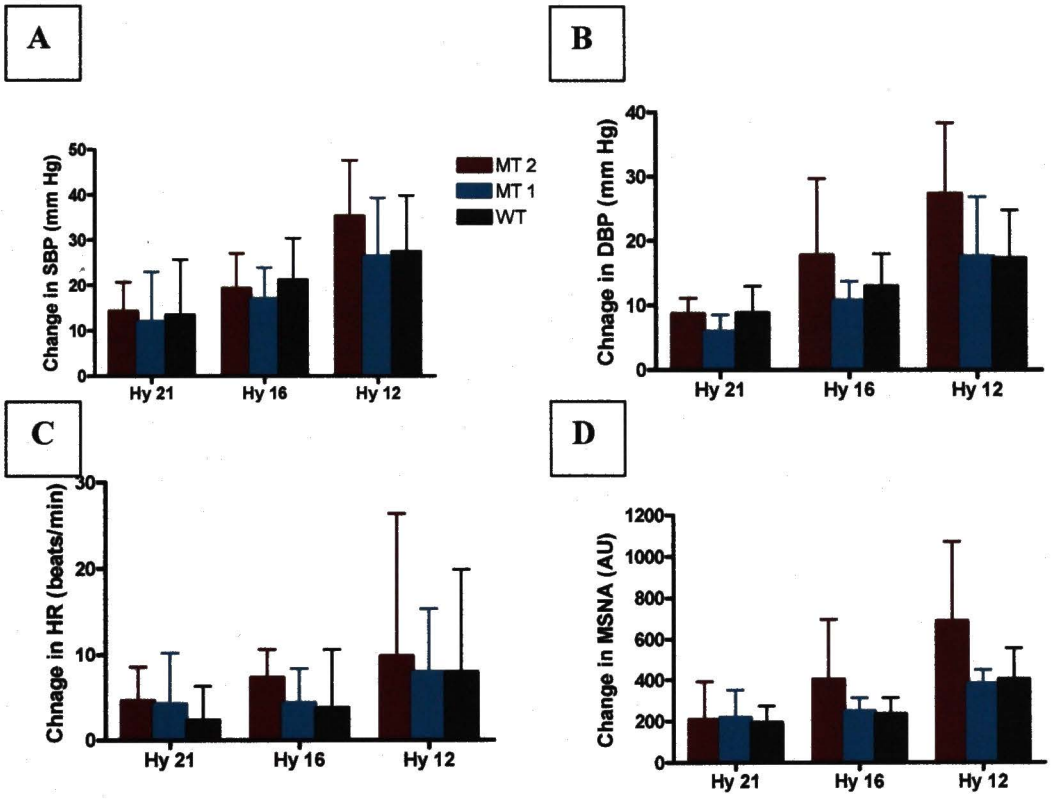

A
Change in SBP (mm Hg)
50
40
30
20
10
0
Hy 21
Hy 16
Hy 12
MT 2
MT 1
WT
B
Chnage in DBP (mm Hg)
40
30
20
10
0
Hy 21
Hy 16
Hy 12
C
Chnage in HR (beats/min)
30
20
10
0
Hy 21
Hy 16
Hy 12
D
Change in MSNA (AU)
1200
1000
800
600
400
200
0
Hy 21
Hy 16
Hy 12

Figure 6 A.

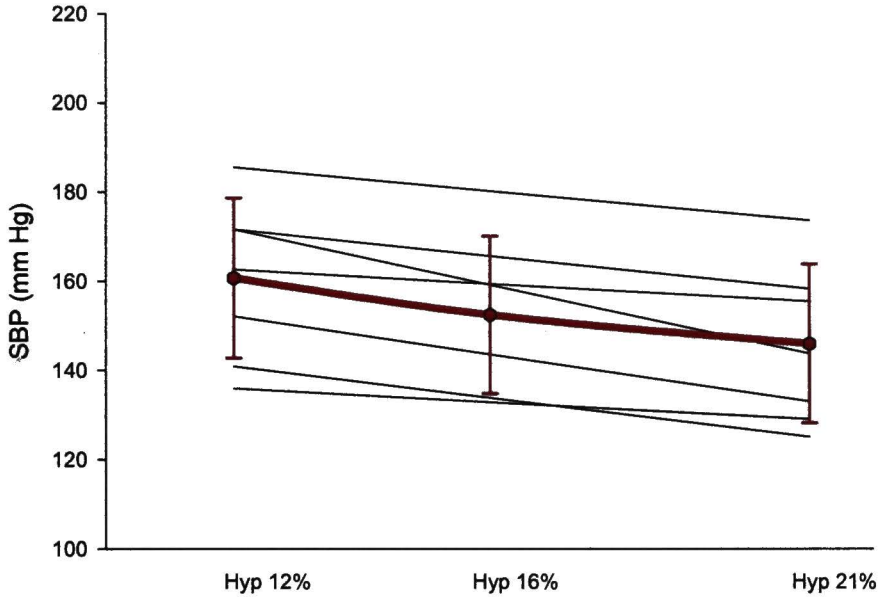


Figure 6 B.

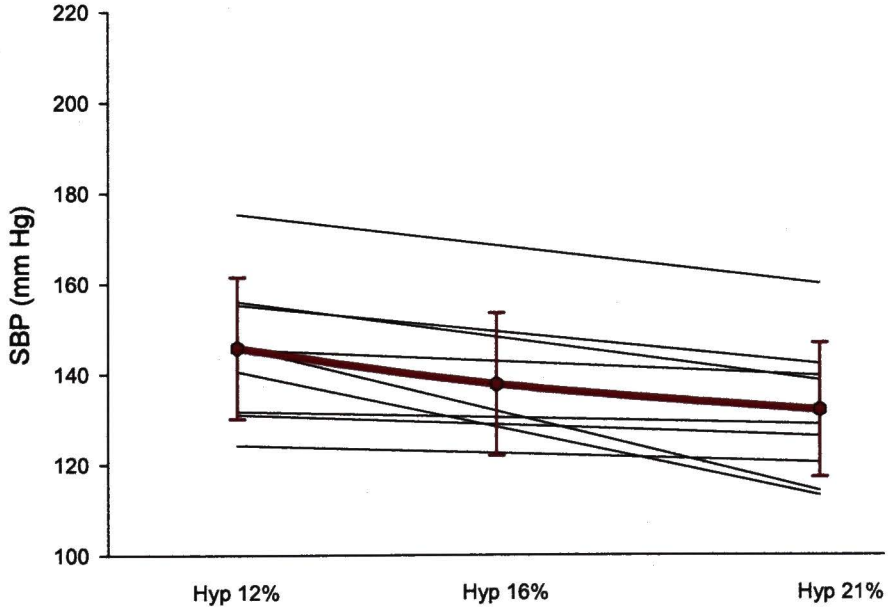


Figure 6 C.

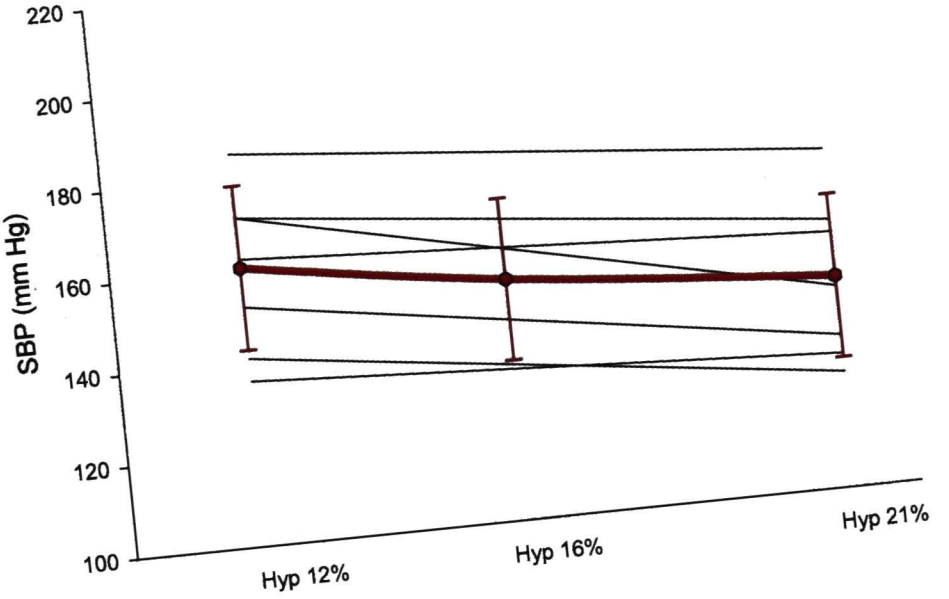
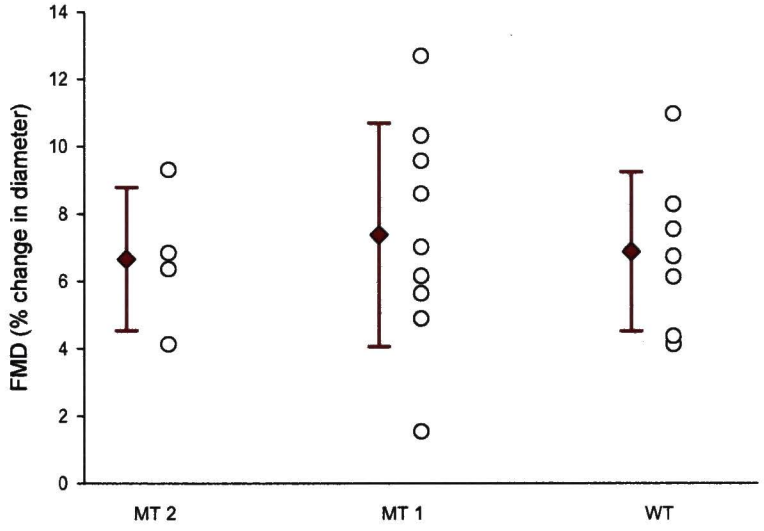


Figure 7.

A.


FMD (% change in diameter)
14
12
10
8
6
4
2
0
MT 2
MT 1
WT

B.

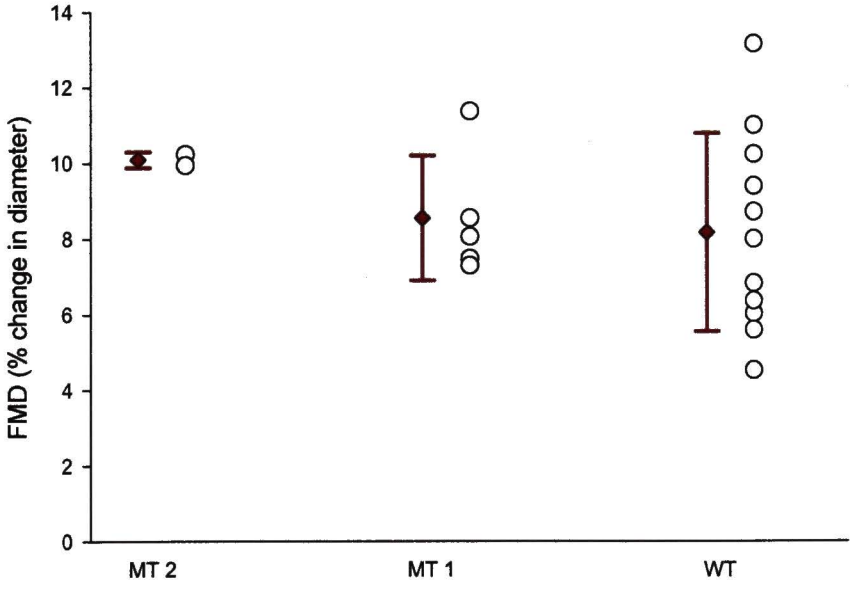

FMD (% change in diameter)
14
12
10
8
6
4
2
0
MT 2
MT 1
WT

Table 1. Table shows baseline cardiovascular variables in the two races. Values are Mean ± S.D.

Cardiovascular variable	African Americans	Caucasian Americans	P
SBP (mm Hg)	132.45 ± 18.69	128.37 ± 7.57	0.42
MAP (mm Hg)	90.03 ± 10.17	85.82 ± 6.20	0.16
DBP (mm Hg)	69.89 ± 10.23	64.54 ± 6.42	0.08
HR (bpm)	69.71 ± 9.86	61.15 ± 10.34	0.01
MSNA (AU/min)	353.96 ± 79.80	385.6 ± 118.88	0.37
CO (L/min)	5.74 ± 1.04	5.16 ± 0.25	0.30
SVR (mm Hg/L.min^{-1})	15.69 ± 4.20	16.35 ± 1.44	0.79

Table 2. Responses of SBP and DBP to cold pressor test and hypoxic apnea in individuals having 0 (MT 2), 1 (MT 1) and 2 (WT) copies of the ancestral GGCC haplotype. The numbers show the difference (mmHg) between the maximum blood pressure observed during stimulus and the mean baseline blood pressure.*

Stimulus	Caucasians				African Americans			
	Copies of GGCC haplotype		P		Copies of GGCC haplotype			P
	WT	MT 1			WT	MT 1	MT 2	
Δ Systolic Blood Pressure								
HY21	10	16	0.19		14	12	13	0.95
HY16	25	18	0.27		19	17	21	0.59
HY12	28	31	0.82		35	26	27	0.49
CP18	18	17	0.56		15	15	11	0.76
CP10	33	30	0.64		29	25	33	0.42
CP2	50	51	0.96		42	46	46	0.94
Δ Diastolic Blood Pressure								
HY21	9	10	0.72		9	6	9	0.17
HY16	18	14	0.44		18	11	13	0.19
HY12	26	24	0.84		27	18	17	0.18
CP18	16	11	0.10		9	8	11	0.75
CP10	31	26	0.58		23	14	24	0.06
CP2	46	40	0.49		37	34	36	0.94

* HY21 – Hypoxic apnea at 21% oxygen, HY16 – Hypoxic apnea at 16% oxygen, HY12 – Hypoxic apnea at 12% oxygen, CP18 – Cold pressor at 18°C, CP10 – Cold pressor at 10°C, CP2 – Cold pressor at 2°C

Table 3. Responses of SBP and DBP to cold pressor test and hypoxic apnea in individuals with 0 (WT), 1 (MT 1) or 2 (MT 2) copies of the CGTT haplotype.The numbers show the difference (mmHg) between the maximum blood pressure observed during stimulus and the mean baseline blood pressure.*

Stimulus	Caucasians				African Americans			
	Copies of CGTT haplotype		P		Copies of CGTT haplotype			P
	WT	MT 1			WT	MT 1	MT 2	
Δ Systolic Blood Pressure								
HY21	17	11	0.32		12	16	11	0.78
HY16	19	26	0.30		19	20	9	0.14
HY12	30	31	0.98		29	27	25	0.88
CP18	18	14	0.58		11	20	14	0.40
CP10	28	34	0.46		39	25	40	0.75
CP2	52	46	0.36		46	39	65	0.73
Δ Diastolic Blood Pressure								
HY21	10	9	0.77		8	7	6	0.55
HY16	15	18	0.51		13	13	5	0.43
HY12	23	27	0.58		18	23	14	0.87
CP18	11	15	0.26		8	12	9	0.97
CP10	25	31	0.50		22	14	19	0.55
CP2	41	45	0.60		88	29	39	0.68

* HY21 – Hypoxic apnea at 21% oxygen, HY16 – Hypoxic apnea at 16% oxygen, HY12 – Hypoxic apnea at 12% oxygen, CP18 – Cold pressor at 18°C, CP10 – Cold pressor at 10°C, CP2 – Cold pressor at 2°C

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

The present investigation is novel in many regards. The study was initiated to expand the results of previous studies by Rankinen *et al* (57) and Glenn *et al* (17) who reported association of RFLPs in the *ATP1a2* gene with hypertension and a greater prevalence of the RFLPs in African Americans. To confirm the results of our RFLP analysis, we sequenced a 200 bp area around this RFLP in the 3'-UTR of the *ATP1a2* gene. In addition to the 3 previously reported SNPs in this area, we discovered 3 novel SNPs, at mRNA positions 3849, 3853 and 3913. The SNP at position 3913 is 100% linked with the previously reported SNP at position 3915. In accord with the findings of Rankinen *et al* (57), we observed that 3'-UTR SNPs of the *ATP1a2* gene influence baseline blood pressure. However, Rankinen *et al* found (57) this effect for systolic blood pressure only, whereas we found that diastolic blood pressure is also elevated. Similarly, we discovered that the prevalence of the mutant alleles for this SNP is higher in African Americans as compared to Caucasian Americans. Another important discovery of this investigation was that haplotypes constructed from these SNPs influenced blood pressure in an ethnic-specific manner. The ancestral GGCC haplotype was associated with lower blood pressure in AAs and higher blood pressure in CAs, suggesting some form of differential evolutionary pressure in this genetic locus in the two races.

Given the association of these SNPs and haplotypes with basal blood pressure, we sought to investigate the mechanistic reasons behind this association. This is the first human study to investigate the link between the physiology of blood pressure control and its link with SNPs in this gene. In this study, we sought to determine whether these basal blood pressure effects are associated with impairment of neural reflex control and endothelial function. Reflex control was tested by studying responses of blood pressure and MSNA to two different pressor stimuli, cold pressor test and hypoxic apnea, since exaggerated responses to these stimuli suggest altered blood pressure control mechanism. Endothelial function was tested using ultrasound-derived measures of flow-mediated dilation in the brachial artery. Endothelial dysfunction is another potential mechanism behind altered blood pressure control. In this group of subjects studied, the physiologic responses to the two pressor stimuli were not greater in the groups who had higher basal blood pressure, suggesting that reflex control of blood pressure was normal. Therefore, the most likely mechanism for higher basal blood pressure in these groups is an increase in basal vascular tone, as evidenced by a tendency in these individuals to have a higher resting systemic vascular resistance. This hypothesis is in agreement with the physiologic role of the Na^+-K^+-ATPase in maintaining ionic equilibrium in cells. The Na^+-K^+-ATPase is functionally connected to the Na^+-Ca^{++}-exchanger. Depression in activity of the former pump decreases the capability of the latter to extrude Ca^{++} out of the cell and leads to an increased intracellular Ca^{++} concentration. This may lead to an increased baseline vascular tone and consequent increase in baseline blood pressures.

The results from this study raise a number of important questions that need to be addressed in future studies:

1. *What is the mechanism for the increased basal blood pressure associated with these SNPs and haplotypes?* As mentioned above, the most likely mechanism is an increased systemic vascular resistance as a consequence of increased basal vascular tone. As noted in the limitations, these data are not sufficient to definitively prove this hypothesis. Therefore, a larger sample of subjects needs to be studied to test whether basal SVR is indeed increased in association with certain *ATP1a2* haplotypes. Based on our power calculations for SVR, we need to study at least 4 more individuals who are homozygous mutants for the GGCC haplotype. Given the prevalence of the homozygous mutant GGCC haplotype was 0.19 among the AAs in this study, we need to recruit at least another 22 AAs and test their baseline SVR.

   At the cellular level, it is not known whether these 3'-UTR SNPs have an effect on Na^+-K^+-ATPase expression. The 3′ UTRs of genes determine such properties as mature mRNA stability/degradation, nuclear export, subcellular localization and translation efficiency (7-9). They are rich in regulatory elements and are involved in regulating gene expression at the pre-mRNA level. (8). Thus, conceivably, these SNPs can affect the expression and stability of Na^+-K^+-ATPase. Future studies need to address whether this is indeed the case. Studies are also needed to address whether these SNPs affect

ionic concentrations inside the cells, particularly the intracellular concentrations of Na^+ and Ca^{++}.

2. *Are the effects of these SNPs and haplotypes different in older individuals?* The subjects for this study were drawn from a young healthy population. It is possible that while only basal blood pressure seemed to be affected in these young individuals, even the responses to pressor stimuli could be exaggerated in an older population, representing a progression of the disease process. It is conceivable that baseline blood pressure is affected by these SNPs at a young age and that reflex control of blood pressure is affected later as a consequence of this inciting event. Similarly, the lack of endothelial dysfunction in association with these genotypes could possibly be because of the young age of the individuals studied. It is possible that subjects with higher basal blood pressure will develop endothelial dysfunction at a later age. The reason to examine the putative association between these *ATP1a2* haplotypes and response to stressor stimuli was to assess for clues to an altered risk of future hypertension. Lack of a longitudinal follow-up in our study does not permit us to directly address this objective and, based on our results, the question of future risk of hypertension remains open. Therefore, longitudinal studies on individuals carrying these SNPs need to be done starting at a young age and continuing into ages in which hypertension is prevalent.

3. *What is the prevalence and effect of these SNPs in hypertensive individuals?* As part of the study design, we recruited only normotensive individuals and compared the prevalence of the SNPs in the *ATP1a2* gene in these individuals. Future studies need to compare prevalence of these SNPs in hypertensive individuals to determine whether these SNPs are more prevalent in hypertensive individuals as compared with normotensive individuals. Studies also need to compare physiologic responses to pressor stimuli in hypertensive individuals and test whether these responses are different in individuals carrying the SNPs than in individuals not carrying the SNPs.

4. *What is the prevalence of these SNPs in races other than African Americans and Caucasian Americans?* Data on the prevalence of these SNPs are available only in African Americans and Caucasian Americans. The prevalence of these SNPs in other races needs to be determined. The effects of these SNPs with basal blood pressure need to be studied in other racial groups. This is important because a significant finding of the current study was that the ancestral haplotype GGCC affected basal blood pressure in an ethnic-specific manner, suggesting an evolutionary pressure on this genetic locus.

5. *What is the association of these SNPs with family history of hypertension?* Given the well-known fact that hypertension runs in families and the finding of association of these SNPs with blood pressure, it is likely that there is an

association of these SNPs with a family history of hypertension. Carefully designed future studies need to test this hypothesis by recruiting families and studying the prevalence of hypertension and of these SNPs in these families.

REFERENCES

1. **Bao G, Metreveli N, Li R, Taylor A, and Fletcher EC.** Blood pressure response to chronic episodic hypoxia: role of the sympathetic nervous system. *J Appl Physiol* 83: 95-101, 1997.

2. **Brooks D, Horner RL, Kimoff RJ, Kozar LF, Render-Teixeira CL, and Phillipson EA.** Effect of obstructive sleep apnea versus sleep fragmentation on responses to airway occlusion. *Am J Respir Crit Care Med* 155: 1609-1617, 1997.

3. **Brownley KA, Hurwitz BE, and Schneiderman N.** Ethnic variations in the pharmacological and nonpharmacological treatment of hypertension: biopsychosocial perspective. *Hum Biol* 71: 607-639, 1999.

4. **Calhoun DA and Mutinga ML.** Race, family history of hypertension, and sympathetic response to cold pressor testing. *Blood Press* 6: 209-213, 1997.

5. **Calhoun DA, Mutinga ML, Collins AS, Wyss JM, and Oparil S.** Normotensive blacks have heightened sympathetic response to cold pressor test. *Hypertension* 22: 801-805, 1993.

6. **Celentano A, Galderisi M, Garofalo M, Mureddu GF, Tammaro P, Petitto M, Di Somma S, and de Divitiis O.** Blood pressure and cardiac morphology in young children of hypertensive subjects. *J Hypertens Suppl* 6: S107-109, 1988.

7. **Corvol P, Jeunemaitre X, Charru A, and Soubrier F.** Can the genetic factors influence the treatment of systemic hypertension? The case of the renin-angiotensin-aldosterone system. *Am J Cardiol* 70: 14D-20D, 1992.

8. **Cui ZH, Ikeda K, Kawakami K, Gonda T, Masuda J, and Nabika T.** Exaggerated response to cold stress in a congenic strain for the quantitative trait locus for blood pressure. *J Hypertens* 22: 2103-2109, 2004.

9. **Cutler MJ, Swift NM, Keller DM, Wasmund WL, and Smith ML.** Hypoxia-mediated prolonged elevation of sympathetic nerve activity after periods of intermittent hypoxic apnea. *J Appl Physiol* 96: 754-761, 2004.

10. **Deal JE, Shah V, Goodenough G, and Dillon MJ.** Red cell membrane sodium transport: possible genetic role and use in identifying patients at risk of essential hypertension. *Arch Dis Child* 65: 1154-1157, 1990.

11. **Duey WJ, Bassett DR, Jr., Walker AJ, Torok DJ, Howley ET, Ely D, and Pease MO.** Cardiovascular and plasma catecholamine response to static exercise in normotensive blacks and whites. *Ethn Health* 2: 127-136, 1997.

12. **Fagius J, Karhuvaara S, and Sundlof G.** The cold pressor test: effects on sympathetic nerve activity in human muscle and skin nerve fascicles. *Acta Physiol Scand* 137: 325-334, 1989.

13. **Fletcher EC.** Effect of episodic hypoxia on sympathetic activity and blood pressure. *Respir Physiol* 119: 189-197, 2000.

14. **Fletcher EC, Lesske J, Culman J, Miller CC, and Unger T.** Sympathetic denervation blocks blood pressure elevation in episodic hypoxia. *Hypertension* 20: 612-619, 1992.

15. **Garcia-Rio F, Racionero MA, Pino JM, Martinez I, Ortuno F, Villasante C, and Villamor J.** Sleep apnea and hypertension. *Chest* 117: 1417-1425, 2000.

16. **Garcia N, Hopkins SR, Elliott AR, Aaron EA, Weinger MB, and Powell FL.** Ventilatory response to 2-h sustained hypoxia in humans. *Respir Physiol* 124: 11-22, 2001.

17. **Glenn BS, Stewart WF, Schwartz BS, and Bressler J.** Relation of alleles of the sodium-potassium adenosine triphosphatase alpha 2 gene with blood pressure and lead exposure. *Am J Epidemiol* 153: 537-545, 2001.

18. **Grobbee DE.** Predicting hypertension in childhood: value of blood pressure measurement and family history. *J Am Coll Nutr* 11 Suppl: 55S-59S, 1992.

19. **Grover AK, Kwan CY, and Samson SE.** Effects of peroxynitrite on sarco/endoplasmic reticulum Ca2+ pump isoforms SERCA2b and SERCA3a. *Am J Physiol Cell Physiol* 285: C1537-1543, 2003.

20. **Guo SW and Thompson EA.** Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* 48: 361-372, 1992.

21. **Hamlyn JM, Hamilton BP, and Manunta P.** Endogenous ouabain, sodium balance and blood pressure: a review and a hypothesis. *J Hypertens* 14: 151-167, 1996.

22. **Hartford AK, Messer ML, Moseley AE, Lingrel JB, and Delamere NA.** Na,K-ATPase alpha 2 inhibition alters calcium responses in optic nerve astrocytes. *Glia* 45: 229-237, 2004.

23. **Heagerty AM, Milner M, Bing RF, Thurston H, and Swales JD.** Leucocyte membrane sodium transport in normotensive populations: dissociation of abnormalities of sodium efflux from raised blood-pressure. *Lancet* 2: 894-896, 1982.

24. **Hedner JA, Wilcox I, Laks L, Grunstein RR, and Sullivan CE.** A specific and potent pressor effect of hypoxia in patients with sleep apnea. *Am Rev Respir Dis* 146: 1240-1245, 1992.

25. **Herrera VL, Xie HX, Lopez LV, Schork NJ, and Ruiz-Opazo N.** The alpha1 Na,K-ATPase gene is a susceptibility hypertension gene in the Dahl salt-sensitiveHSD rat. *J Clin Invest* 102: 1102-1111, 1998.

26. **Ikeda K, Onimaru H, Yamada J, Inoue K, Ueno S, Onaka T, Toyoda H, Arata A, Ishikawa TO, Taketo MM, Fukuda A, and Kawakami K.** Malfunction of respiratory-related neuronal activity in Na+, K+-ATPase alpha2 subunit-deficient mice is attributable to abnormal Cl- homeostasis in brainstem neurons. *J Neurosci* 24: 10693-10701, 2004.

27. **Imholz BP, van Montfrans GA, Settels JJ, van der Hoeven GM, Karemaker JM, and Wieling W.** Continuous non-invasive blood pressure monitoring: reliability of Finapres device during the Valsalva manoeuvre. *Cardiovasc Res* 22: 390-397, 1988.

28. **Iwamoto T and Kita S.** Hypertension, Na+/Ca2+ exchanger, and Na+, K+-ATPase. *Kidney Int* 69: 2148-2154, 2006.

29. **Jen JC, Klein A, Boltshauser E, Cartwright MS, Roach ES, Mamsa H, and Baloh RW.** Prolonged hemiplegic episodes in children due to mutations in ATP1A2. *J Neurol Neurosurg Psychiatry* 78: 523-526, 2007.

30. **Kara T, Narkiewicz K, and Somers VK.** Chemoreflexes--physiology and clinical implications. *Acta Physiol Scand* 177: 377-384, 2003.

31. **Kazantsev A, Yamaoka LH, and Roses AD.** A dinucleotide repeat polymorphism in the human Na+,K+ ATPase, alpha subunit (ATP1A3) gene. *Nucleic Acids Res* 20: 1164, 1992.

32. **Kelsey RM, Alpert BS, Patterson SM, and Barnard M.** Racial differences in hemodynamic responses to environmental thermal stress among adolescents. *Circulation* 101: 2284-2289, 2000.

33. **Kramer HJ, Glanzer K, Freitag T, Schonfeld J, Sorger M, Schlebusch H, Dusing R, and Kruck F.** Studies on the role of sodium- and potassium-activated adenosine triphosphatase inhibition in the pathogenesis of human hypertension. Changes in vascular and cardiac function following inhibition of the sodium pump in normotensive subjects and effects of calcium entry blockade. *Klin Wochenschr* 63: 32-36, 1985.

34. **Lafleche AB, Pannier BM, Laloux B, and Safar ME.** Arterial response during cold pressor test in borderline hypertension. *Am J Physiol* 275: H409-415, 1998.

35. **Lecuona E, Luquin S, Avila J, Garcia-Segura LM, and Martin-Vasallo P.** Expression of the beta 1 and beta 2(AMOG) subunits of the Na,K-ATPase in neural tissues: cellular and developmental distribution patterns. *Brain Res Bull* 40: 167-174, 1996.

36. **Lee YH, Tokraks S, Pratley RE, Bogardus C, and Permana PA.** Identification of differentially expressed genes in skeletal muscle of non-diabetic insulin-resistant and insulin-sensitive Pima Indians by differential display PCR. *Diabetologia* 46: 1567-1575, 2003.

37. **Levene H.** On a matching problem arising in genetics. *Annals of Mathematical Statistics* 20: 91-94, 1949.

38. **Lijnen P, M'Buyamba-Kabangu JR, Fagard R, Staessen J, and Amery A.** Erythrocyte concentrations and transmembrane fluxes of sodium and potassium in essential hypertension: role of intrinsic and environmental factors. *Cardiovasc Drugs Ther* 4 Suppl 2: 321-333, 1990.

39. **Lim LC and Gill M.** A complex dinucleotide repeat polymorphism in the human Na+K+ATPase alpha subunit (ATP1A2) gene. *Hum Mol Genet* 2: 616, 1993.

40. **Linder L, Kiowski W, Buhler FR, and Luscher TF.** Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation in vivo. Blunted response in essential hypertension. *Circulation* 81: 1762-1767, 1990.

41. **Lingrel J, Moseley A, Dostanic I, Cougnon M, He S, James P, Woo A, O'Connor K, and Neumann J.** Functional roles of the alpha isoforms of the Na,K-ATPase. *Ann N Y Acad Sci* 986: 354-359, 2003.

42. **Livshits G, Ginsburg E, and Kobyliansky E.** Heterogeneity of genetic control of blood pressure in ethnically different populations. *Hum Biol* 71: 685-708, 1999.

43. **Luscher TF.** Endothelium-derived relaxing and contracting factors: potential role in coronary artery disease. *Eur Heart J* 10: 847-857, 1989.

44. **Luscher TF, Yang ZH, Diederich D, and Buhler FR.** Endothelium-derived vasoactive substances: potential role in hypertension, atherosclerosis, and vascular occlusion. *J Cardiovasc Pharmacol* 14 Suppl 6: S63-69, 1989.

45. **Markovitz JH, Raczynski JM, Wallace D, Chettur V, and Chesney MA.** Cardiovascular reactivity to video game predicts subsequent blood pressure increases in young men: The CARDIA study. *Psychosom Med* 60: 186-191, 1998.

46. **Masharani U and Frossard PM.** MspI and HindIII restriction fragment length polymorphisms at the human Na,K-ATPase beta-subunit (ATP1B) gene locus. *Hum Genet* 80: 308, 1988.

47. **Matsukawa T, Gotoh E, Uneda S, Miyajima E, Shionoiri H, Tochikubo O, and Ishii M.** Augmented sympathetic nerve activity in response to stressors in young borderline hypertensive men. *Acta Physiol Scand* 141: 157-165, 1991.

48. **Matthews KA, Salomon K, Brady SS, and Allen MT.** Cardiovascular reactivity to stress predicts future blood pressure in adolescence. *Psychosom Med* 65: 410-415, 2003.

49. **Mazzanti L, Rabini RA, Testa I, Coppa GV, Catassi C, Cecconi M, and Giorgi PL.** Sodium metabolism in offspring of hypertensive parents. *Biochem Med Metab Biol* 45: 181-187, 1991.

50. **Naber CK and Siffert W.** Genetics of human arterial hypertension. *Minerva Med* 95: 347-356, 2004.

51. **Neutel JM and Smith DH.** Hypertension control: multifactorial contributions. *Am J Hypertens* 12: 164S-169S, 1999.

52. **Panza JA, Quyyumi AA, Brush JE, Jr., and Epstein SE.** Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *N Engl J Med* 323: 22-27, 1990.

53. **Parati G, Casadei R, Groppelli A, Di Rienzo M, and Mancia G.** Comparison of finger and intra-arterial blood pressure monitoring at rest and during laboratory testing. *Hypertension* 13: 647-655, 1989.

54. **Peng L, Martin-Vasallo P, and Sweadner KJ.** Isoforms of Na,K-ATPase alpha and beta subunits in the rat cerebellum and in granule cell cultures. *J Neurosci* 17: 3488-3502, 1997.

55. **Poulter NR.** Major risk factors for coronary heart disease in hypertension: implications for management. *J Hum Hypertens* 4 Suppl 3: 3-6, 1990.

56. **Preik M, Kelm M, Schoebel F, Schottenfeld Y, Leschke M, and Strauer BE.** Selective impairment of nitric oxide dependent vasodilation in young adults with hypercholesterolaemia. *J Cardiovasc Risk* 3: 465-471, 1996.

57. **Rankinen T, Perusse L, Deriaz O, Theriault G, Chagnon M, Nadeau A, and Bouchard C.** Linkage of the Na,K-ATPase alpha 2 and beta 1 genes with resting and exercise heart rate and blood pressure: cross-sectional and longitudinal observations from the Quebec Family Study. *J Hypertens* 17: 339-349, 1999.

58. **Rea RF and Hamdan M.** Baroreflex control of muscle sympathetic nerve activity in borderline hypertension. *Circulation* 82: 856-862, 1990.

59. **Rodrigo R, Bachler JP, Araya J, Prat H, and Passalacqua W.** Relationship between (Na + K)-ATPase activity, lipid peroxidation and fatty acid profile in erythrocytes of hypertensive and normotensive subjects. *Mol Cell Biochem*, 2007.

60. **Rousset F and Raymond M.** Testing heterozygote excess and deficiency. *Genetics* 140: 1413-1419, 1995.

61. **Rowell LB, Johnson DG, Chase PB, Comess KA, and Seals DR.** Hypoxemia raises muscle sympathetic activity but not norepinephrine in resting humans. *J Appl Physiol* 66: 1736-1743, 1989.

62. **Seals DR.** Sympathetic activation during the cold pressor test: influence of stimulus area. *Clin Physiol* 10: 123-129, 1990.

63. **Shelly DA, He S, Moseley A, Weber C, Stegemeyer M, Lynch RM, Lingrel J, and Paul RJ.** Na(+) pump alpha 2-isoform specifically couples to contractility in vascular smooth muscle: evidence from gene-targeted neonatal mice. *Am J Physiol Cell Physiol* 286: C813-820, 2004.

64. **Shull MM, Pugh DG, Lane LK, and Lingrel JB.** MspI and PvuII polymorphisms in the Na,K-ATPase beta subunit gene ATP1B1. *Nucleic Acids Res* 18: 1087, 1990.

65. **Shull MM, Pugh DG, and Lingrel JB.** The human Na, K-ATPase alpha 1 gene: characterization of the 5'-flanking region and identification of a restriction fragment length polymorphism. *Genomics* 6: 451-460, 1990.

66. **Skoczynska A, Juzwa W, Smolik R, Szechinski J, and Behal FJ.** Response of the cardiovascular system to catecholamines in rats given small doses of lead. *Toxicology* 39: 275-289, 1986.

67. **Skrabal F, Hamberger L, Cerny E, Schonegger J, and Gernhold M.** Raised sodium pump activity and a circulating sodium transport inhibitor demonstrated on red blood cells of patients with untreated essential hypertension: correlation of pump activity with potassium permeability. *Klin Wochenschr* 63 Suppl 3: 117-121, 1985.

68. **Slatkin M.** Linkage disequilibrium in growing and stable populations. *Genetics* 137: 331-336, 1994.

69. **Slatkin M and Excoffier L.** Testing for linkage disequilibrium in genotypic data using the Expectation-Maximization algorithm. *Heredity* 76 (Pt 4): 377-383, 1996.

70. **Smith ML, Niedermaier ON, Hardy SM, Decker MJ, and Strohl KP.** Role of hypoxemia in sleep apnea-induced sympathoexcitation. *J Auton Nerv Syst* 56: 184-190, 1996.

71. **Somers VK, Mark AL, and Abboud FM.** Interaction of baroreceptor and chemoreceptor reflex control of sympathetic nerve activity in normal humans. *J Clin Invest* 87: 1953-1957, 1991.

72. **Somers VK, Mark AL, and Abboud FM.** Potentiation of sympathetic nerve responses to hypoxia in borderline hypertensive subjects. *Hypertension* 11: 608-612, 1988.

73. **Somers VK, Mark AL, Zavala DC, and Abboud FM.** Contrasting effects of hypoxia and hypercapnia on ventilation and sympathetic activity in humans. *J Appl Physiol* 67: 2101-2106, 1989.

74. **Songu-Mize E, Alpert BS, and Willey ES.** Race, sex, and family history of hypertension and erythrocyte sodium pump [3H]ouabain binding. *Hypertension* 15: 146-151, 1990.

75. **Songu-Mize E, Liu X, Stones JE, and Hymel LJ.** Regulation of Na+,K+-ATPase alpha-subunit expression by mechanical strain in aortic smooth muscle cells. *Hypertension* 27: 827-832, 1996.

76. **Stein CM, Lang CC, Singh I, He HB, and Wood AJ.** Increased vascular adrenergic vasoconstriction and decreased vasodilation in blacks. Additive mechanisms leading to enhanced vascular reactivity. *Hypertension* 36: 945-951, 2000.

77. **Stewart JC and France CR.** Cardiovascular recovery from stress predicts longitudinal changes in blood pressure. *Biol Psychol* 58: 105-120, 2001.

78. **Sweadner KJ.** Isozymes of the Na+/K+-ATPase. *Biochim Biophys Acta* 988: 185-220, 1989.

79. **Sweadner KJ, Herrera VL, Amato S, Moellmann A, Gibbons DK, and Repke KR.** Immunologic identification of Na+,K(+)-ATPase isoforms in myocardium. Isoform change in deoxycorticosterone acetate-salt hypertension. *Circ Res* 74: 669-678, 1994.

80. **Tian G, Dang C, and Lu Z.** The change and significance of the Na+-K+-ATPase alpha-subunit in ouabain-hypertensive rats. *Hypertens Res* 24: 729-734, 2001.

81. **Treiber FA, Turner JR, Davis H, and Strong WB.** Prediction of resting cardiovascular functioning in youth with family histories of essential hypertension: a 5-year follow-up. *Int J Behav Med* 4: 278-291, 1997.

82. **Vallbo AB, Hagbarth KE, and Wallin BG.** Microneurography: how the technique developed and its role in the investigation of the sympathetic nervous system. *J Appl Physiol* 96: 1262-1269, 2004.

83. **Vanmolkot KR, Kors EE, Turk U, Turkdogan D, Keyser A, Broos LA, Kia SK, van den Heuvel JJ, Black DF, Haan J, Frants RR, Barone V, Ferrari MD, Casari G, Koenderink JB, and van den Maagdenberg AM.** Two de novo mutations in

the Na,K-ATPase gene ATP1A2 associated with pure familial hemiplegic migraine. *Eur J Hum Genet* 14: 555-560, 2006.

84. **Victor RG, Leimbach WN, Jr., Seals DR, Wallin BG, and Mark AL.** Effects of the cold pressor test on muscle sympathetic nerve activity in humans. *Hypertension* 9: 429-436, 1987.

85. **von Brevern M, Zeise D, Neuhauser H, Clarke AH, and Lempert T.** Acute migrainous vertigo: clinical and oculographic findings. *Brain* 128: 365-374, 2005.

86. **Wennmalm A.** Endothelial nitric oxide and cardiovascular disease. *J Intern Med* 235: 317-327, 1994.

87. **Yang ZH, Diederich D, Schneider K, Siebenmann R, Stulz P, von Segesser L, Turina M, Buhler FR, and Luscher TF.** Endothelium-derived relaxing factor and protection against contractions induced by histamine and serotonin in the human internal mammary artery and in the saphenous vein. *Circulation* 80: 1041-1048, 1989.

88. **Ziegler MG, Nelesen R, Mills P, Ancoli-Israel S, Kennedy B, and Dimsdale JE.** Sleep apnea, norepinephrine-release rate, and daytime hypertension. *Sleep* 20: 224-231, 1997.