DIETARY SODIUM RESTRICTION AND VASCULAR DYSFUNCTION WITH AGING

by

KRISTEN LYNN JABLONSKI

B.S., Marquette University, 2005

M.S., University of Colorado, 2008

A dissertation submitted to the

Faculty of the Graduate School of the

University of Colorado in partial fulfillment

of the requirement for the degree of

Doctor of Philosophy

Department of Integrative Physiology

2011

This dissertation entitled:

Dietary Sodium Restriction and Vascular Dysfunction with Aging
written by Kristen Lynn Jablonski
has been approved for the Department of Integrative Physiology

_____

Dr. Douglas R. Seals

_____

Dr. Robert Mazzeo

Date _____

This final copy of this thesis has been examined by the signatories, and we
Find that both the content and the form meet acceptable presentation standards
Of scholarly work in the above mentioned discipline.

IRB protocols # 0603.12, 0304.17, 0404.04, 0505.18, 0608.12

Jablonski, Kristen Lynn (Ph.D., Integrative Physiology)
Dietary Sodium Restriction and Vascular Dysfunction with Aging
Thesis directed by Professor Douglas R. Seals

Endothelial dysfunction, commonly assessed as a decline in endothelium-dependent dilation (EDD), is a clinically important predictor of future cardiovascular diseases (CVD). EDD is reduced with age and elevated systolic blood pressure (SBP), whether assessed as brachial artery flow-mediated dilation (FMD_{BA}) or as the forearm blood flow response to acetylcholine (FBF_{ACh}). Thus, establishing the efficacy of interventions that may preserve EDD in this population is a high biomedical research priority. Dietary sodium restriction (DSR) is one potential intervention. While the influence of sodium on BP is widely documented and appreciated, high dietary sodium has other adverse effects on cardiovascular health, independent of its effect on BP. Accordingly, the purpose of these studies was to determine a) if reduced sodium intake is associated with enhanced EDD in middle-aged and older adults (MA/O) with moderately elevated SBP; b) if DSR improves EDD in this group; and c) the physiological mechanisms mediating any improvements.

In cross-sectional analyses, FMD_{BA} was 52% higher in subjects self-reporting low sodium intake (73±6 mmol/d), without differences in endothelium-independent dilation (EID), SBP or other subject characteristics. In the entire cohort, FMD_{BA} was inversely related to dietary sodium intake (%Δ r=-0.53, $p<0.01$).

In the intervention study, EDD was 71% (FMD_{BA}) and 53% (peak FBF_{ACh}) higher following 4 weeks of low sodium (70$\pm$8 mmol/day) vs. normal sodium (153$\pm$7 mmol/day) intake ($p<0.05$; randomized cross-over design), whereas EID did not change. The improvements in EDD remained significant when correcting for SBP, and other subject characteristics/dietary factors did not change. Functional improvements were mediated by increased nitric oxide (NO) bioavailability (significant reduction in FBF_{ACh} with co-infusion of an endothelial nitric oxide

synthase inhibitor), increased tetrahydrobiopterin (BH_4) bioavailability (FMD_{BA} no longer improved with oral BH_4) and reduced oxidative stress (FMD_{BA} and FBF_{ACh} no longer improved with ascorbic acid infusion; reduced endothelial cell nitrotyrosine abundance).

In conclusion, DSR reverses endothelial dysfunction in MA/O with moderately elevated SBP by enhancing NO and BH_4 bioavailability and reducing oxidative stress. Thus, the physiological mechanisms modulating benefits of DSR are beyond BP lowering alone, and the potential impact of DSR upon CVD risk may be even greater than previously appreciated.

DEDICATION

This work is dedicated to my loving husband, Ken. You have been my greatest source of support through these past 5 ½ years, and I am eternally grateful that you entered my life during my graduate training. At last, we both made it!!!

ACKNOWLEDGEMENTS

I would like to thank Dr. Douglas Seals for his mentorship. I am so incredibly fortunate to have received such excellent training over the past five and a half years.

Thank you to the members of the Integrative Physiology of Aging Lab, past and present, for all of the training, mentorship, support, encouragement and assistance over the years. In particular thank you to Tony Donato, my M.S. intermediary mentor, who has taught me more over the years than can I could ever verbalize. Thank you for the informal mentorship that I have received from other past members of the lab, including Gary Pierce, Lisa Lesniewski, Phil Gates and Ashley Walker. I am incredibly grateful to Candace Geolfos, Matt Racine and Keri Nelson for the recruitment of subjects, scheduling and running of visits, and for all the other "behind the scenes" work they have done. I could not have done it without your help. Thank you to the many other members of the lab who have helped with this research over the years, including Brooke Lawson, Molly Russell, Elena Pellicer, Eric Chung, Kate Howell, Melanie Zigler, Tom LaRocca, Brad Fleenor, Allison DeVan, Forrest Brooks, Rhea Chiang, Rachelle Kaplon, Lindsey Gano, Laura Liu, Hylke Sneider, Muhammad Hamza and Cindy Seals.

Thank you to my other committee members, especially Dr. Michel Chonchol for serving as the physician of record for the research and always responding incredibly rapidly to our inquiries about subject enrollment and study design. I am also most appreciative of the assistance of Dr. Matthew McQueen with the statistical analyses.

Many thanks to the staff of the Clinical and Translational Research Center (CTRC), particularly the bionutrition support of Kathleen Farrell and Becky Staley. Importantly, thank you

to my subjects for their dedication to a low sodium diet. This research would not have been possible without them.

Last, my deepest thanks to my family and friends for a lifetime of support. Thank you to my parents for fostering my desire to learn and discover and for the opportunity to pursue the career path best suited for me. Thank you Mom and Kate for your unending support, as well as the rest of the Jablonski, Kelly and Nowak families. And above all, thank you to my wonderful husband Ken. You are my rock, and I could not have made it without you.

Financial support was provided by NIH pre-doctoral fellowship AG033994, as well as NIH RR025780, AG013038, AG006537, AG03114, AG03167, AG22241 and AG029337.

CONTENTS

TABLES

Chapter IV

Chapter V

FIGURES

Chapter IV

Chapter V

CHAPTER I

List of Publications

Research Articles

Jablonski KL, Racine ML, Geolfos CJ, Gates PE, Chonchol M, McQueen MB & Seals DR. Dietary sodium restriction reverses vascular endothelial dysfunction in middle-aged and older adults with moderately elevated systolic blood pressure. To be submitted to *Circulation*.

Jablonski KL, Donato AJ, Fleenor BS, Russell MJ, Walker AE & Seals DR. Absence of nuclear factor-κB-mediated increase in large elastic artery stiffness in middle-aged and older adults who habitually perform aerobic exercise. In preparation.

Jablonski KL, Racine ML, Geolfos CJ, DeVan AE, Gates PE, Chonchol M & Seals DR. Mechanisms of reduced arterial stiffness with dietary sodium restriction in middle-aged and older adults with moderately elevated systolic blood pressure. In preparation.

DeVan AE, Eskurza I, Pierce GL, Walker AE, **Jablonski KL**, Kaplon RE & Seals DR. Impaired fasting blood glucose-related exacerbation of age-associated vascular endothelial dysfunction: protective effect of regular aerobic exercise *(in preparation)*

DeVan AE, Pierce GL, Walker AE, **Jablonski KL** & Seals DR. Vascular endothelial function and systemic markers of oxidative modification, antioxidants and inflammation in adults without clinical disease. In preparation.

Pierce GL, **Jablonski KL**, Walker AE, Seibert S, DeVan AE & Seals DR. Reduced tetrahydrobiopterin bioactivity contributes to decreased carotid artery compliance in middle-aged and older adults. In revision, *Am J Physiol Heart and Circ Physiol*.

Jalal D, **Jablonski K**, McFann K, Chonchol M & Seals DR. Uric acid as a predictor of vascular endothelial function in healthy middle-aged and older adults. In press, *Am J Hypertens*.

Jablonski KL, Chonchol M, Pierce GL, Walker AE & Seals DR. 25-Hydroxyvitamin D deficiency is associated with inflammation-linked vascular endothelial dysfunction in middle-aged and older adults. *Hypertension* 57:63-69, 2011.

Seals DR, **Jablonski KL** & Donato AJ. Aging and vascular endothelial function in humans. *Clin Sci (Lond)* 120:357-75, 2011.

Jablonski KL, Gates PE, Pierce, GL, Eskurza I & Seals DR. Low dietary sodium intake is associated with enhanced vascular endothelial function in middle-aged and older adults with elevated baseline systolic blood pressure. *Ther Adv Cardiovasc Dis* 3: 347-356, 2009.

Donato AJ, Gano LB, Eskurza I, Silver AE, Gates,PE, **Jablonski KL** & Seals DR. Age-associated vascular endothelial dysfunction is related to increased endothelial expression of endothelin-1 in healthy humans. *Am J Physiol Heart and Circ Physiol* 297: H425-H432, 2009.

Donato AJ, AD, Black, AD**, Jablonski KL**, Gano LB & Seals DR. Aging is associated with greater nuclear NFκB, reduced IκBα and increased expression of proinflammatory cytokines in vascular endothelial cells of healthy humans. *Aging Cell* 7: 805-812, 2008.

Donato AJ, Eskurza I, **Jablonski KL**, Gano LB, Lawson BL, Pierce GL & Seals DR. Increased Cytochrome P450 2C9 signaling does not contribute to vascular endothelial dysfunction in healthy older adults. *Journal of Applied Physiology* 105: 1359-1363, 2008.

Jablonski KL, Seals DR, Eskurza I, Monahan KD & Donato AJ. High-dose ascorbic acid infusion abolishes chronic vasoconstriction and restores leg blood flow in healthy older men. *Journal of Applied Physiology* 103: 1715-1721, 2007.

Abstracts

Jablonski KL, Donato AJ, Fleenor BS, Russell MJ, Walker AE & Seals DR. Absence of nuclear factor-κB- mediated increase in large elastic artery stiffness with aging in exercising adults. Submitted to the Annual Experimental Biology Meeting, 2012.

Racine ML, Geolfos CJ, DeVan AE, Gates PE, Chonchol M, Seals DR & **Jablonski KL**. Mechanisms of improved carotid artery compliance with dietary sodium restriction in middle-aged and older adults with moderately elevated systolic blood pressure. Submitted to the Annual Experimental Biology Meeting, 2012.

Pierce GL, Donato AJ, LaRocca TJ, **Jablonski KL** & Seals DR. Endothelium-dependent dilation is inversely related to hematocrit among healthy young and older adults. Submitted to the Annual Experimental Biology Meeting, 2012.

DeVan AE, Eskurza I, Pierce GL, Walker AE, **Jablonski KL**, Kaplon RE & Seals DR. Impaired fasting blood glucose-related exacerbation of age-associated vascular endothelial dysfunction: protective effect of regular aerobic exercise. Submitted to the Annual Experimental Biology Meeting, 2012.

Jalal D, **Jablonski KL**, McFann K, Chonchol M & Seals DR. Uric acid is associated with systemic inflammation and reduced MnSOD expression in endothelial cells of healthy adults. To be presented at the Annual American Society of Nephrology Renal Week, 2011.

Pierce GL, **Jablonski KL**, Walker AE, Siebert SM, Black SM, Shruti S & Seals DR. Reduced tetrahydrobiopterin bioactivity contributes to decreased carotid artery compliance with aging in healthy adults. *Circulation* 2011;P319.

Jablonski KL, Racine ML, Gates PE, Chonchol M, Howell KL & Seals DR. Dietary sodium restriction improves vascular endothelial dysfunction in middle-aged and older adults with moderately elevated systolic blood pressure. *FASEB J*. 25: 818.3, 2011.

DeVan AE, Pierce GL, Walker AE, **Jablonski KL** & Seals DR. Vascular endothelial function and systemic markers of oxidative modification, antioxidants and inflammation in adults without clinical disease. *FASEB J*. 25: 818.8, 2011.

Jalal D, **Jablonski K**, McFann K, Chonchol M & Seals DR. Uric acid as a predictor of vascular endothelial function in healthy middle-aged and older adults. *J Am Soc Nephrol*. 21: F-PO1725, 2010.

Jablonski KL, Pierce GL, Walker AE, Chonchol M & Seals DR. 25-Hydroxyvitamin D deficiency is associated with vascular endothelial dysfunction in middle-aged and older adults. *FASEB J.* 24: 1039.7, 2010.

Jablonski KL, Gates PE, Pierce, GL, Eskurza I Seals DR. Low dietary sodium intake is associated with enhanced vascular endothelial function in older adults with elevated baseline systolic blood pressure. *FASEB J*. 23: 1017.5, 2009.

Jablonski KL, Gano LB, Lawson BL, Pierce GL, Eskurza I, Seals DR & Donato AJ. Increased cytochrome P450 2C9 signaling does not contribute to vascular endothelial dysfunction in healthy older adults. *FASEB J* 22: 967.1, 2008.

Donato AJ, Lawson BR, **Jablonski KL**, Gano LB & Seals DR. Age-related vascular endothelial dysfunction is associated with altered regulation of nuclear factor κ B and increased pro-inflammatory cytokines in humans. *FASEB J.* 22: 964.12, 2008.

Gano LB, Magerko K, Roeca C, Lawson B, **Jablonski KL**, Seals DR & Donato AJ. Inflammatory circulating mononuclear cell phenotype in healthy older adults with low-grade systemic inflammation and endothelial dysfunction. *FASEB J.* 22: 1155.4, 2008.

Jablonski KL, Eskurza I, Monahan KD, Seals DR & Donato AJ. Oxidative stress contributes to the age related decline in basal leg blood flow in sedentary men. *FASEB J.* 21: A1238, 2007.

CHAPTER II

Introduction

Cardiovascular diseases (CVD) remain the leading cause of mortality in modern societies. Importantly, as much as 80% of all CVD are associated with dysfunction and disorders of arteries (107). Advancing age is a/the major risk factor for CVD (73). Thus, aging adversely affects arteries, which increases risk of developing CVD (74). One of the major contributors to the increased risk of CVD with aging is vascular endothelial dysfunction, most commonly assessed in humans as endothelium-dependent dilation (EDD) of a) peripheral conduit arteries in response to a mechanical (shear) stress (i.e. brachial artery flow-mediated dilation; FMD_{BA}) or b) resistance vessels in response to a chemical stimulus (i.e. forearm blood flow to acetylcholine; FBF_{ACh}). Both measures of EDD are clinically important predictors of future CVD and/or CV events (13, 63, 130).

Systolic blood pressure (SBP) increases progressively with age, whereas diastolic BP increases until ~age 50 and then levels off or even declines at older ages (74). The increase in SBP with aging is associated with a marked increase in the prevalence of systolic essential hypertension (SBP$\geq$140 mmHg) (74). 50% of adults $\geq$60 years of age and 75% of adults $\geq$70 years of age have essential hypertension, and this is primarily the result of systolic hypertension (24). Middle-aged and older (MA/O) adults with elevated SBP have a greater risk of CVD compared with their non-hypertensive peers (120). Thus, elevated SBP/systolic hypertension is the primary BP-related risk factor for adults $\geq$50 years of age. The combined effect of age and elevated SBP on CVD risk is of particular importance for the present analyses, which sought to establish the efficacy of treatment of vascular endothelial dysfunction in this setting.

Dietary sodium restriction (DSR) is a lifestyle intervention that may improve vascular endothelial function in MA/O adults with elevated SBP. Blood pressure sensitivity to sodium

intake (“salt sensitivity”) increases with age (129, 138) and is present in >50% of patients with hypertension (129). Indeed, excess sodium intake is considered a causal factor in the development of systolic hypertension with aging (24). High sodium intake also is associated with greater increases in aortic pulse-wave velocity (aPWV) with aging (5), and DSR reduces large elastic artery stiffness and improves carotid artery compliance in MA/O adults with elevated baseline SBP (59, 114). In addition to large elastic artery stiffness, excessive sodium intake causes vascular endothelial dysfunction in peripheral arteries of experimental animals and humans (11, 123, 141). However, it is unknown if low dietary sodium modulates vascular endothelial function in MA/O adults with moderately elevated SBP.

In Chapter IV, I tested the hypothesis that dietary sodium intake is related to EDD in MA/O adults with moderately elevated SBP. This study established that subjects reporting lower dietary sodium intake have greater EDD than their age-matched peers consuming a more typical American diet. However, the study design could only identify an association and could not determine cause-and-effect.

To test the efficacy of DSR for improving vascular endothelial function in this population, I performed a randomized, placebo-controlled, cross-over intervention. In Chapter V, I tested the hypothesis that a low sodium diet (~50 mmol/day) would improve EDD compared to a “normal” (i.e. typical American) sodium diet (~150 mmol/day). Subjects were placed on a low sodium diet for 10 weeks, and sodium was added-back during one half of the 10-week period using slow-release sodium tablets in a randomized, placebo-controlled design.

In humans, impaired EDD with aging is mediated primarily by reduced nitric oxide (NO) bioavailability, as indicated by the absence of age group differences when NO production by endothelial nitric oxide synthase (eNOS) is inhibited (118). The reductions in NO bioavailability are, in turn, mediated by oxidative stress, as indicated by improvement or restoration of EDD with acute infusion of supra-physiological concentrations of the antioxidant, ascorbic acid (vitamin C) (51, 118). Oxidative stress reduces NO bioavailability via excessive production of

reactive oxygen species, including superoxide, which reacts with NO to form peroxynitrite. Peroxynitrite, in turn: a) causes nitration of tyrosine residues on amino acids of proteins (nitrotyrosine), providing a useful cellular marker of oxidative stress (78, 105); and b) oxidizes tetrahydrobiopterin (BH_4), an essential cofactor for NO synthesis by eNOS, converting BH_4 to its inactive form, BH_2. The consequent reduction in bioavailability of BH_4 leads to the "uncoupling" of eNOS, which produces more superoxide and less NO in a vicious cycle that further reduces NO bioavailability (16, 61, 75).

There is much less information as to how excessive sodium intake causes impaired EDD, especially in humans. However, as with aging, the available data from rodents support the idea that sodium suppresses EDD by reducing NO bioavailability as the result of oxidative stress (97, 140, 141), possibly via oxidation and reduced bioavailability of BH_4 (98). Thus, in Chapter V, I also tested the hypothesis that the improvements in EDD with DSR are mediated by increased NO bioavailability, increased BH_4 bioavailability and reduced oxidative stress.

Specific Aims

Specific Aim 1 (Chapter IV): To determine if reduced sodium intake is associated with enhanced EDD in MA/O adults with moderately elevated SBP

Specific Aim 2 (Chapter V): To determine if DSR improves EDD in MA/O adults with moderately elevated SBP

Specific Aim 3 (Chapter V): To determine if the improvements in vascular endothelial function with DSR are mediated by NO and BH_4 bioavailability, and reduced oxidative stress

CHAPTER III

Portions of this chapter were published previously in Clin Sci (Lond) 120:357-75, 2011 (DR Seals, KL Jablonski and AJ Donato)

Review of Literature

Endothelium-Dependent Dilation

***Clinical Significance*.** Despite reductions in death rates from cardiovascular diseases (CVD) over the last 4 decades, CVD remain the leading cause of morbidity and mortality in modern societies. What is less appreciated, perhaps, is that the great majority of CVD (as much as 80%) are associated with dysfunction and disorders of arteries (85). The vascular endothelium is a critical site for development of future CVD (13, 27, 130). The endothelium is a single layer of cells lining the lumen of the vessel wall that interfaces with the blood. Once believed to be merely a structural filter for the passage of molecules between the circulation and vascular wall, it is now known that the vascular endothelium secretes a variety of biologically active compounds that regulate the function and health of arteries. Vascular endothelial dysfunction is viewed as a key antecedent of clinical arterial diseases and serves as a marker of the inherent risk of developing future CVD (62, 130). Given its central role in the development of clinical coronary, cerebrovascular and peripheral artery diseases, vascular endothelial dysfunction is considered an important therapeutic target for reducing the risk of CVD morbidity and mortality (26, 130).

***EDD and Aging*.** Impaired endothelium-dependent dilation (EDD) is the central feature of vascular endothelial dysfunction and is an independent predictor of future clinical atherosclerotic disease and related events (13, 63, 130). In the absence of clinical disease, healthy aging is associated with impaired conduit artery and microvascular EDD (22, 44, 51,

120). In addition, systolic blood pressure (SBP) increases progressively with age, and the age-associated impairments in EDD are exacerbated by the presence of systolic hypertension (37, 113, 119). The combined effect of age and elevated SBP on CVD risk is of particular importance for the present work, which sought to establish the efficacy of treatment of vascular endothelial dysfunction in this setting.

Measurement of EDD in Humans. Although coronary EDD has been assessed in patients with heart disease and in subjects undergoing diagnosis for coronary disease (29, 86, 137), in general, EDD of peripheral arteries has been used to assess vascular endothelial function in humans (13, 34). There are limited data suggesting that peripheral EDD correlates with EDD measured in the coronary arteries (2, 121) and, thus, may reflect disease processes in the coronary circulation. Peripheral artery EDD is assessed by two primary methods: forearm blood flow (FBF) response to endothelium-dependent dilators and flow-mediated dilation (FMD).

The FBF technique uses a chemical stimulus to evoke EDD (58). A pharmacological agonist for nitric oxide (NO) synthesis and release from the vascular endothelium (most often acetylcholine (ACh)) is infused into an artery of a limb (usually the brachial artery) and the consequent increase blood flow to the distal portion of the limb (usually the forearm) is measured using venous occlusion plethysmography (70, 131). A dose-response relation is established and group- or condition-differences are identified either by the slopes of the dose-response curves or the peak blood flows attained. The increase in blood flow reflects the dilation occurring in the resistance vessels (arterioles) of the distal limb. Thus, this technique measures EDD of peripheral resistance vessels in response to a chemical stimulus. The advantage of this methodology is that it allows for “pharmaco-dissection” of physiological mechanisms, such as the role of NO by inhibiting endothelial nitric oxide synthase (eNOS) with N^G-monomethyl-L-arginine (L-NMMA).

The other method, FMD, uses a mechanical stimulus to evoke an EDD (60). This approach involves inflating a cuff on a limb (typically the upper forearm) to a supra-systolic external pressure for several minutes and measuring the dilation in a segment of an artery (typically the brachial artery) proximal to the occlusion in response to the acute increase in blood flow produced by rapid deflation of the cuff (21). The ischemia-evoked dilation of resistance vessels distal to the occlusion produces a marked temporary increase in blood flow ("hyperemic stimulus") in the proximal conduit arteries that, in turn, causes a "flow-mediated dilation" (FMD) of those arteries. Thus, this procedure assesses the ability of peripheral conduit arteries to dilate in response to the physiological stimulus of an acute increase in intravascular shear produced by an increase in blood flow (71). Unlike the FBF technique that assesses EDD of resistance vessels, FMD assesses EDD of conduit arteries.

Experiments performed in animals have established that the responses evoked by both approaches are "endothelium-dependent" because they are abolished after removal of the vascular endothelium (58, 104). Moreover, the responses are primarily (although not completely) mediated by vascular endothelial production and release of NO because they are markedly attenuated by administration of agents that inhibit NO synthesis by eNOS, such as L-NMMA (83, 96).

In both experimental approaches, the possibility that group or condition differences observed are due to other (i.e., "endothelium-independent") mechanisms can be assessed by determining the vasodilatory responses to intra-arterial infusion of sodium nitroprusside (i.a. infusion model) or sublingual administration of nitroglycerin (FMD model). These drugs serve as "NO donors", thus providing a measure of the sensitivity of the vascular smooth muscle cells in the arterial wall to NO (94, 104). An absence of group or condition differences in response to endothelium-independent stimuli in the presence of clear differences in endothelium-dependent responses are interpreted as indicative of vascular endothelium-specific dysfunction in vasodilatory responsiveness.

Mechanisms of Impaired EDD with Aging. Both animal and human data support that physiological mechanisms contributing to the age-associated reduction in EDD include reduced NO bioavailability, reduced tetrahydrobiopterin (BH_4) bioactivity and increased oxidative stress (113). The reduction in bioavailable NO with aging is supported by the fact that the reduction in EDD induced by pharmacological inhibition of NO production by eNOS is smaller with advancing age, with age group differences in EDD no longer existing in the absence of NO synthesis by eNOS (47, 81, 96). This may be the result of either decreased stimulus-evoked NO production, increased NO removal, or both.

Despite consistent observations of reduced NO bioavailability, analysis of arterial tissue in experimental animals indicates decreased, increased and unchanged eNOS expression and/or activation (i.e., phosphorylation at serine 1177) with aging (23, 47, 106, 127). In healthy humans, eNOS protein expression tends to be greater in vascular endothelial cells obtained from the brachial artery of older compared with young adults (46), whereas eNOS phosphorylated at serine 1177 is significantly increased, suggesting a greater state of activation of the enzyme with aging (46). If so, such activation with age in healthy adults may represent an attempt to compensate for low NO bioavailability.

Tetrahydrobiopterin (BH_4) is an essential co-factor for NO synthesis by eNOS (111). When bioavailability of BH_4 is inadequate, eNOS becomes “uncoupled”, resulting in increased synthesis of the superoxide anion instead of NO (28, 75, 128). Administration of BH_4 to young and older humans causes a selective improvement in EDD in older adults (52, 66), which is abolished by L-NMMA (66). This suggests that the improvements are mediated by augmented NO bioavailability. The mechanism responsible for the reduction in BH_4 bioactivity with aging is unclear. In rodents, BH_4 concentrations in arteries have been reported to be either reduced (35, 116) or unchanged (9) with aging. However, consistent with observations in humans, augmenting vascular BH_4 bioavailability in skeletal muscle arterioles of old rats restores NO-mediated flow-induced dilation (35).

The available evidence suggests that the development of oxidative stress also contributes to vascular endothelial function with aging. Nitration of tyrosine residues on proteins (nitrotyrosine) can be taken as a maker of oxidant damage, and nitrotyrosine increases in endothelial cells of humans (45) and arterioles from rodents (30, 47, 81, 106). In healthy adults varying in age, brachial artery FMD is inversely related to circulating markers of oxidative stress (49, 53), as well as to nitrotyrosine staining in vascular endothelial cells (45). An acute reduction in oxidative stress via infusion of antioxidants such as ascorbic acid selectively improves or restores EDD in older adults (51, 118).

The mechanisms contributing to arterial oxidative stress-associated vascular endothelial dysfunction with aging appear to involve increased production of reactive oxygen species in the face of unchanged or reduced antioxidant defenses. Superoxide and other free radical bioactivity are increased with aging in skeletal muscle of humans (6) and in arteries of rodents (18, 64, 106). Antioxidant enzyme expression in vascular endothelial cells is not different in young and older healthy adults (45), whereas expression and activity of these enzymes generally are unchanged or reduced in arteries of experimental animals (36, 47, 106, 132). That superoxide dismutase mimetics restore EDD in arteries of old rodents (81, 106, 122) supports a key role for increased superoxide in age-associated vascular endothelial dysfunction.

The sources of superoxide mediating impaired EDD with aging include upregulation of the oxidant enzyme nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, uncoupling of eNOS and increased mitochondrial production (45, 47, 126, 134). In contrast, the available evidence does not support a role for the oxidant enzymes xanthine oxidase or cytochrome *P*-450 epoxygenase2C9, at least in humans (44, 49). Increased superoxide could reduce NO bioavailability and impair EDD with aging by reacting with NO to form peroxynitrite. In turn, peroxynitrite oxidizes BH_4 to its inactive form, which both reduces NO production and increases superoxide production by eNOS (75).

Dietary Sodium

***Clinical Significance*.** Perhaps the most widely documented and appreciated influence of dietary sodium intake in humans is upon blood pressure, particularly SBP. Blood pressure sensitivity to sodium intake ("salt sensitivity") increases with age (129, 138) and is present in >50% of patients with hypertension (129). Indeed, excess sodium intake is considered a causal factor in the development of systolic hypertension with aging (24), whereas evidence across various cultures supports that low dietary sodium intake slows the age-associated rise in blood pressure (91). Restricting dietary sodium intake has been repeatedly demonstrated to lower blood pressure, particularly SBP (31, 65, 87, 109). However, as discussed below, dietary sodium exerts cardiovascular effects beyond influencing blood pressure alone.

Lifestyle modifications remain the initial strategy for the prevention of age-associated CVD (24, 55, 112). Dietary sodium restriction (DSR) is one such modification with enormous potential. New evidence indicates that DSR may be an even more effective intervention for prevention of CVD and CV events than previously recognized (3, 8, 54, 117). These data project that even modest reductions in sodium intake could reduce the number of new cases of coronary heart disease by 60,000 to 120,000 per year and, over the lifetime of adults aged 40-85, avert 500,000 strokes and 480,000 myocardial infarctions, while increasing quality adjusted life-years by 2.1 million. The CV benefits of DSR would be equivalent to or greater than those previously established for reductions in tobacco use, obesity and cholesterol.

Dietary sodium restriction is a promising lifestyle modification for preventing CVD. As recently stressed by the American Heart Association, the projected health and societal benefits resulting from population-wide dietary sodium restriction are substantial (8). To date, such projections have focused on the effects of sodium restriction on blood pressure. However, independent of its effects on blood pressure, high dietary sodium contributes to other adverse cardiovascular outcomes (39, 57, 110). If DSR improves vascular endothelial function via

physiological mechanisms other than blood pressure lowering alone, the potential impact of reducing dietary sodium consumption upon risk of CVD may be even greater than presently appreciated.

Dietary Sodium and Vascular Function. High sodium intake also is associated with greater increases in aortic pulse-wave velocity (a measure of arterial stiffness) with aging (5). Two previous intervention studies performed in the Seals laboratory have shown that dietary sodium restriction (50-60% reductions from baseline sodium intake to 50-60 mmol/d) reduces large elastic artery stiffness/increases compliance in MA/O adults with elevated baseline SBP, in the absence of changes in body mass or composition, metabolic risk factors or other dietary factors (59, 114). In addition to large elastic artery stiffness, excessive sodium intake also causes vascular endothelial dysfunction in peripheral arteries of experimental animals and humans (11, 39, 123, 141). Individuals with salt sensitive hypertension demonstrate decreased EDD compared to those with salt resistant hypertension, suggesting a causal role of sodium in endothelial dysfunction (15, 77, 92). Thus, reducing sodium intake in MA/O adults with elevated baseline SBP may improve EDD, consistent with observations of increased FMD in overweight/obese adults following sodium restriction (40).

Mechanisms Linking Dietary Sodium and Vascular Function. The adverse influence of high dietary sodium on arterial function is at least in part independent of blood pressure (57, 115, 136). In the most recent DSR intervention study published from the Seals laboratory, the improvements in vascular function remained significant after correcting for reductions in blood pressure using the beta-stiffness index expression of arterial compliance (59). Other evidence in animals and humans also indicates that dietary sodium exerts cardiovascular effects beyond traditionally appreciated influences on blood pressure. Negative consequences of high dietary sodium include left ventricular hypertrophy, fibrosis of the heart, kidney and arteries, functional and other structural changes to target organs, and increased oxidative stress (4, 56, 91).

Rodents resistant to changes in blood pressure in response to increased dietary sodium nevertheless develop vascular endothelial dysfunction (12, 80, 97, 139), and acute administration of high dietary sodium impairs EDD in healthy adults, independent of changes in blood pressure (39, 123).

There is little information in humans as to how excessive sodium intake causes vascular dysfunction. However, data from rodents strongly support the idea that sodium suppresses EDD by reducing NO bioavailability as the result of oxidative stress (97, 140, 141), possibly via oxidation and reduced bioavailability of BH_4 (98). However, no direct evidence is available from humans as to the mechanisms by which DSR may improve vascular function, which is thus a central focus the focus of the present work.

Chapter IV

Low Dietary Sodium Intake is Associated with Enhanced Vascular Endothelial Function in Middle-Aged and Older Adults with Elevated Systolic Blood Pressure

Kristen L Jablonski[1], Phillip E Gates[2], Gary L Pierce[1],

and Douglas R Seals[1]

[1] Department of Integrative Physiology

University of Colorado

Boulder, CO, 80309

[2] Institute of Biomedical and Clinical Sciences

University of Exeter

Exeter, United Kingdom

Ther Adv Cardiovasc Dis. 2009, 3:347-356.

Abstract

OBJECTIVE: Age and increasing systolic blood pressure (BP) are associated with vascular endothelial dysfunction, but the factors involved are incompletely understood. We tested the hypothesis that vascular endothelial function is related to dietary sodium intake among middle-aged and older adults (MA/O) with elevated systolic BP. METHODS: Data were analyzed on 25 otherwise healthy adults aged 48-73 years with high normal systolic BP or stage I systolic hypertension (130-159 mmHg) and diastolic BP <99 mmHg. Self-reported sodium intake was <100 mmol/d in 12 (7M) subjects (low sodium, 73±6 mmol/d) and between 100-200 mmol/d in 13 (9M) subjects (normal sodium, 144±6 mmol/d). RESULTS: Groups did not differ in other dietary factors, age, body weight and composition, BP, metabolic risk factors, physical activity and maximal aerobic capacity. Plasma concentrations of norepinephrine, endothelin-1, oxidized LDL, antioxidant status and inflammatory markers did not differ between groups. Brachial artery FMD was 42% (mmΔ) to 52% (%Δ) higher in the low vs. normal sodium group ($P<0.05$). In the entire cohort, brachial artery FMD was inversely related to dietary sodium intake (FMD mmΔ $r=-0.40$, $P<0.05$; %Δ $r=-0.53$, $P<0.01$). Brachial artery FMD was not related to any other variable. In contrast, endothelium-independent dilation did not differ between groups ($P\geq0.24$) and was not related to sodium intake in the overall group ($P\geq0.29$). CONCLUSIONS: Low sodium intake is associated with enhanced brachial artery FMD in MA/O with elevated systolic BP. These results suggest that dietary sodium restriction may be an effective intervention for improving vascular endothelial function in this high-risk group.

Introduction

Cardiovascular diseases (CVD) remain the leading cause of death in modern societies and this is attributable in large part to dysfunction of the endothelial layer of arteries (13, 130). Aging is associated with development of vascular endothelial dysfunction and is a major risk factor for CVD (21, 74, 120). Systolic blood pressure increases with age and contributes to the age-associated increase in CVD (74, 108). Middle-aged and older adults with elevated systolic blood pressure demonstrate greater vascular endothelial dysfunction and risk of CVD than their peers with normal systolic pressure (118, 120). Thus, identifying factors that influence vascular endothelial function in middle-aged and older adults with elevated systolic blood pressure has important clinical implications for preventing age-associated CVD.

Indirect evidence suggests that dietary sodium intake may be one such factor. Self-reported sodium intake is positively related to CV outcomes among middle-aged and older adults (125) and blood pressure sensitivity to sodium increases with age in healthy adults (48, 138). In rodents, increased sodium intake causes impaired endothelium-dependent dilation, the most common measure of vascular endothelial dysfunction, independent of effects on arterial blood pressure (11, 97, 141). In humans, impaired endothelium-dependent dilation is observed in young healthy men in response to acute sodium loading (123) as well as in patients with essential hypertension who demonstrate blood pressure sensitivity to changes in sodium compared with patients who are resistant to sodium (15, 77, 92). Taken together, these observations suggest that middle-aged and older adults with lower dietary sodium intake may have enhanced vascular endothelial function compared with their peers with higher intake of sodium. However, presently there are no data addressing this issue.

To test this hypothesis, we compared groups of unmedicated middle-aged and older adults with elevated baseline systolic blood pressure who consumed a diet either low or within the average range for sodium intake in the U.S. The groups did not differ in blood pressure and

other subject characteristics, risk factors and diet composition (e.g., potassium intake) that could independently affect vascular endothelial function, allowing us, as much as is possible in an initial cross-sectional investigation, to isolate the effects of dietary sodium intake. Brachial artery flow-mediated dilation (FMD), a measure of endothelium-dependent dilation, was used to assess vascular endothelial function. Endothelium-independent dilation also was assessed as a control to ensure that any effect of dietary sodium intake on brachial FMD reflected an endothelium-dependent influence.

Methods

Subjects. Subjects were 25 middle-aged and older (48-73 years) men (n=16) and postmenopausal women (n=9) with high normal systolic blood pressure or stage I systolic hypertension (130-159 mmHg). Subjects had a diastolic blood pressure <99 mmHg and were otherwise free of cardiovascular diseases, diabetes and other clinical disorders as assessed by medical history, physical examination, blood chemistries and resting and exercise ECG. All subjects were non-smokers, not taking medications (prescription or over the counter) or dietary supplements (including those with antioxidant properties) and not regularly exercising. All procedures were approved by the Human Research Committee of the University of Colorado at Boulder. The nature, benefits and risks of the study were explained to the volunteers and their written informed consent was obtained prior to participation.

Study Procedures. All measurements were performed at the University of Colorado at Boulder General Clinical Research Center (GCRC) after an overnight fast (water only) and a 24-hour abstention from alcohol and vigorous physical activity.

Group Selection. Initially, subjects were identified from our laboratory database according to the inclusion/exclusion criteria described above. Among subjects meeting those

criteria, individuals then were identified as having dietary sodium intake <100 mmol/d (low sodium) or between 100-200 mmol/d (normal sodium). The upper cutoff for the low sodium group was based in part on the Dietary Reference Intake tolerable upper limit for sodium intake of 100 mmol/d (1) and to achieve a low sodium group mean value as close as possible to the Dietary Approaches to Stop Hypertension (DASH) recommended level of ~60 mmol/d (109). The range of 100-200 mmol/d was selected to provide a normal sodium group mean value consistent with the average American dietary sodium intake of ~150 mmol/d (133).

Dietary Analysis. Diet composition and caloric intake were estimated from 3-day food intake records (The Food Processor 8.2, ESHA Research) (99) (n=15, 7 low sodium/8 normal sodium) or food-frequency questionnaires (FFQ) (NHANES III food-intake database) (52) (n=10, 5 low sodium/5 normal sodium) previously analyzed by a GCRC bionutritionist.

Subject Characteristics and Blood Assays. Body mass index (BMI) was calculated from height and weight to the nearest 0.1 kg, and waist and hip circumferences were measured by anthropometry (25). Total body fat was determined using dual energy x-ray absorptiometry (DPX-IQ, GE/Lunar, Inc.) (25). Arterial blood pressure was measured over the brachial artery during seated rest using a semi-automated device (Dynamap XL, Johnson and Johnson) (93). Habitual physical activity was assessed from estimates of daily energy expenditure using the Stanford Physical Activity Questionnaire as previously described (45). Peak oxygen consumption was determined with on-line, computer-assisted, open-circuit spirometry and was used as a measure of maximal aerobic exercise capacity (38).

All assays were performed by the University of Colorado GCRC core laboratory. Plasma total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), very low-density lipoprotein cholesterol (VLDL-C), triglycerides, glucose and insulin were determined using standard assays. The homeostasis model of insulin resistance (HOMA-

IR) was calculated by the formula: [fasting glucose (mg/dl) x fasting insulin μU/L)]/405 (14). Plasma norepinephrine concentrations were measured by high performance liquid chromatography. Serum endothelin-1 (Peninsula Laboratories, Inc), oxidized LDL (Alpco, Inc), total antioxidant status (Randox Laboratories, Inc.), interleukin-6 (IL-6; R&D Systems, Inc) and tumor-necrosis factor-α (TNF-α) were measured by ELISA. Serum concentrations of C-reactive protein were measured using a high-sensitivity Chemistry Immuno Analyzer (AU400e, Olympus America, Inc.).

Brachial Artery FMD and Endothelium-Independent Dilation. Brachial artery FMD (occlusion cuff on the upper forearm), peak shear rate during FMD, and endothelium-independent dilation (brachial artery dilation in response to sublingual nitroglycerin) were determined using duplex ultrasonography (Power Vision 6000, Toshiba) with a linear array transducer as described previously by our laboratory (99, 102). Responses are expressed as mm and percent change from baseline diameter (42). Measurements were performed within two weeks of establishing baseline subject characteristics.

Statistics. Statistical analyses were performed with SPSS (version 16.0). Group differences were determined by t-tests for independent sample comparisons. Pearson correlation analysis was used to assess bivariate relations of interest. Statistical significance for all analyses was set at $P < 0.05$.

Results

Subject Characteristics and Circulating Humoral Factors. Characteristics of the groups are shown in Table 1. There were no differences in age, body mass, BMI, waist:hip ratio, percent total body fat, systolic and diastolic blood pressure, plasma lipids and lipoproteins, plasma glucose and insulin, HOMA, physical activity and peak oxygen consumption between

groups (all P≥0.11). Circulating humoral factors for the groups are shown in Table 2. Plasma norepinephrine, endothelin-1, oxidized LDL, total antioxidant status, C-reactive protein, IL-6 and TNF-α did not differ between groups (all P≥0.16).

Table 1: Subject Characteristics

Table 1. Subject Characteristics

	Normal Na+	Low Na+	P-value
n (m/f)	13 (9/4)	12 (7/5)	
Age (years)	60±2	62±1	0.44
Body Mass (kg)	84±3	80±7	0.61
BMI (kg/m^2)	28.1±0.7	26.3±1.3	0.22
Waist:Hip Ratio	0.94±0.03	0.87±0.03	0.11
Total Body Fat (%)	34±2	34±2	0.90
Systolic BP (mmHg)	138±2	138±1	0.93
Diastolic BP (mmHg)	81±2	79±2	0.68
Total Cholesterol ($mg \cdot dL^{-1}$)	197±7	214±9	0.16
LDL-C ($mg \cdot dL^{-1}$)	121±7	135±9	0.19
HDL-C ($mg \cdot dL^{-1}$)	50±4	50±4	0.92
VLDL-C ($mg \cdot dL^{-1}$)	26±3	28±5	0.71
Triglycerides ($mg \cdot dL^{-1}$)	128±17	142±25	0.62
Glucose ($mg \cdot dL^{-1}$)	95±3	95±3	0.91
Insulin (μU/L)	7.2±1.6	8.3±1.9	0.66
HOMA	1.76±0.46	2.08±0.58	0.67
Physical Activity (MET $hrs \cdot wk^{-1}$)	78±23	42±19	0.24
Peak VO_2 ($ml \cdot kg^{-1} \cdot min^{-1}$)	26±1	25±1	0.82

Data are mean ± S.E.; BMI, body mass index; BP, blood pressure; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; VLDL-C, very low-density lipoprotein cholesterol; HOMA, insulin sensitivity index; (homeostasis model assessment);

physical activity, average daily leisure and occupational activity; MET, metabolic equivalent; VO_2, volume of oxygen consumption

Table 2: Circulating Humoral Factors

Table 2. Circulating Humoral Factors

	Normal Na+	Low Na+	P-value
Norepinephrine (pg/ml)	271±17	336±46	0.16
ET-1 (pg/ml)	6.2±0.4	6.9±0.4	0.26
Oxidized LDL (U/L)	56.1±3.0	63.9±6.2	0.25
Total Antioxidant Status (mmol/L)	1.40±0.09	1.18±0.08	0.24
C-reactive protein (mg/L)	0.76±0.06	0.84±0.22	0.68
IL-6 (pg/ml)	1.8±0.4	1.3±0.1	0.23
TNF-α (pg/ml)	1.7±0.2	1.4±0.1	0.24

Data are mean ± S.E. ET-1, endothelin-1; IL-6, interleukin-6; TNF-alpha, tumor necrosis factor-α

Diet. Diet composition of the groups is shown in Table 3. Sodium intake was 144±6 and 73±6 mmol/d for the normal and low sodium groups, respectively ($P<0.0001$). Potassium and calcium intake, and carbohydrate, fat and protein composition did not differ between groups (all $P\geq0.53$). Total energy intake tended to be lower in the low sodium group ($P=0.07$).

Table 3: Subject Diet Composition

Table 3. Subject Diet Composition

	Normal Na+	Low Na+	P-value
Sodium Intake (mmol/d)	144±6	73±6	<0.01
Potassium Intake (mmol/d)	66±8	64±9	0.89
Calcium (mmol/d)	21±2	20±3	0.75
Carbohydrates (% of total kilojoules/day)	52±3	53±2	0.69
Fat (% of total kilojoules/day)	32±2	31±1	0.53
Protein (% of total kilojoules/day)	16±3	16± 2	0.94
Total Kilojoules/day	8709±475	7140±697	0.07

Data are mean ± S.E.

Brachial Artery FMD and Endothelium-Independent Dilation. Baseline brachial artery diameter was smaller in the low compared with the normal sodium intake group (3.46±0.17 vs. 3.96±0.16 mm, P<0.05), whereas peak shear rate during FMD was similar in the two groups (392±57 vs. 384±42 sec^{-1}, P=0.91). Brachial artery FMD was 42% (mm∆) to 52% (%∆) higher in the low sodium compared with the normal sodium group (both P<0.05) (Figure 1). In contrast, endothelium-independent dilation did not differ between groups (P≥0.24) (Figure 1). In all subjects, brachial artery FMD was inversely related to dietary sodium intake (%∆ r=-0.53, P<0.01; mm∆ r=-0.40, P<0.05) (Figure 2). Sodium intake was not related to endothelium-independent dilation (P≥0.29). FMD and endothelium-independent dilation were not related to any other variable, including baseline brachial artery diameter and total kilojoules/day.

Figure 1.

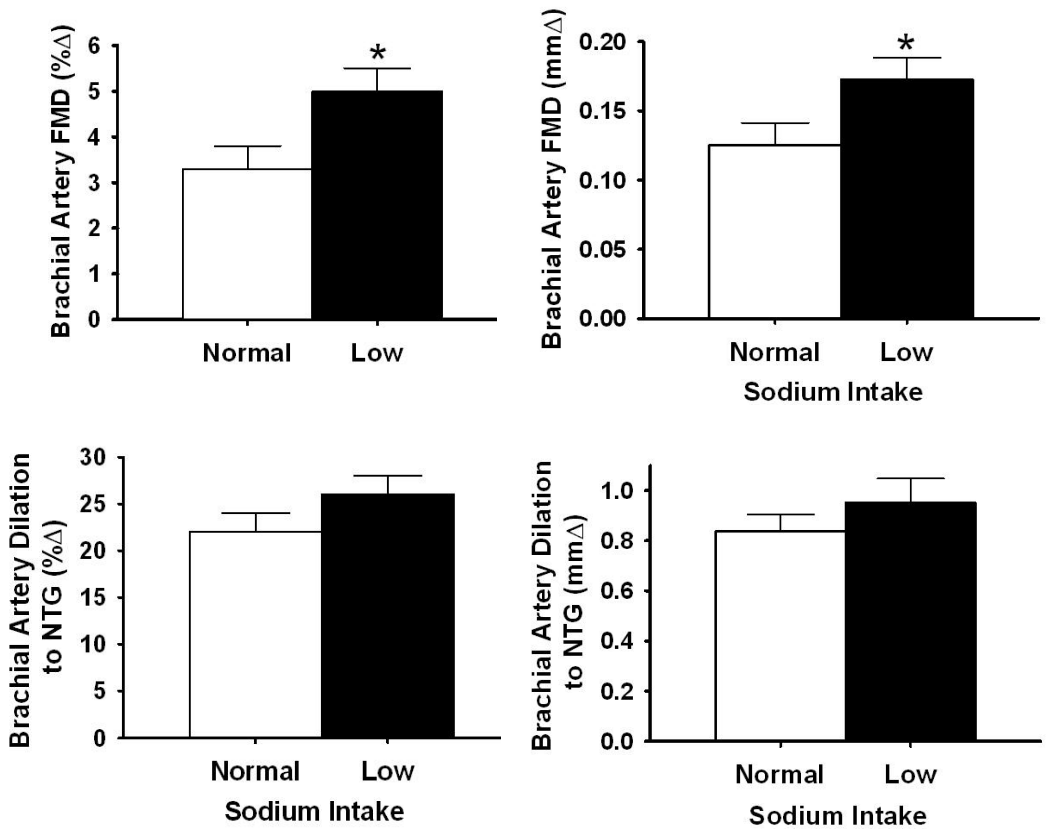

Figure 1. Endothelium-dependent dilation (brachial artery FMD; percentage change (%Δ), absolute change (mmΔ); top) in normal and low dietary sodium groups, and endothelium-independent dilation (brachial artery dilation in response to sublingual nitroglycerin (NTG); bottom) in normal and low dietary sodium groups. Values are mean ± S.E. **P*<0.05 vs normal sodium group.

Figure 2.

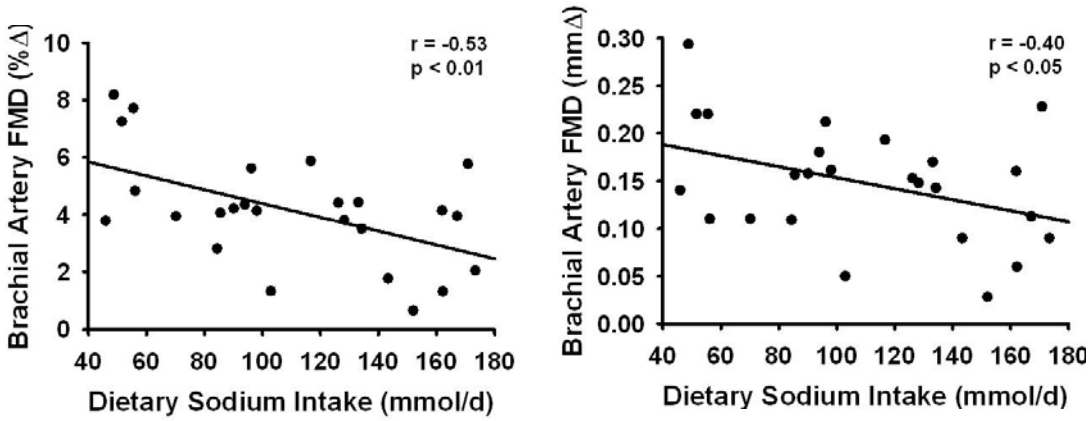

Figure 2. Relation between brachial artery flow-mediated dilation (FMD; percentage change (%Δ); left, absolute change (mmΔ); right) and daily dietary sodium (Na+) intake.

Discussion

To our knowledge this is the first study to report an association between vascular endothelial function and the normal dietary sodium intake of humans. We found that in middle-aged and older adults with elevated baseline systolic blood pressure, brachial artery FMD, a well-established measure of endothelium-dependent dilation and vascular endothelial function, was greater in those with low compared with average sodium intake based on U.S. norms. Consistent with these group differences, brachial FMD was inversely related to dietary sodium intake among individuals in the entire sample. In contrast, no such relations were observed with endothelium-independent dilation. Considered together, these findings indicate that sodium intake in the diet may exert an important influence on vascular endothelial function in this group.

Biomedical Significance. Recent evidence suggests that self-reported sodium intake is positively related to the risk of CV events among middle-aged and older adults (125), suggesting a link with age-associated CVD. Previous investigations in rodents and young adult

humans have established that experimentally increasing sodium intake or sodium loading results in impaired vasodilatory responses to the endothelium-dependent dilator, acetycholine (11, 97, 123).

The present findings extend and complement these earlier observations by demonstrating that in middle-aged and older adults with elevated systolic blood pressure, a group with baseline vascular endothelial dysfunction and increased risk of CVD, brachial artery FMD is inversely related to self-reported dietary sodium intake. Importantly, our results show that the relation between sodium intake and vasodilatory responsiveness is specific to the vascular endothelium, as endothelium-independent dilation did not differ between the low and normal sodium intake groups, nor was it related to sodium intake among individual subjects. Our findings also are important because brachial artery FMD predicts future risk of CV events in middle-aged and older adults (130, 135). Overall, the present results provide new clinical insight that vascular endothelial dysfunction is a possible intermediary pathophysiological mechanism linking dietary sodium intake to future CV events in middle-aged and older adults (125).

Findings from studies in which endothelium-dependent dilation was assessed under experimentally controlled sodium intake conditions in humans have been inconsistent. No effects of dietary sodium restriction on the forearm blood flow responses to acetylcholine was observed in patients with sodium-sensitive or -resistant forms of essential hypertension (67, 92), whereas brachial artery FMD was improved in overweight and obese adults with normal blood pressure (40). The results of the present study are in agreement with the latter investigation, which, together, support an association between dietary sodium intake and brachial artery FMD in groups with impaired baseline function.

Physiological Influence of Sodium Intake. The influence of sodium intake on brachial artery FMD in our study appears to be physiologically significant. Depending on which of the two standard clinical expressions of FMD are used (mmΔ or %Δ), the low vs. normal sodium intake group differences were 42-52%. Moreover, a moderately strong correlation coefficient of r=-0.53 was observed for the relation between brachial FMD and sodium intake among all subjects. As with most correlations, this correlation coefficient likely significantly underestimates the true physiological relation between endothelium-dependent dilation and dietary sodium intake because of the inherent measurement error associated with each of the variables.

In a recent investigation by our laboratory using the same procedures as in the present study, we reported a mean %Δ brachial FMD of 7.2% in healthy young adult men and women with normal blood pressure (n=62) (102). In comparison, the values for the low and normal sodium intake groups in the present study were 5.0% and 3.3%, i.e., 30% vs. 55% lower, respectively, than the values for young healthy controls. Together, these observations suggest that although brachial artery FMD is impaired in middle-aged and older adults with elevated systolic blood pressure regardless of dietary sodium intake, those consuming a diet <100 mmol/d in sodium demonstrate less impairment than those consuming even average amounts of sodium.

Strengths of the Study Design. A major strength of our study was that the low and normal sodium intake groups were carefully selected for similar subject characteristics (e.g., body fatness, physical activity, maximal aerobic capacity) and coronary risk factors (e.g., plasma lipids and lipoproteins, fasting glucose and insulin) (Table 1) that could independently influence brachial FMD. This allowed us to isolate the effects of sodium intake as much as is possible using cross-sectional group comparisons. Consistent with observations in rodents (12, 84) , arterial blood pressure was similar in our two groups and, thus, cannot explain the differences in brachial artery FMD. Several other dietary factors that could influence

endothelium-dependent dilation also were controlled for, including potassium intake (Table 3). This experimental approach resulted in smaller group sizes, but allowed much clearer interpretation of our results than would otherwise be possible. Although estimated total energy intake and brachial artery baseline diameter tended to be or were significantly different, respectively, in the 2 groups, neither was related to FMD.

Limitations of the Study. There are several limitations of our study that we wish to emphasize. We only can provide limited insight into the mechanisms by which low sodium intake was associated with enhanced vascular endothelial function. Results from studies in rodents suggest that sodium intake may be positively related to oxidative stress, which limits nitric oxide bioavailability and impairs endothelium-dependent dilation (97, 140, 141). In the present study, plasma oxidized LDL, an indirect measure of oxidative stress, and plasma total antioxidant status did not differ between groups. Plasma concentrations of norepinephrine, a measure of sympathetic nervous system activity, endothelin-1, a potent vasoconstrictor produced by the vascular endothelium, and the inflammatory markers C-reactive protein, IL-6 and TNF-α also did not differ between groups. However, these plasma measures do not necessarily reflect differences in the vascular endothelium that would directly influence FMD, particularly among a relatively homogeneous group of adults such as those studied here.

Our subjects were otherwise healthy middle-aged and older adults with elevated systolic blood pressure and, therefore, likely to be more sensitive to sodium than younger adults with normal blood pressure. As a result, the relation we observed between brachial FMD and sodium intake may not be apparent in younger adults, as sodium-sensitivity increases with age (48, 138).

In the present study we were able to determine the influence of low compared with average sodium intake. However, we did not have sufficient data on subjects with higher

sodium intake who fit our eligibility requirements to compare with the low and average sodium intake groups. As such, we do not know if higher than average sodium intake is associated with greater impairments in brachial FMD in this population.

Baseline brachial artery diameter was smaller in the low sodium group, likely because of the slightly higher percentage of female subjects compared with the normal sodium group. However, it is unlikely that this confounded the interpretation of our results. First, FMD was not related to baseline diameter. Second, if smaller baseline diameter *per se* contributed to greater FMD we also would expect to see this effect in endothelium-independent dilation, which was not the case. Last, FMD in percent change accounts for differences in baseline diameter and group differences in FMD also were observed with this expression.

Shear rate produced by the hyperemic stimulus also influences FMD. We do not believe this factor explains our group differences in FMD as shear rate did not differ between groups and was not related to FMD among individuals. Moreover, Donald et al. (42) recently established that percent and absolute change in FMD are the most sensitive and reproducible expressions of FMD for depicting differences among groups.

Abdominal fat generally is inversely related to FMD (17) and there was a non-significant (P=0.11) trend for waist:hip ratio to be smaller in our low sodium group (possibly due to a slightly higher percentage of females). However, waist:hip ratio was not related to FMD. Percent body fat was identical in the two groups.

Finally, the available methods of assessing dietary sodium intake in our subjects were limited because of the retrospective nature of the data analysis. Sodium intake was assessed using two different methods, and urinary sodium excretion, an objective measure of sodium intake (32), was available on only a small number of the total subjects (n=6). Despite these limitations, we were able to show associations between brachial artery FMD and sodium intake

in group comparisons and among individuals. Diet composition assessed using FFQ and 3-day diet records generally are in agreement (10, 88, 89), and the % of subjects in each group assessed with these instruments was similar in the present study. Moreover, sodium intake measured by these self-report instruments correlates with urinary sodium excretion (19, 33, 79, 90). In a separate group of 32 middle-aged and older adults (mean 60±1 years, 17M/15F) in whom we previously had obtained one of these self-report measures of sodium intake and urinary sodium excretion, we found surprisingly similar mean values of 126±10 vs. 127±10 mmol/d, respectively, between the two methods.

***Summary and Conclusions*.** In summary, the results of our study suggest that low sodium intake is associated with enhanced brachial artery FMD in middle-aged and older adults with elevated systolic blood pressure. As such, our findings provide evidence for a possible link between dietary sodium intake, the development of vascular endothelial dysfunction and increased risk of CVD in this group. Importantly, our results and those of others (40) provide an experimental basis for conducting an intervention trial aimed at determining the efficacy of dietary sodium restriction for improving vascular endothelial function in this group, as previously demonstrated for large elastic artery stiffness (59).

Chapter V

Dietary sodium restriction reverses vascular endothelial dysfunction in middle-aged and older adults with moderately elevated systolic blood pressure

Kristen L. Jablonski[1], Matthew L. Racine[1], Candace J. Geolfos[1], Phillip E. Gates[2], Michel Chonchol[3], Matthew B. McQueen[4], Douglas R. Seals[1]

[1]Department of Integrative Physiology, University of Colorado, Boulder, CO 80309

[2]Institute of Biomedical and Clinical Sciences, University of Exeter, Exeter, United Kingdom

[3]Division of Renal Diseases and Hypertension, University of Colorado Denver Health Sciences Center, Denver, CO 80262

[4]Department of Integrative Physiology and Institute for Behavioral Genetics, University of Colorado, Boulder, CO 80309

Abstract

Background: Vascular endothelial dysfunction increases with advancing age and elevated systolic blood pressure (SBP), thus interventions that may improve function in this group are clinically significant. I tested the hypothesis that dietary sodium restriction would improve vascular endothelial dysfunction (assessed by endothelium-dependent dilation, EDD) in middle-aged and older adults with moderately elevated SBP (130-159 mmHg) by increasing nitric oxide (NO) and tetrahydrobiopterin (BH_4) bioavailability and reducing oxidative stress. **Methods and Results:** EDD, measured by brachial artery flow-mediated dilation (FMD_{BA}) and forearm blood flow to acetylcholine (FBF_{ACh}), was 71% and 53% (peak FBF_{ACh}) higher following 4 weeks of a low sodium (70$\pm$8 mmol/day) vs. normal sodium (153$\pm$7 mmol/day) diet ($p<0.05$; randomized, cross-over design; 9M/5F; 61$\pm$2 yrs), whereas endothelium-independent dilation (brachial artery dilation to nitroglycerin and FBF to sodium nitroprusside) did not differ between conditions ($p\geq0.50$). Compared to normal sodium, low sodium increased NO bioavailability (significant reduction in FBF_{ACh} with co-infusion of an endothelial nitric oxide synthase inhibitor), increased BH_4 bioavailability (FMD_{BA} no longer improved with oral BH_4) and reduced oxidative stress (FMD_{BA} and FBF_{ACh} no longer improved with ascorbic acid infusion; reduced endothelial cell nitrotyrosine abundance) ($p\leq0.06$). These differences remained when correcting for SBP, and other subject characteristics/dietary factors did not change across conditions. **Conclusions:** These results indicate that dietary sodium restriction reverses vascular endothelial dysfunction in middle-aged and older adults with moderately elevated SBP by enhancing NO and BH_4 bioavailability and reducing oxidative stress, thus the physiological mechanisms modulating benefits are beyond BP lowering alone.

Introduction

Cardiovascular diseases (CVD) remain the leading cause of death in the United States, and risk of CVD increases progressively with age (73, 107). Endothelial dysfunction, commonly assessed as a decline in endothelium-dependent dilation (EDD), is a clinically important predictor of future CVD and/or CV events (13, 63, 130). Whether assessed as brachial artery flow-mediated dilation (FMD_{BA}) or as the forearm blood flow response to acetylcholine (FBF_{ACh}), EDD is reduced in middle-aged and older adults (22, 120). Systolic blood pressure (SBP) also increases with advancing age (74), and the age-associated reduction in EDD is even greater in individuals with essential hypertension (37, 113, 119). Thus, interventions that may improve vascular endothelial dysfunction in middle-aged and older adults with moderately elevated SBP are clinically significant.

Dietary sodium restriction is a promising lifestyle modification for preventing CVD. As recently stressed by the American Heart Association, the projected health and societal benefits resulting from population-wide dietary sodium restriction are substantial (8). To date, such projections have focused on the effects of sodium restriction on blood pressure. However, independent of its effects on blood pressure, high dietary sodium contributes to other adverse cardiovascular outcomes (39, 57, 110). The evidence supporting the relation between sodium and endothelial function has been largely based on animal literature or acute salt loading/cross-sectional observations in humans (11, 39, 69, 123, 141). It is presently unknown if dietary sodium restriction can attenuate the age- and elevated blood pressure-associated decline in vascular endothelial function.

The physiological mechanisms by which dietary sodium restriction may modulate vascular endothelial function in humans are also widely unknown. The available data from rodents support the idea that sodium suppresses EDD by reducing nitric oxide (NO) bioavailability as the result of oxidative stress (97, 140, 141), possibly via oxidation and reduced bioavailability of tetrahydrobiopterin (BH_4) (98). Thus, dietary sodium restriction may improve

EDD by modulating one or more of these factors. If sodium restriction indeed improves vascular endothelial function via physiological mechanisms other than blood pressure lowering alone, the potential impact of reducing dietary sodium consumption upon risk of CVD may be even greater than presently appreciated.

Thus, the aim of the present study was to determine if dietary sodium restriction improves EDD in middle-aged and older adults with moderately elevated SBP and the physiological mechanisms modulating any functional improvements. Using a double-blind, placebo-controlled, randomized, crossover design, I assessed EDD and associated physiological mechanisms under conditions of low and normal dietary sodium intake. A low sodium target of 1,200 mg/day (50 mmol/day) was set, with the anticipation that actual mean intake would fall at ~1,500 mg/d (65 mmol/day), consistent with sodium intake in the Dietary Approaches to Stop Hypertension (DASH) diet (109), as well as recent AHA recommendations (4). This was compared to a typical (e.g. normal sodium) American intake of 3,600 mg/day (150 mmol/day), similar to recent National Health and Nutrition Examination Survey (NHANES) mean intake data (124). I present here novel findings that a low sodium diet reverses endothelial dysfunction by increasing NO and BH_4 bioavailability and reducing oxidative stress.

Methods

Subjects. Subjects were 14 middle-aged and older (51-77 years) men (n=9) and postmenopausal women (n=5). Resting SBP was 130-159 mmHg (i.e. high normal or stage I hypertension) and diastolic blood pressure was <99 mmHg, verified on a minimum of two occasions. Those taking antihypertensive medications were included in the study if blood pressure still fell within the inclusion range despite medication use, because dietary sodium restriction reduces SBP even in subjects on blood pressure lowering medications (103). Thus, it was reasonable to assume that such individuals were as likely to benefit from the present

intervention as those not on antihypertensive medications. All subjects were otherwise free of CVD, diabetes, kidney disease and other clinical disorders as assessed by medical history, physical examination, ankle-brachial index ($\leq$0.9), blood chemistries and resting and exercise ECG. All subjects were non-smokers, had a body-mass index (BMI) <40 and were not taking hormone replacement therapy or dietary supplements known to influence vascular function (including those with antioxidant properties). Subjects were either sedentary or recreationally active, but not aerobically trained. All procedures were approved by the Institutional Review Board of the University of Colorado at Boulder. The nature, benefits and risks of the study were explained to the volunteers and their written informed consent was obtained prior to participation.

Experimental Design. I used a double-blind, placebo-controlled, randomized, crossover design originally described by Cappuccio et. al. (20) and more recently by our laboratory (59). During a ten week intervention period, subjects reduced dietary sodium intake to a target of ~50 mmol/day and were asked to take a total of ten tablets spread across the day with meals. For five of the weeks the tablets were placebo pills, and for the other five weeks the tablets were slow-release sodium chloride tablets (10 mmol (0.23 g) per tablet) (HK Pharma, UK). The slow-release salt tablets aimed to return sodium intake to ~150 mmol/day, consistent with a “normal” sodium intake for an American diet (124). Weekly visits were made during the study to the University of Colorado at Boulder Clinical and Translational Research Center (CTRC) for nutritional consultation to assure adherence to the diet. Vascular measurements were performed after four weeks (i.e. during the fifth week) of each condition following an overnight fast (water only) and a 48-hour abstention from non-prescription medications and a 24-hour abstention from alcohol, vigorous physical activity and prescription medications (including antihypertensive medications). Results are presented for all fourteen subjects except when indicated (due to failed procedures such as arterial catheterizations, venous blood draw,

etc.). Subjects completing a given procedure at one time-point (i.e. low sodium or normal sodium condition) only were included in analyses in accordance with intent to treat.

***Dietary Sodium Restriction*.** Following screening, subjects were provided with comprehensive dietary education and counseling by a CTRC bionutritionist to reduce dietary sodium intake without changing caloric intake, dietary composition or potassium intake. After the initial education session, subjects met weekly throughout the ten week intervention period with the bionutritionist. Each week, blood pressure, body mass and 24-hour urinary sodium and potassium excretion were assessed.

Casual Blood Pressure Measures. Resting casual brachial artery blood pressure measurements were made in a supine position using a semi-automated device (Dynamap XL, Johnson and Johnson) after $\geq$1 hr abstention from caffeine and exercise, in accordance with the Joint National Committee (24), as described previously (59, 68). The average of three readings within 5 mmHg following 15 minutes of rest was recorded. Blood pressure was assessed weekly during the first 4 weeks of each condition and on three separate days during each testing week (the mean value across all three days is reported). We have previously shown that reductions in blood pressure with sodium restriction are the same whether assessed with random zero sphygmomanometry or using a semi-automated device, and that ambulatory blood pressure is also reduced (59).

***Dietary Analysis*.** Subjects were instructed how to keep 3-day dietary records by a CTRC bionutritionist. Records were analyzed using Food Processor software (ESHA Research) at baseline and at the end of each condition of the intervention, as described previously (59).

Subject Characteristics and Blood Assays. To explore any influence of body composition upon responsiveness to a low sodium diet, body-mass index (BMI) was determined at baseline using height and weight to the nearest 0.1 kg, waist and hip circumferences were

measured by anthropometry and total body fat was determined using dual energy x-ray absorptiometry (DPX-IQ, GE/Lunar, Inc.) (99). As we have previously demonstrated that a low sodium diet does not alter body composition (59), only BMI and not total body fat measurements were repeated during the intervention period.

Subjects were asked not to alter their usual physical activity during the study. Habitual physical activity was assessed during each sodium condition using a) estimates of daily energy expenditure using the Stanford Physical Activity Questionnaire as previously described (45) and b) weekly kilocalorie expenditure measured by ActiGraph accelerometers (Model GT3X, ActiGraph, LLC, Fort Walton Beach, FL) recording physical activity at a rate of 30 HZ (60 second epochs) (n=7).

24-hr urinary sodium and potassium excretion were measured by Boulder Community Hospital once a week during the first four weeks and twice (the mean is presented) at baseline and during the testing week of each sodium condition. All blood assays were performed by the University of Colorado CTRC core laboratories or Boulder Community Hospital (n=13). Plasma total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides, glucose, insulin and plasma sodium were determined using standard assays. Estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease (MDRD) formula: $186.3 \times (\text{serum creatinine})^{-1.154} \times \text{age}^{-0.203} \times (0.742 \text{ for women})$, with serum creatinine measured using an enzymatic assay (Beckman Coulter Inc.) (82).

Glutathione peroxidase was determined with the Mira method and total antioxidant status was measured using a commercial kit (both Randox Laboratories, Inc.). Serum oxidized LDL (Mercodia), interleukin-6 (IL-6; R&D Systems, Inc), tumor-necrosis factor-α (TNF-α, R&D Systems, Inc) and endothelin-1 (n=7) (R&D Systems, Inc), were measured by ELISA.

Extracellular superoxide dismutase (ecSOD) activity was measured in the plasma at rest using a commercially available colimetric kit (Cell Technology), as described previously (n=10 low sodium / n=9 normal sodium) (76). Serum concentrations of high sensitivity C-reactive protein were measured using the immunoturbidimetric method (Beckman Coulter). Angiotensin II (Evaluating Data), plasma renin activity (DiaSorin) and aldosterone (Diagnostic Products, Corp.) were measured using conventional radioimmunoassays. Plasma norepinephrine concentrations were measured by high performance liquid chromatography (BioRad Laboratories). Cystatin C was measured using a commercially available particle-enhanced immunonephelometric assay (Dade-Behring).

Brachial Artery FMD and Endothelium-Independent Dilation. Brachial artery flow-mediated dilation (FMD_{BA}) (occlusion cuff on the upper forearm), peak shear rate during FMD_{BA} and endothelium-independent dilation (brachial artery dilation in response to 0.4 mg of sublingual nitroglycerin; NTG) were determined using duplex ultrasonography (Power Vision 6000, Toshiba) with a linear array transducer as described previously by our laboratory (68, 99, 100, 102). Baseline, occlusion (i.e. the average diameter for the 15 s immediately before rapid cuff deflation) and peak brachial artery diameters were analyzed using commercially available software (Vascular Research Tools 5.0; Medical Imaging Applications) by the same investigator, who was blinded to the subject group assignment. Pulsed Doppler signals were recorded at an angle of insonation of $\leq 68^{\circ}$ with a sample volume the entire width of the artery, as described previously (100). Time-averaged peak velocity was obtained from recording the first ten velocity envelopes. Brachial artery peak hyperemic shear rate was calculated as 8 times (due to wide sample volume) the peak velocity immediately following 5 min of forearm occlusion, divided by occlusion diameter (n=11). Responses are expressed as mm and percent change from baseline diameter (42).

Tetrahydrobiopterin (BH_4) modulation of EDD was determined by measuring FMD_{BA} ~3 hrs after oral administration of BH_4 or placebo tablets, when BH_4 reaches its maximal plasma concentration, as described previously (52, 101) (n=12 low sodium / n=13 normal sodium). Because the key comparison was the BH_4-placebo difference in FMD_{BA} during low vs. normal dietary sodium intake states, the order of the placebo (first) and BH_4 (second) sessions was standardized (blinded to subject). Standardizing the order, under these particular conditions, eliminated the measurement variability that would be introduced by randomization. BH_4 is presently only available for oral administration, thus was used to assess modulation of FMD_{BA} but not local FBF_{ACh}. Endothelium-independent dilation to sublingual NTG was also assessed during BH_4 and placebo sessions (n=14 low sodium / n=13 normal sodium, placebo; n=8 low sodium / n=6 normal sodium, BH_4).

The influence of oxidative stress on FMD_{BA} on each of these days (BH_4 and placebo) was determined by infusing a supraphysiological dose of ascorbic acid (American Regent Laboratories Inc., Shirley, NY) or isovolumic saline as described previously by our laboratory and others (50, 51, 95, 118). A priming bolus of 0.06 g ascorbic acid/kg fat-free mass dissolved in 100 mL of saline was infused intravenously at 5 mL/min for 20 min, followed immediately by a "drip-infusion" of 0.02 g/kg fat-free mass dissolved in 30 mL of saline administered over 60 min at 0.5 mL/min. Vascular measurements were made at the end of the 20 min bolus during the "drip infusion", when peak plasma concentrations of ascorbic acid occur (51) (n=14 low sodium / n=13 normal sodium, placebo; n=10 low sodium / n=12 normal sodium, BH_4).

Forearm Blood Flow Assessment of EDD and Endothelium-Independent Dilation. FBF was measured in the experimental (catheterized) and the control forearm with strain-gauge venous occlusion plethysmography (AI6 Arterial Inflow System, Hokanson), as previously described (38, 44). Briefly, a mercury-Silastic strain gauge was placed around the forearm, and a cuff was placed around each upper arm and repeatedly inflated to 60 mmHg to occlude

venous outflow for 7 s. A second cuff was placed around the wrist and inflated to suprasystolic pressures to exclude the hand circulation during FBF measures. Flows were measured during the last minute of each 3-min dose, and the mean value is reported. All FBF values are presented as percent increase in flow (ml · 100 ml forearm tissue^{-1} · min^{-1}) compared to saline control. Forearm vascular conductance (FVC) was calculated from intra-arterial blood pressure and FBF (FVC=FBF/mean arterial pressure) to account for any differences in arterial blood pressure between sodium conditions.

Forearm volume was determined by the water displacement method. Drug infusion rates were normalized per 100 ml of forearm tissue and infused at <4 ml/min by a syringe pump (Harvard). EDD and endothelium-independent dilation were determined as the FBF responses to an incremental intrabrachial artery infusion of acetylcholine (ACh) (1.0, 2.0, 4.0, and 8.0 μg · 100 ml forearm tissue^{-1} · min^{-1}) and sodium nitroprusside (0.5, 1.0, and 2.0 μg · 100 ml forearm tissue^{-1} · min^{-1}), respectively (n=11 low sodium / n=12 normal sodium). To determine the effect of dietary sodium restriction on the nitric oxide (NO) contribution to EDD, NG monomethyl-L-arginine (L-NMMA, 5 mg/min, 10-min loading dose) was co-infused with ACh (same doses as above). This dose of L-NMMA inhibits NO production by competitive inhibition of endothelial NO synthase (eNOS) in the human forearm without causing systemic effects (41). As L-NMMA must be infused locally to avoid systemic changes in blood pressure, it was only co-administered during assessment of FBF_{ACh} and not during measurement of FMD_{BA}.

Endothelial Cell Protein Expression. The procedures used for collection of arterial endothelial cells and measurement of protein expression using immunofluorescence have been described in detail previously by our laboratory (43, 45, 68, 102). Briefly, J-wires were advanced into the brachial artery ~4 cm beyond the tip an 18-guage catheter and withdrawn. Cells were recovered by washing and centrifugation, fixed with 3.7% formaldehyde and plated on slides. Non-specific binding sites were blocked with 5% donkey serum (Jackson

Immunoresearch) and cells were incubated with monoclonal antibodies for nitrotyrosine (Abcam), total eNOS and eNOS phosphorylated at Ser 1177 (BD Biosciences) (n=11 low sodium / n=12 normal sodium).

Slides were systematically viewed with a fluorescence microscope (Eclipse 600, Nikon, Melville, NY) to identify endothelial cells (positive staining for VE cadherin) and nuclear integrity was confirmed with DAPI (4',6'-diamidino-2-phenylindole hydrochloride) staining. Images were then captured and analyzed with Metamorph Software (Universal Imaging Corp, Downington, PA) to quantify the intensity of CY3 staining (i.e., average pixel intensity). The values for each protein are reported as a ratio of arterial endothelial cell to human umbilical vein endothelial cell (HUVEC) average pixel intensity, which minimizes the possible confound of differences in staining intensity among different staining sessions. A single investigator analyzed each batch of cells and was blinded to subject identification.

Statistics. The changes in EDD in response to dietary sodium restriction (normal vs. low sodium intake) were analyzed in a mixed-effects regression model with factors for condition (normal vs. low sodium), drug (ascorbic acid, BH_4 (FMD_{BA}); ascorbic acid, L-NMMA (FBF_{ACh})), and dose (FBF_{ACh} only). There was no evidence of a carryover effect, thus sequence of the intervention (low vs. normal sodium first) was not included in the model. Covariates, such as systolic blood pressure, were included in the model as appropriate. Differences in other outcomes and characteristics between sodium conditions/vs. baseline were assessed using paired t-tests (between sodium conditions) or repeated measures ANOVA with post-hoc Bonferonni corrected comparisons (sodium conditions vs. baseline). C-reactive protein, IL-6 and TNF-α were logarithmically transformed to improve the normality of these distributions. The bivariate relations between FMD_{BA} and urinary sodium excretion and SBP were determined using Pearson correlation coefficient. Statistical significance for all analyses was set at $P < 0.05$. All data are reported as means $\pm$ S.E.

Results

Subject Characteristics and Dietary Sodium Restriction. Subject characteristics prior to the start of the dietary intervention are presented in Table 1. 86% of the subjects were non-Hispanic Caucasian and 14% were Asian. Three of the fourteen subjects were on an anti-hypertensive medication. Subjects successfully reduced sodium intake (152±11 mmol/d, baseline; 153±7 mmol/d, normal sodium condition (week 5); 70±8 mmol/d, low sodium condition (week 5)) without altering potassium intake (Figure 1). As anticipated, SBP was reduced during the low vs. normal sodium condition, but other characteristics did not change (Table 2; values are for week 5). The low sodium condition also reduced SBP as assessed during the first four weeks of the intervention ($p<0.05$ vs. NS; not shown). Dietary composition other than sodium intake did not change (Table 3). Circulating markers of oxidative stress, inflammation, the renin angiotensin aldosterone system, endothelin-1, norepinephrine, cystatin-C and insulin were also unchanged with the low sodium diet (Table 4).

Table 1: Baseline Characteristics

Baseline Characteristics	**Baseline**
n (m/f)	14 (9/5)
Age (years)	61±2
Body Fat (%)	29±3
Waist Circumference (cm)	88±3
Waist:Hip (ratio)	0.87±0.03
Medication Use	Hypothyroidism drugs (n=3) ACE-inhibitor (n=1) Ca^{2+} channel blocker (n=1) Diuretic (n=1) Anti-depressant (n=1)

Data are mean ± S.E. ACE, angiotensin converting enzyme.

Figure 1.

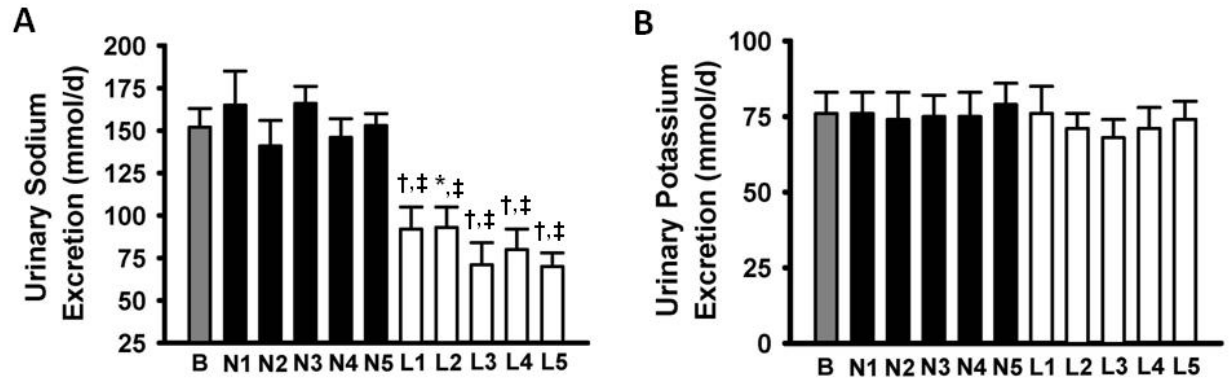

Figure 1. 24-hour urinary excretion of sodium (panel A) and potassium (panel B) at baseline (B), weeks 1-5 of the normal sodium condition (N1-N5) and weeks 1-5 of the low sodium condition (L1-L5). Values are mean ± S.E.; * P<0.05 vs. normal sodium of same week; † P<0.01 vs. normal sodium of same week; ‡ P<0.01 vs. baseline.

Table 2: Subject Characteristics

Subject Characteristics	**Baseline**	**LS**	**NS**
Body Mass (kg)	78.4±4.3	77.2±4.3	77.6±4.3
BMI ($kg \cdot m^2$)	26.6±1.0	25.9±1.1	26.0±1.1
Systolic Blood Pressure (mmHg)	139±2	128±3*	140±4
Diastolic Blood Pressure (mmHg)	83±2	79±2	81±2
Total Cholesterol ($mg \cdot dL^{-1}$)	194±8	184±8	193±6
LDL Cholesterol ($mg \cdot dL^{-1}$)	121±6	114±6	125±7
HDL-Cholesterol ($mg \cdot dL^{-1}$)	52±5	50±5	50±4
Triglycerides ($mg \cdot dL^{-1}$)	110±18	99±12	93±13
Fasting Glucose ($mg \cdot dL^{-1}$)	83±5	84±4	82±4
24-hr Urinary Creatinine Excretion ($mg \cdot d^{-1}$)	1382±78	1304±85	1297±103
PA (Actigraph) ($kcals \cdot wk^{-1}$)	N/A	3137±290	3129±360
PA (questionnaire) (MET $hrs \cdot wk^{-1}$)	40±11	28±8	29±8
Plasma Sodium (mmol)	139.2±0.7	137.9±0.9	139.0±0.5
MDRD eGRF ($mL \cdot min^{-1} \cdot 1.73m^2$)	83±4	83±4	88±6

Data are mean ± S.E. * P<0.05 vs. baseline or NS. BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; PA, physical activity; MET, metabolic equivalent; eGFR, estimated glomerular filtration rate (Modification of Diet in Renal Disease (MDRD) Study Equation).

Table 3: Diet Composition

Diet Composition	**Baseline**	**LS**	**NS**
Total kcals per day	2100±112	2037±146	1989±122
Carbohydrates (g·day^{-1})	249±12	258±21	247±16
Protein (g·day^{-1})	88±6	76±6	83±4
Fat (g·day^{-1})	76±8	69±10	71±12
Calcium (mmol·day^{-1})	23±2	18±2	20±1
Magnesium (mmol·day^{-1})	11±1	11±1	10±1
Potassium (mmol·day^{-1})	63±5	65±5	67±5
Sodium (mmol·day^{-1}) †	130±11	56±4*	59±3*
Fruits and Vegetables (cups·day^{-1})	3.7±0.6	3.8±0.4	4.1±0.5

Data are mean ± S.E; * P<0.01 vs. baseline. † Note, sodium intake is based on 3-day diet records and excludes sodium added back in pill form.

Table 4: Circulating Humoral Factors

Circulating Humoral Factors	**LS**	**NS**	**P-value**
Glutathione Peroxidase ($U \cdot L^{-1}$)	8032±861	7769±742	0.82
Oxidized LDL ($U \cdot L^{-1}$)	43.6±2.6	40.0±2.5	0.32
Total Antioxidant Status ($mmol \cdot L^{-1}$)	1.45±0.03	1.42±0.03	0.34
ecSOD Activity (% inhibition rate)	59.7±1.8	57.2±1.6	0.32
Log C-Reactive Protein ($mg \cdot L^{-1}$)	0.27±0.15	0.13±0.15	0.53
Log Interleukin-6 ($pg \cdot mL^{-1}$)	0.11±0.06	0.05±0.09	0.61
Log Tumor Necrosis Factor α ($pg \cdot mL^{-1}$)	0.07±0.03	0.08±0.04	0.87
Angiotensin II ($pg \cdot mL^{-1}$)	5.3±0.7	4.6±0.6	0.44
Plasma Renin Activity ($ng \cdot mL^{-1} \cdot hr^{-1}$)	0.76±0.3	0.49±0.1	0.46
Aldosterone ($ng \cdot dL^{-1}$)	4.4±0.7	3.0±0.5	0.11
Endothelin-1 ($pg \cdot mL^{-1}$)	5.8±0.5	6.8±0.5	0.19
Norepinephrine ($pg \cdot mL^{-1}$)	398±51	406±40	0.91
Cystatin C ($mg \cdot L^{-1}$)	0.71±0.03	0.72±0.03	0.75
Insulin ($\mu U \cdot mL^{-1}$)	11.1±1.2	9.0±0.9	0.20

Data are mean ± S.E. LDL, low-density lipoprotein; ecSOD, extracellular superoxide dismutase.

Dietary Sodium Restriction Improved FMD_{BA} without Changing Endothelium-Independent Dilation to Sublingual NTG. FMD_{BA} (%Δ) was 71% greater under the low sodium vs. normal sodium condition (6.11±0.62 vs. 3.57±0.44; $p<0.01$; Figure 2, panel A). Results were similar when expressed as mm change (0.23±0.02 vs. 0.14±0.02; $p<0.01$). The individual subject response to dietary sodium restriction is shown in Figure 2, panel B, demonstrating improved FMD_{BA} in 12 of the 14 subjects. Panel C displays a significant inverse relation between sodium excretion and FMD_{BA} with data points across the two sodium conditions pooled ($r=-0.51$, $p<0.01$). Baseline diameter and shear rate did not differ across sodium conditions or with ascorbic acid and/or BH_4 administration ($p\geq0.21$; not shown). There was a significant interaction term between sodium condition and the following characteristics: gender, medication use and anti-hypertensive medication use. Females and those on medications/blood pressure lowering medications tended to be more responsive to dietary sodium restriction; $p<0.01$. When co-varying for all of these factors, FMD_{BA} was still significantly greater during the low sodium condition ($p<0.01$). The difference across sodium conditions remained significant when SBP was added as a covariate ($p<0.01$), and the change in SBP with sodium restriction was not correlated with the change in FMD_{BA} ($r=-0.05$, $p=0.86$). In contrast to FMD_{BA}, endothelium-independent dilation to sublingual NTG was unchanged by dietary sodium restriction (Figure 2, panel D; %Δ; mmΔ not shown; $p\geq0.50$).

Figure 2.

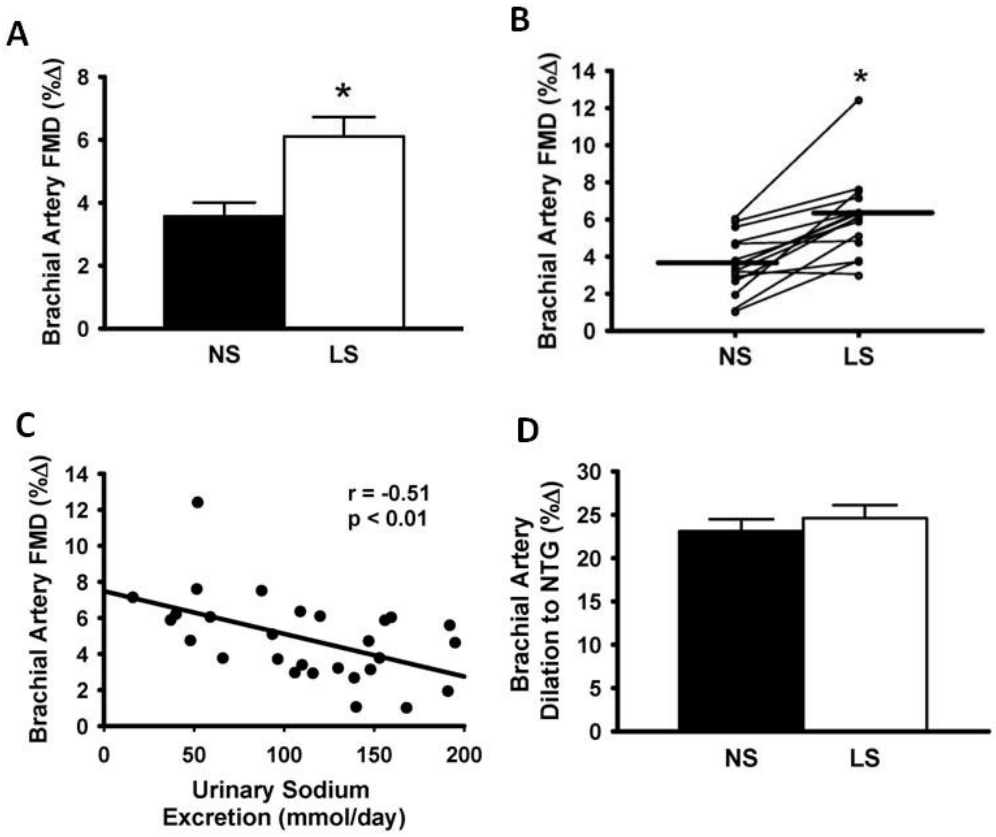

Figure 2. Brachial artery FMD (percent change (%Δ), panel A) during the normal vs. low sodium condition; individual subject FMD (%Δ) response between sodium conditions (panel B); relation between 24-hour urinary sodium excretion and FMD (%Δ) with pooled sodium conditions (panel C); brachial artery endothelium-independent dilation to sublingual nitroglycerin (NTG) during the normal vs. low sodium condition (percent change (%Δ), panel D). Values are mean ± S.E.; NS, normal sodium; LS, low sodium; *P<0.01 vs. NS.

Dietary Sodium Restriction improved FBF_{ACh} without Changing in Endothelium-Independent Dilation (FBF to Sodium Nitroprusside). The low sodium condition improved EDD to ACh (% increase vs. saline control), whether expressed as FBF (53% increase at peak dose) or as the blood pressure corrected expression, forearm vascular conductance (68%

increase at peak dose) (Figure 3; panels A/B; both $p<0.05$). In contrast, FBF and forearm vascular conductance in response to the endothelium-independent dilator sodium nitroprusside did not change across sodium conditions (Figure 3; panels C/D, $p \geq 0.80$). Results for all panels were similar when area under the curve was compared across sodium conditions (panels A/B, $p<0.05$; panels C/D, $p \geq 0.94$). FBF in the control arm did not change with sodium condition or drug infusions (not shown).

Figure 3.

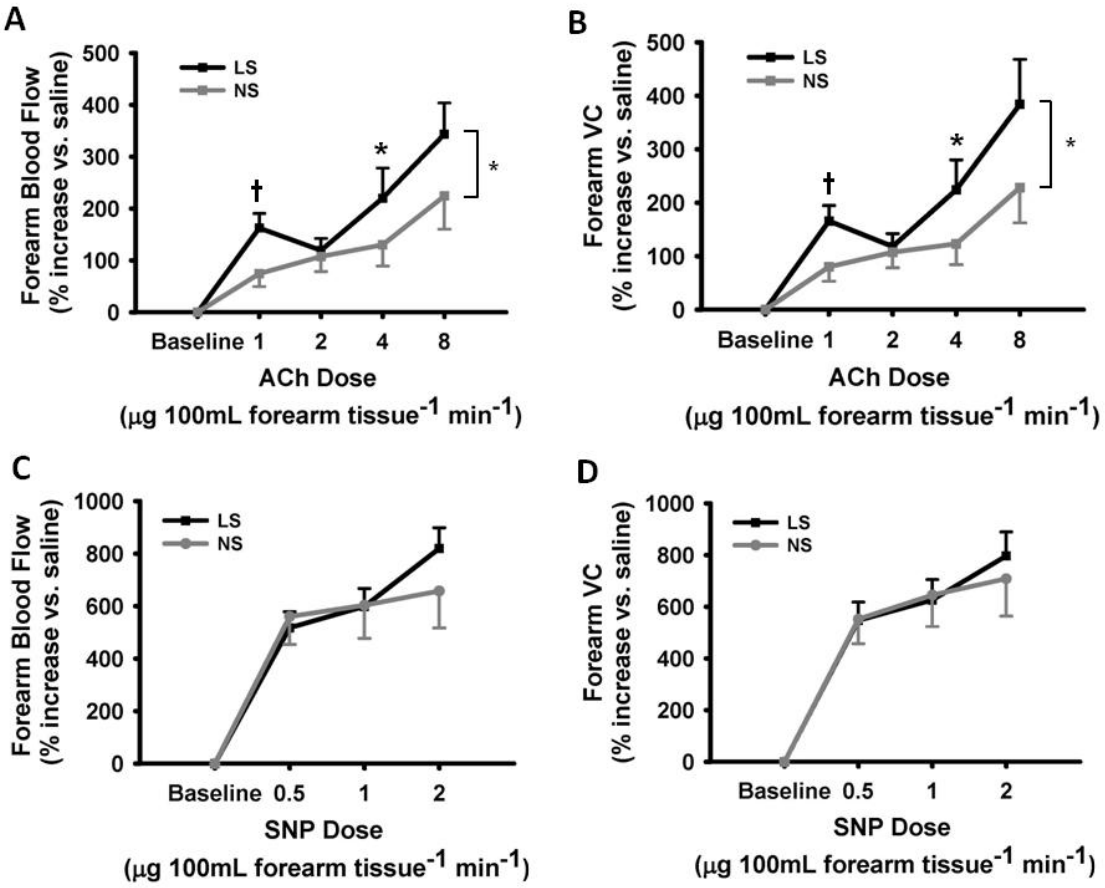

Figure 3. Forearm blood flow (panel A) and forearm vascular conductance (VC) (panel B) response to increasing doses of the endothelium-dependent dilator acetylcholine (ACh) (% increase vs. saline control) during the normal vs. low sodium condition; forearm blood flow (panel C) and forearm vascular conductance (VC) (panel D) response to increasing doses of the

endothelium-independent dilator sodium nitroprusside (SNP) (% increase vs. saline control) during the normal vs. low sodium condition. Values are mean ± S.E.; Bracketed * refers to the overall response across all doses; NS, normal sodium; LS, low sodium; * P<0.05 LS vs. NS; † P<0.01 LS vs. NS.

The Improvement in EDD was Mediated by Increased NO Bioavailability. Co-infusion of the eNOS inhibitor L-NMMA reduced FBF_{ACh} (50% decrease at peak ACh dose) during the low sodium (Figure 4B; p<0.01) but not the normal sodium condition (Figure 4A; p=0.90), indicating increased NO bioavailability during the low sodium condition. Results were similar when area under the curve was compared between the two conditions (panel A, p=0.98; panel B, p=0.07) and when EDD was expressed as forearm vascular conductance instead of FBF_{ACh} (not shown). However, arterial endothelial cell protein expression of eNOS and PeNOS were unchanged across sodium conditions (Figures 4C and 4D; $p \geq 0.41$).

Figure 4.

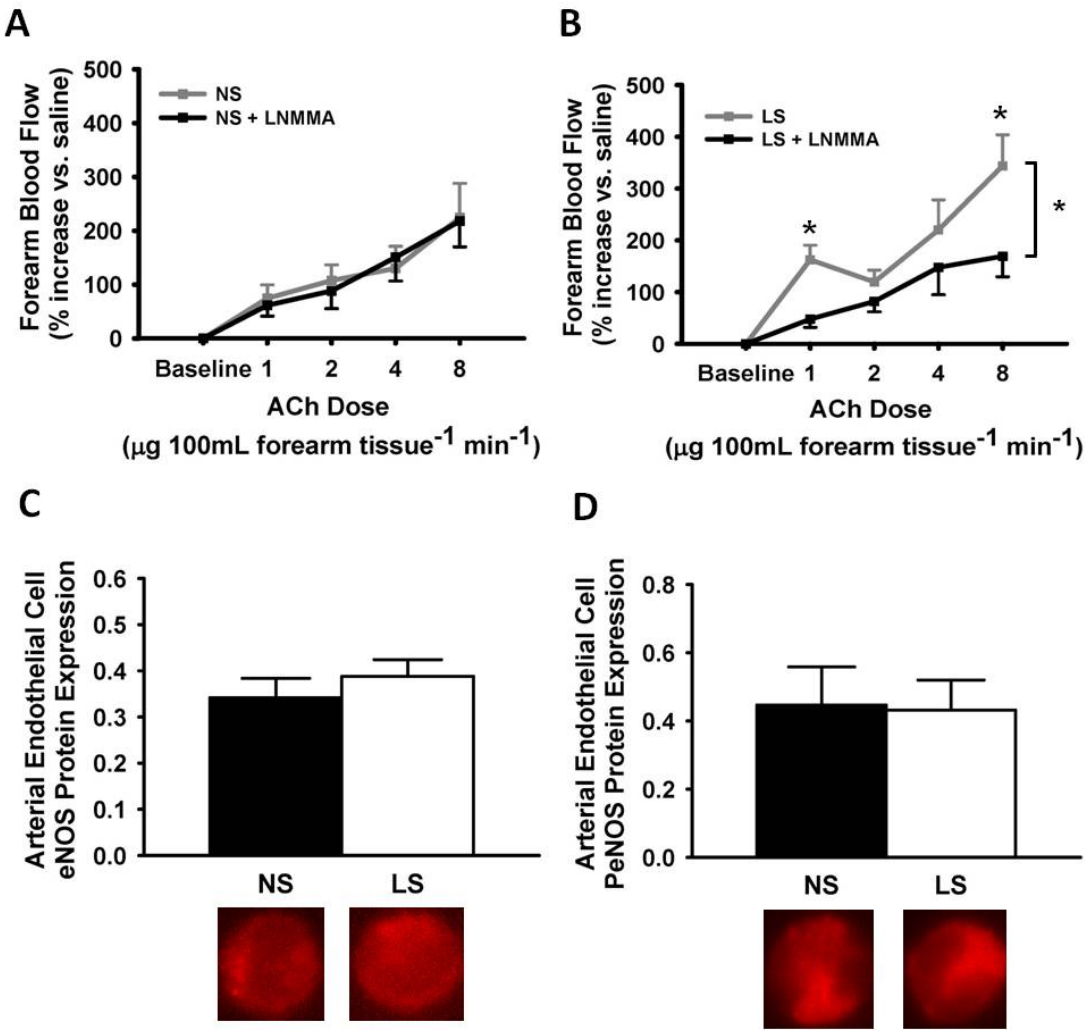

Figure 4. Forearm blood flow to increasing doses of acetylcholine (ACh) at baseline (gray line; ACh only) and during co-infusion of *N*G monomethyl-L-arginine (black line; L-NMMA) under the normal sodium (NS; panel A) and low sodium (LS; panel B) condition (% increase vs. saline control); arterial endothelial cell protein expression of endothelial nitric oxide synthase (eNOS; panel C) and eNOS phosphorylated at Ser 1177 (PeNOS; panel D) according to sodium condition (relative to human umbilical vein endothelial cell (HUVEC) control; representative images shown below). Values are mean ± S.E; bracketed comparison refers to the overall response across all doses; * P<0.01.

The Improvement in EDD was Mediated by Increased BH_4 Bioavailability and Decreased Oxidative Stress. FMD_{BA} was improved with a systemic infusion of ascorbic acid during the normal sodium (46% increase) but not low sodium condition (Figure 5A; $p<0.01$ for repeated measures of saline vs. ascorbic acid). Similarly, oral BH_4 improved FMD_{BA} under the normal sodium (66% increase) but not low sodium condition (Figure 5A; $p<0.01$ for repeated measures of saline vs. BH_4), without altering SBP during either sodium condition ($p\geq0.40$). Improvements in FMD_{BA} were similar when ascorbic acid and BH_4 were co-administered (Figure 5A; $p<0.01$ for repeated measures of saline vs. ascorbic acid + BH_4). These results persisted when covarying for SBP ($p<0.01$ for all three FMD_{BA} comparisons above). Endothelium-independent dilation to NTG did not change with ascorbic acid + BH_4 during either sodium condition ($p\geq0.40$; not shown). Co-infusion of ascorbic acid also tended to improve FBF_{ACh} (50% increase at peak ACh dose) under the normal sodium (Figure 5B; $p=0.05$) but not low sodium condition (Figure 5C; $p=0.20$). Results were similar when area under the curve was compared between the two conditions (panel B, $p<0.01$; panel C, $p=0.60$) and when EDD was expressed as forearm vascular conductance instead of FBF_{ACh} (not shown). Brachial artery endothelial cell nitrotyrosine abundance, a marker of oxidative damage, tended to be reduced with low dietary sodium (Figure 5D; $p=0.06$).

Figure 5.

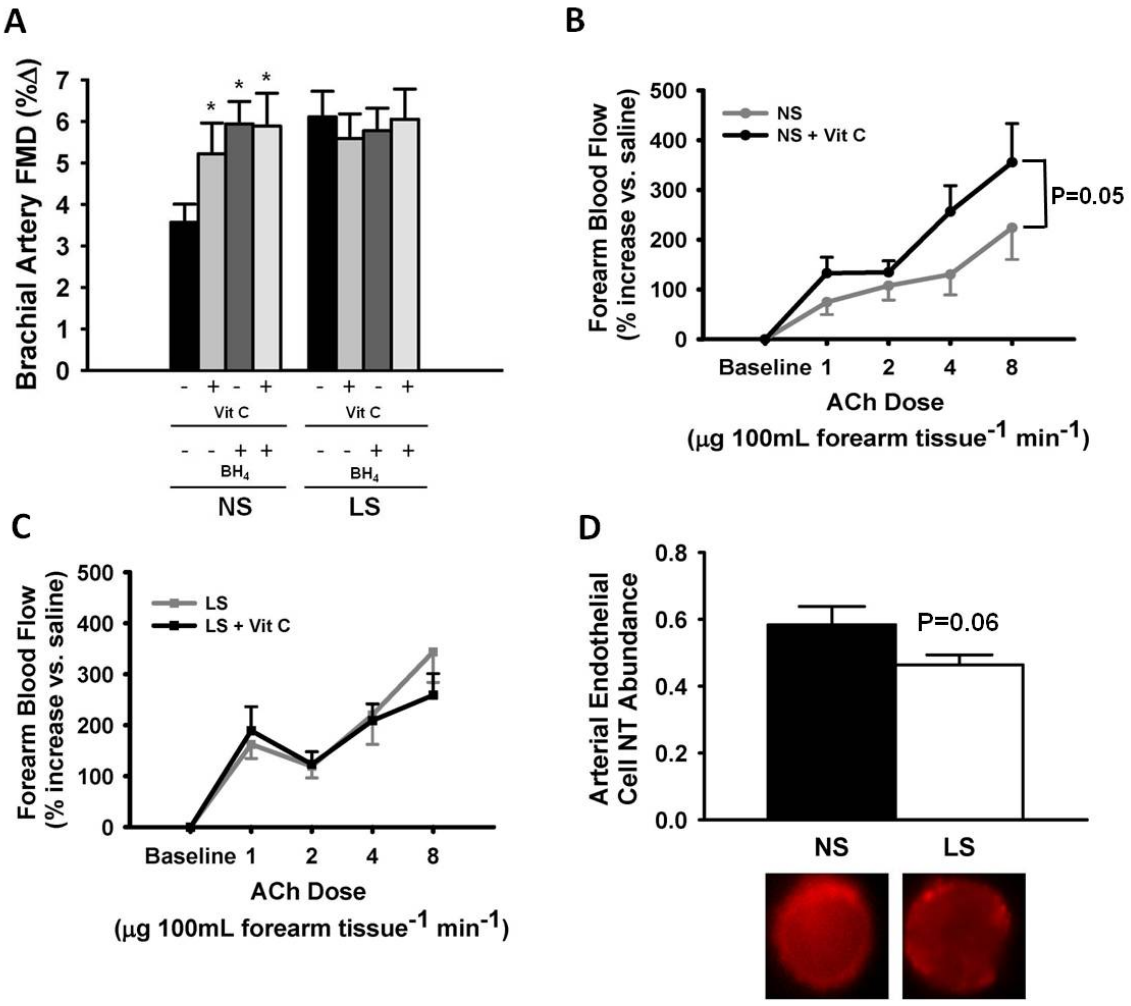

Figure 5. Brachial artery flow-mediated dilation (FMD, %Δ) during the normal vs. low sodium condition according to co-administration of ascorbic acid (Vit C) and/or tetrahydrobiopterin (BH_4) (panel A); forearm blood flow response to increasing doses of acetylcholine (ACh) at baseline (ACh only) and during co-infusion of ascorbic acid (Vit C) under the normal sodium (NS; panel B) and low sodium (LS; panel C) condition (% increase vs. saline control); arterial endothelial cell nitrotyrosine (NT) abundance according to sodium condition (relative to human umbilical vein endothelial cell (HUVEC) control; representative images shown below) (panel D). Values

are mean ± S.E; bracketed comparison refers to the overall response across all doses; * P<0.01.

Discussion

The findings of the present study are the first to show that restricting dietary sodium intake to a level consistent with the DASH diet and AHA recommendations improves EDD in middle-aged and older adults with moderately elevated SBP, whether assessed as FMD_{BA} or FBF_{ACh}. I have also provided the first insight in humans regarding the physiological mechanisms contributing to improvements in vascular endothelial function with dietary sodium restriction. These results demonstrate that the mechanisms linking sodium to cardiovascular risk are likely far more complex than via blood pressure modulation alone, as dietary sodium restriction increased NO bioavailability, increased BH_4 bioavailability and reduced oxidative stress.

Dietary Sodium Restriction and Vascular Endothelial Function with Aging/Elevated SBP. My results demonstrate that dietary sodium restriction improves EDD as assessed by FMD_{BA}, a measure of peripheral conduit artery dilation to a mechanical (shear) stress, and FBF_{ACh}, an assessment of resistance vessel dilation in response to a chemical stimulus. These findings are consistent with impairment in EDD to either a shear stress or chemical stimulus in animals (11, 12) and humans (39, 123) following a high sodium load. The demonstrated benefits of dietary sodium restriction upon vascular function extend previous findings of reduced arterial stiffness (59, 114) and improved FMD_{BA} (40) in select populations. Importantly, the magnitude of improvement in both FMD_{BA} (71%) and peak FBF_{ACh} (53%) was quite large, similar to or greater than functional improvements we have found in our laboratory with aerobic exercise (FMD_{BA}, 54% (100); peak FBF_{ACh}, 30% (38)) and weight loss (FMD_{BA}, 30%; peak FBF_{ACh}, 26% (99)) interventions. The improvements in vascular endothelial

function with dietary sodium restriction do not appear to have been mediated by changes in body composition, blood lipids, glucose, kidney function, physical activity, diet composition or circulating humoral factors, as these values did not change between sodium conditions.

Dietary Sodium Restriction, EDD and Physiological Mechanisms. I have demonstrated that the improvement in EDD with dietary sodium restriction is mediated by increased NO and BH_4 bioavailability and reduced oxidative stress. Importantly, the reduction in EDD with advancing age is mediated in part by decreased NO (44, 118) and BH_4 (52, 66) bioavailability and increased oxidative stress (51, 118). Thus, dietary sodium restriction may be an effective strategy to attenuate these age-related changes. While this is the first available mechanistic insight into functional improvements in EDD with sodium restriction in humans, the results are consistent with findings in animals and non-intervention studies in humans.

High salt feeding in rodents reduces NO bioavailability, which contributes to impaired EDD (11, 141). In humans, salt loading acutely impairs EDD in young normotensive adults also by reducing NO bioavailability (123), and the impairment in EDD in salt-sensitive compared to salt-resistant adults with hypertension is associated with reductions in bioavailable NO (15, 77). In the present study, I have shown that low dietary sodium increases NO bioavailability without altering arterial eNOS expression. Thus, it is reasonable to hypothesize that the demonstated improvements in BH_4 bioavailability are contributing to the increase in NO. Due to limitations in the delivery of BH_4 (presently only available orally, thus cannot be used during assessment of FBF_{ACh}) and L-NMMA (can only be infused locally, or else systemic blood pressure would be altered), this concept could not be directly evaluated in the present work.

The finding that sodium modulates BH_4 bioavailability is novel, though consistent with a recent study in high salt fed mice (98). High dietary sodium in rodents also impairs EDD by increasing oxidative stress, indicated by increased oxidative damage (7, 72), increased superoxide production (7, 97), reduced antioxidant activity (80) and improved EDD with the addition of an antioxidant (97). In the present study, subjects no longer demonstrated functional

improvements in FMD_{BA} and FBF_{ACh} with administration of ascorbic acid under the low sodium condition, indicating reduced oxidative stress compared to the normal sodium condition. The lack of significant change in circulating markers of oxidative stress, in conjunction with a reduction in vascular endothelial cell nitrotyrosine abundance, support the concept that dietary sodium restriction reduces oxidative stress locally at the vascular endothelium.

The reduction in SBP between sodium conditions, 12 mmHg, was very similar to our previous studies on dietary sodium restriction (59, 114). In contrast to our previous work, I allowed inclusion of subjects on antihypertensive medications and still demonstrated similar reductions in SBP. Importantly, the improvement in FMD_{BA} remained significantly different between sodium conditions after statistically correcting for SBP, and the change in FMD_{BA} was not correlated with the change in SBP. Similarly, FBF_{ACh} was significantly improved during the low sodium condition when evaluated as the blood pressure corrected expression forearm vascular conductance. Thus, dietary sodium restriction improved vascular endothelial function beyond its influence on SBP alone.

The concept that high sodium has adverse cardiovascular effects independent of blood pressure has been advanced in recent years by animal and human literature. High dietary sodium has been repeatedly shown to impair EDD even in rodents that are salt resistant, thus do not exhibit increases in blood pressure in response to a high sodium diet (11, 80, 84, 98). Acute impairment of FMD_{BA} in normotensive adults following a high salt load is also blood pressure independent (39), and adults with elevated SBP who report lower sodium intake have enhanced FMD_{BA} independent of blood presssure (69). The present findings lend support to an overall hypothesis advanced by Frolich (56) that salt loading not only elevates arterial blood pressure, but also contributes to adverse functional alterations to target organs. Thus, sodium restriction may help reverse age-associated alterations in vascular function, beyond its benefit of blood pressure lowering.

Limitations. Due to complexity of the study design, including multiple measures of vascular function and pharmacodissection of contributing physiological mechanisms, there was a relatively small number of study participants. Thus, results may not be extrapolated to all individuals and future work should explore the efficacy of dietary sodium restriction for improving vascular function in other populations. While the interaction between gender/medication use and responsiveness to dietary sodium restriction was intruiging, the study was not powered to perform sub-group analyses. Future work should follow-up on the significant interaction term found between these factors and sodium condition.

Conclusions. The results of the present study support the hypothesis that dietary sodium restriction to a level consistent with the DASH diet and AHA recommendations improves vascular endothelial function, as assessed by FMD_{BA} or FBF_{ACh}. A low sodium diet improves EDD via mechanisms independent of/in addition to reductions in SBP, including an increase in NO and BH_4 bioavailability and reduction in oxidative stress.

Perspectives. Dietary sodium intake in modern societies such as the United States greatly exceeds current recommendations (4, 124). Sodium restriction improves reverses vascular endothelial dysfunction, and the physiological mechanisms modulating this improvement are beyond blood pressure lowering alone. Thus, the potential impact of lowering dietary sodium upon risk of CVD may be even greater than previously appreciated. These findings lend support to the American Heart Association's recent call to action for a population-wide effort to reduce sodium consumption (4).

CHAPTER VI

Conclusions

I used two different approaches to examine the modulation of vascular endothelial function by dietary sodium intake in middle-aged and older adults with moderately elevated systolic blood pressure (SBP). First, I found that lower self-reported sodium intake is associated with enhanced endothelium-dependent dilation (EDD), as assessed by brachial artery flow-mediated dilation (FMD_{BA}). Second, I used a randomized, placebo-controlled cross-over intervention to demonstrate that dietary sodium restriction improves EDD, as assessed by both FMD_{BA} and the forearm blood flow response to acetylcholine (FBF_{ACh}). I also showed that enhancements in nitric oxide (NO) and tetrahydrobiopterin (BH_4) bioavailability and reductions in oxidative stress mediated the improvements in EDD with a low sodium diet. NO bioavailability was increased with dietary sodium restriction, as co-infusion the endothelial nitric oxide synthase (eNOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA) reduced FBF_{ACh} when subjects were undergoing the low sodium, but not normal sodium condition. Consistent with aging studies, subjects demonstrated improvements in EDD with acute administration of BH_4 and ascorbic acid while on the normal sodium diet. However, BH_4 and/or ascorbic acid no longer improved EDD during the low sodium condition, indicating that reduced oxidative stress and increased BH_4 bioavailability contributed to the improvement in EDD. Oxidative damage, as measured by endothelial cell nitrotyrosine abundance, was also reduced with the low sodium diet. The functional improvements in EDD were independent of reductions in SBP.

In summary, dietary sodium restriction improves EDD in middle-aged and older adults with moderately elevated SBP. The physiological mechanisms mediating reductions in vascular dysfunction are independent of/in addition to reductions in SBP, including increased NO and

BH_4 bioavailability and decreased oxidative stress. Thus, the potential impact of lowering dietary sodium upon risk of cardiovascular diseases may be even greater than previously appreciated. As dietary sodium intake in modern societies such as the United States greatly exceeds current recommendations, these findings lend support to the American Heart Association's recent call to action for a population-wide effort to reduce dietary sodium consumption.

CHAPTER VII

Bibliography

1. Dietary Reference Intakes for Water, Potassium, Sodium, Chloride, and Sulfate. Washington, DC: National Academies of Science 2004.

2. **Anderson TJ, Uehata A, Gerhard MD, Meredith IT, Knab S, Delagrange D, Lieberman EH, Ganz P, Creager MA, Yeung AC, and et al.** Close relation of endothelial function in the human coronary and peripheral circulations. J Am Coll Cardiol 26: 1235-1241, 1995.

3. **Appel LJ and Anderson CA.** Compelling evidence for public health action to reduce salt intake. N Engl J Med 362: 650-652, 2010.

4. **Appel LJ, Frohlich ED, Hall JE, Pearson TA, Sacco RL, Seals DR, Sacks FM, Smith SC, Jr., Vafiadis DK, and Van Horn LV.** The Importance of population-wide sodium reduction as a means to prevent cardiovascular disease and stroke: A call to action from the American Heart Association. Circulation 123: 1138-1143, 2011.

5. **Avolio AP, Deng FQ, Li WQ, Luo YF, Huang ZD, Xing LF, and O'Rourke MF.** Effects of aging on arterial distensibility in populations with high and low prevalence of hypertension: comparison between urban and rural communities in China. Circulation 71: 202-210, 1985.

6. **Bailey DM, McEneny J, Mathieu-Costello O, Henry RR, James PE, McCord JM, Pietri S, Young IS, and Richardson RS.** Sedentary aging increases resting and exercise-induced intramuscular free radical formation. J Appl Physiol 109: 449-456, 2010.

7. **Bayorh MA, Ganafa AA, Socci RR, Silvestrov N, and Abukhalaf IK.** The role of oxidative stress in salt-induced hypertension. Am J Hypertens 17: 31-36, 2004.

8. **Bibbins-Domingo K, Chertow GM, Coxson PG, Moran A, Lightwood JM, Pletcher MJ, and Goldman L.** Projected effect of dietary salt reductions on future cardiovascular disease. N Engl J Med 362: 590-599, 2010.

9. **Blackwell KA, Sorenson JP, Richardson DM, Smith LA, Suda O, Nath K, and Katusic ZS.** Mechanisms of aging-induced impairment of endothelium-dependent relaxation: role of tetrahydrobiopterin. Am J Physiol Heart Circ Physiol 287: H2448-2453, 2004.

10. **Block G, Woods M, Potosky A, and Clifford C.** Validation of a self-administered diet history questionnaire using multiple diet records. J Clin Epidemiol 43: 1327-1335, 1990.

11. **Boegehold MA.** Effect of dietary salt on arteriolar nitric oxide in striated muscle of normotensive rats. Am J Physiol 264: H1810-1816, 1993.

12. **Boegehold MA.** Flow-dependent arteriolar dilation in normotensive rats fed low- or high-salt diets. Am J Physiol 269: H1407-1414, 1995.

13. **Bonetti PO, Lerman LO, and Lerman A.** Endothelial dysfunction: a marker of atherosclerotic risk. Arterioscler Thromb Vasc Biol 23: 168-175, 2003.

14. **Bonora E, Targher G, Alberiche M, Bonadonna RC, Saggiani F, Zenere MB, Monauni T, and Muggeo M.** Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. Diabetes Care 23: 57-63, 2000.

15. **Bragulat E, de la Sierra A, Antonio MT, and Coca A.** Endothelial dysfunction in salt-sensitive essential hypertension. Hypertension 37: 444-448, 2001.

16. **Brocq ML, Leslie SJ, Milliken P, and Megson IL.** Endothelial dysfunction: from molecular mechanisms to measurement, clinical implications, and therapeutic opportunities. Antioxid Redox Signal 10: 1631-1674, 2008.

17. **Brook RD, Bard RL, Rubenfire M, Ridker PM, and Rajagopalan S.** Usefulness of visceral obesity (waist/hip ratio) in predicting vascular endothelial function in healthy overweight adults. Am J Cardiol 88: 1264-1269, 2001.

18. **Brown KA, Chu Y, Lund DD, Heistad DD, and Faraci FM.** Gene transfer of extracellular superoxide dismutase protects against vascular dysfunction with aging. Am J Physiol Heart Circ Physiol 290: H2600-2605, 2006.

19. **Caggiula AW, Wing RR, Nowalk MP, Milas NC, Lee S, and Langford H.** The measurement of sodium and potassium intake. Am J Clin Nutr 42: 391-398, 1985.

20. **Cappuccio FP, Markandu ND, Carney C, Sagnella GA, and MacGregor GA.** Double-blind randomised trial of modest salt restriction in older people. Lancet 350: 850-854, 1997.

21. **Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, Lloyd JK, and Deanfield JE.** Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet 340: 1111-1115, 1992.

22. **Celermajer DS, Sorensen KE, Spiegelhalter DJ, Georgakopoulos D, Robinson J, and Deanfield JE.** Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. J Am Coll Cardiol 24: 471-476, 1994.

23. **Cernadas MR, Sanchez de Miguel L, Garcia-Duran M, Gonzalez-Fernandez F, Millas I, Monton M, Rodrigo J, Rico L, Fernandez P, de Frutos T, Rodriguez-Feo JA, Guerra J, Caramelo C, Casado S, and Lopez F.** Expression of constitutive and inducible nitric oxide synthases in the vascular wall of young and aging rats. Circ Res 83: 279-286, 1998.

24. **Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL, Jr., Jones DW, Materson BJ, Oparil S, Wright JT, Jr., and Roccella EJ.** Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. Hypertension 42: 1206-1252, 2003.

25. **Christou DD, Gentile CL, DeSouza CA, Seals DR, and Gates PE.** Fatness is a better predictor of cardiovascular disease risk factor profile than aerobic fitness in healthy men. Circulation 111: 1904-1914, 2005.

26. **Cooke JP.** The endothelium: a new target for therapy. Vasc Med 5: 49-53, 2000.

27. **Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, and Vogel R.** Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. J Am Coll Cardiol 39: 257-265, 2002.

28. **Cosentino F, Barker JE, Brand MP, Heales SJ, Werner ER, Tippins JR, West N, Channon KM, Volpe M, and Luscher TF.** Reactive oxygen species mediate endothelium-dependent relaxations in tetrahydrobiopterin-deficient mice. Arterioscler Thromb Vasc Biol 21: 496-502, 2001.

29. **Cox DA, Vita JA, Treasure CB, Fish RD, Alexander RW, Ganz P, and Selwyn AP.** Atherosclerosis impairs flow-mediated dilation of coronary arteries in humans. Circulation 80: 458-465, 1989.

30. **Csiszar A, Ungvari Z, Edwards JG, Kaminski P, Wolin MS, Koller A, and Kaley G.** Aging-induced phenotypic changes and oxidative stress impair coronary arteriolar function. Circ Res 90: 1159-1166, 2002.

31. **Cutler JA, Follmann D, and Allender PS.** Randomized trials of sodium reduction: an overview. Am J Clin Nutr 65: 643S-651S, 1997.

32. **Dahl LK.** Sodium intake of the American male: implications on the etiology of essential hypertension. Am J Clin Nutr 6: 1-7, 1958.

33. **Day N, McKeown N, Wong M, Welch A, and Bingham S.** Epidemiological assessment of diet: a comparison of a 7-day diary with a food frequency questionnaire using urinary markers of nitrogen, potassium and sodium. Int J Epidemiol 30: 309-317, 2001.

34. **Deanfield JE, Halcox JP, and Rabelink TJ.** Endothelial function and dysfunction: testing and clinical relevance. Circulation 115: 1285-1295, 2007.

35. **Delp MD, Behnke BJ, Spier SA, Wu G, and Muller-Delp JM.** Ageing diminishes endothelium-dependent vasodilatation and tetrahydrobiopterin content in rat skeletal muscle arterioles. J Physiol 586: 1161-1168, 2008.

36. **Demaree SR, Lawler JM, Linehan J, and Delp MD.** Ageing alters aortic antioxidant enzyme activities in Fischer-344 rats. Acta Physiol Scand 166: 203-208, 1999.

37. **Deng YB, Wang XF, Le GR, Zhang QP, Li CL, and Zhang YG.** Evaluation of endothelial function in hypertensive elderly patients by high-resolution ultrasonography. Clin Cardiol 22: 705-710, 1999.

38. **DeSouza CA, Shapiro LF, Clevenger CM, Dinenno FA, Monahan KD, Tanaka H, and Seals DR.** Regular aerobic exercise prevents and restores age-related declines in endothelium-dependent vasodilation in healthy men. Circulation 102: 1351-1357, 2000.

39. **Dickinson KM, Clifton PM, and Keogh JB.** Endothelial function is impaired after a high-salt meal in healthy subjects. Am J Clin Nutr 93: 500-505, 2011.

40. **Dickinson KM, Keogh JB, and Clifton PM.** Effects of a low-salt diet on flow-mediated dilatation in humans. Am J Clin Nutr 89: 485-490, 2009.

41. **Dietz NM, Engelke KA, Samuel TT, Fix RT, and Joyner MJ.** Evidence for nitric oxide-mediated sympathetic forearm vasodiolatation in humans. J Physiol 498 (Pt 2): 531-540, 1997.

42. **Donald AE, Halcox JP, Charakida M, Storry C, Wallace SM, Cole TJ, Friberg P, and Deanfield JE.** Methodological approaches to optimize reproducibility and power in clinical studies of flow-mediated dilation. J Am Coll Cardiol 51: 1959-1964, 2008.

43. **Donato AJ, Black AD, Jablonski KL, Gano LB, and Seals DR.** Aging is associated with greater nuclear NFkappaB, reduced IkappaBalpha, and increased expression of proinflammatory cytokines in vascular endothelial cells of healthy humans. Aging Cell 7: 805-812, 2008.

44. **Donato AJ, Eskurza I, Jablonski KL, Gano LB, Pierce GL, and Seals DR.** Cytochrome P-450 2C9 signaling does not contribute to age-associated vascular endothelial dysfunction in humans. J Appl Physiol 105: 1359-1363, 2008.

45. **Donato AJ, Eskurza I, Silver AE, Levy AS, Pierce GL, Gates PE, and Seals DR.** Direct evidence of endothelial oxidative stress with aging in humans: relation to impaired endothelium-dependent dilation and upregulation of nuclear factor-kappaB. Circ Res 100: 1659-1666, 2007.

46. **Donato AJ, Gano LB, Eskurza I, Silver AE, Gates PE, Jablonski K, and Seals DR.** Vascular endothelial dysfunction with aging: endothelin-1 and endothelial nitric oxide synthase. Am J Physiol Heart Circ Physiol 297: H425-432, 2009.

47. **Durrant JR, Seals DR, Connell ML, Russell MJ, Lawson BR, Folian BJ, Donato AJ, and Lesniewski LA.** Voluntary wheel running restores endothelial function in conduit arteries of old mice: direct evidence for reduced oxidative stress, increased superoxide dismutase activity and down-regulation of NADPH oxidase. J Physiol 587: 3271-3285, 2009.

48. **Elliott P, Stamler J, Nichols R, Dyer AR, Stamler R, Kesteloot H, and Marmot M.** Intersalt revisited: further analyses of 24 hour sodium excretion and blood pressure within and across populations. Intersalt Cooperative Research Group. BMJ 312: 1249-1253, 1996.

49. **Eskurza I, Kahn ZD, and Seals DR.** Xanthine oxidase does not contribute to impaired peripheral conduit artery endothelium-dependent dilatation with ageing. J Physiol 571: 661-668, 2006.

50. **Eskurza I, Monahan KD, Robinson JA, and Seals DR.** Ascorbic acid does not affect large elastic artery compliance or central blood pressure in young and older men. Am J Physiol Heart Circ Physiol 286: H1528-1534, 2004.

51. **Eskurza I, Monahan KD, Robinson JA, and Seals DR.** Effect of acute and chronic ascorbic acid on flow-mediated dilatation with sedentary and physically active human ageing. J Physiol 556: 315-324, 2004.

52. **Eskurza I, Myerburgh LA, Kahn ZD, and Seals DR.** Tetrahydrobiopterin augments endothelium-dependent dilatation in sedentary but not in habitually exercising older adults. J Physiol 568: 1057-1065, 2005.

53. **Franzoni F, Ghiadoni L, Galetta F, Plantinga Y, Lubrano V, Huang Y, Salvetti G, Regoli F, Taddei S, Santoro G, and Salvetti A.** Physical activity, plasma antioxidant capacity, and endothelium-dependent vasodilation in young and older men. Am J Hypertens 18: 510-516, 2005.

54. **Frieden TR and Briss PA.** We can reduce dietary sodium, save money, and save lives. Ann Intern Med 152: 526-527, W182, 2010.

55. **Fries JF.** Measuring and monitoring success in compressing morbidity. Ann Intern Med 139: 455-459, 2003.

56. **Frohlich ED.** The salt conundrum: a hypothesis. Hypertension 50: 161-166, 2007.

57. **Frohlich ED and Varagic J.** The role of sodium in hypertension is more complex than simply elevating arterial pressure. Nat Clin Pract Cardiovasc Med 1: 24-30, 2004.

58. **Furchgott RF and Zawadzki JV.** The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288: 373-376, 1980.

59. **Gates PE, Tanaka H, Hiatt WR, and Seals DR.** Dietary sodium restriction rapidly improves large elastic artery compliance in older adults with systolic hypertension. Hypertension 44: 35-41, 2004.

60. **Gerova M, Gero J, Barta E, Dolezel S, Smiesko V, and Levicky V.** Neurogenic and myogenic control of conduit coronary a.: a possible interference. Basic Res Cardiol 76: 503-507, 1981.

61. **Griendling KK and FitzGerald GA.** Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. Circulation 108: 1912-1916, 2003.

62. **Halcox JP, Schenke WH, Zalos G, Mincemoyer R, Prasad A, Waclawiw MA, Nour KR, and Quyyumi AA.** Prognostic value of coronary vascular endothelial dysfunction. Circulation 106: 653-658, 2002.

63. **Halcox JPJ, Donald AE, Ellins E, Witte DR, Shipley MJ, Brunner EJ, Marmot MG, and Deanfield JE.** Endothelial function predicts progression of carotid intima-media thickness. Circulation 119: 1005-1012, 2009.

64. **Hamilton CA, Brosnan MJ, McIntyre M, Graham D, and Dominiczak AF.** Superoxide excess in hypertension and aging: a common cause of endothelial dysfunction. Hypertension 37: 529-534, 2001.

65. **He FJ and MacGregor GA.** Effect of longer-term modest salt reduction on blood pressure. Cochrane Database Syst Rev: CD004937, 2004.

66. **Higashi Y, Sasaki S, Nakagawa K, Kimura M, Noma K, Hara K, Jitsuiki D, Goto C, Oshima T, Chayama K, and Yoshizumi M.** Tetrahydrobiopterin improves aging-related

impairment of endothelium-dependent vasodilation through increase in nitric oxide production. Atherosclerosis 186: 390-395, 2006.

67. **Higashi Y, Sasaki S, Nakagawa K, Kimura M, Noma K, Sasaki S, Hara K, Matsuura H, Chayama K, and Oshima T.** Sodium chloride loading does not alter endothelium-dependent vasodilation of forearm vasculature in either salt-sensitive or salt-resistant patients with essential hypertension. Hypertens Res 24: 711-716, 2001.

68. **Jablonski KL, Chonchol M, Pierce GL, Walker AE, and Seals DR.** 25-Hydroxyvitamin D deficiency is associated with inflammation-linked vascular endothelial dysfunction in middle-aged and older adults. Hypertension 57: 63-69, 2011.

69. **Jablonski KL, Gates PE, Pierce GL, and Seals DR.** Low dietary sodium intake is associated with enhanced vascular endothelial function in middle-aged and older adults with elevated systolic blood pressure. Ther Adv Cardiovasc Dis, 2009.

70. **Joyner MJ, Dietz NM, and Shepherd JT.** From Belfast to Mayo and beyond: the use and future of plethysmography to study blood flow in human limbs. J Appl Physiol 91: 2431-2441, 2001.

71. **Kelm M.** Flow-mediated dilatation in human circulation: diagnostic and therapeutic aspects. Am J Physiol Heart Circ Physiol 282: H1-5, 2002.

72. **Kitiyakara C, Chabrashvili T, Chen Y, Blau J, Karber A, Aslam S, Welch WJ, and Wilcox CS.** Salt intake, oxidative stress, and renal expression of NADPH oxidase and superoxide dismutase. J Am Soc Nephrol 14: 2775-2782, 2003.

73. **Lakatta EG.** Age-associated cardiovascular changes in health: Impact on cardiovascular disease in older persons. Heart Failure Reviews 7: 29-49, 2002.

74. **Lakatta EG and Levy D.** Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part I: aging arteries: a "set up" for vascular disease. Circulation 107: 139-146, 2003.

75. **Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, and Harrison DG.** Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J Clin Invest 111: 1201-1209, 2003.

76. **Landmesser U, Merten R, Spiekermann S, Buttner K, Drexler H, and Hornig B.** Vascular extracellular superoxide dismutase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation. Circulation 101: 2264-2270, 2000.

77. **Larrousse M, Bragulat E, Segarra M, Sierra C, Coca A, and de La Sierra A.** Increased levels of atherosclerosis markers in salt-sensitive hypertension. Am J Hypertens 19: 87-93, 2006.

78. **Leeuwenburgh C, Hardy MM, Hazen SL, Wagner P, Oh-ishi S, Steinbrecher UP, and Heinecke JW.** Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. J Biol Chem 272: 1433-1436, 1997.

79. **Leiba A, Vald A, Peleg E, Shamiss A, and Grossman E.** Does dietary recall adequately assess sodium, potassium, and calcium intake in hypertensive patients? Nutrition 21: 462-466, 2005.

80. **Lenda DM and Boegehold MA.** Effect of a high salt diet on microvascular antioxidant enzymes. J Vasc Res 39: 41-50, 2002.

81. **Lesniewski LA, Connell ML, Durrant JR, Folian BJ, Anderson MC, Donato AJ, and Seals DR.** B6D2F1 mice are a suitable model of oxidative stress-mediated impaired endothelium-dependent dilation with aging. J Gerontol A Biol Sci Med Sci 64: 9-20, 2009.

82. **Levey A, Greene T, Kusek J, and Beck G.** A simplified equation to predict glomerular filtration rate from serum creatinine. J Am Soc Nephrol 11: A0828, 2000.

83. **Lieberman EH, Gerhard MD, Uehata A, Selwyn AP, Ganz P, Yeung AC, and Creager MA.** Flow-induced vasodilation of the human brachial artery is impaired in patients <40 years of age with coronary artery disease. Am J Cardiol 78: 1210-1214, 1996.

84. **Liu Y, Rusch NJ, and Lombard JH.** Loss of endothelium and receptor-mediated dilation in pial arterioles of rats fed a short-term high salt diet. Hypertension 33: 686-688, 1999.

85. **Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, Ferguson TB, Ford E, Furie K, Gillespie C, Go A, Greenlund K, Haase N, Hailpern S, Ho PM, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, Marelli A, McDermott MM, Meigs J, Mozaffarian D, Mussolino M, Nichol G, Roger VL, Rosamond W, Sacco R, Sorlie P, Roger VL, Thom T, Wasserthiel-Smoller S, Wong ND, and Wylie-Rosett J.** Heart disease and stroke statistics--2010 update: a report from the American Heart Association. Circulation 121: e46-e215, 2010.

86. **Ludmer PL, Selwyn AP, Shook TL, Wayne RR, Mudge GH, Alexander RW, and Ganz P.** Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. N Engl J Med 315: 1046-1051, 1986.

87. **MacGregor GA, Markandu ND, Sagnella GA, Singer DR, and Cappuccio FP.** Double-blind study of three sodium intakes and long-term effects of sodium restriction in essential hypertension. Lancet 2: 1244-1247, 1989.

88. **Mares-Perlman JA, Klein BE, Klein R, Ritter LL, Fisher MR, and Freudenheim JL.** A diet history questionnaire ranks nutrient intakes in middle-aged and older men and women similarly to multiple food records. J Nutr 123: 489-501, 1993.

89. **Margetts BM, Cade JE, and Osmond C.** Comparison of a food frequency questionnaire with a diet record. Int J Epidemiol 18: 868-873, 1989.

90. **McKeown NM, Day NE, Welch AA, Runswick SA, Luben RN, Mulligan AA, McTaggart A, and Bingham SA.** Use of biological markers to validate self-reported dietary intake in a random sample of the European Prospective Investigation into Cancer United Kingdom Norfolk cohort. Am J Clin Nutr 74: 188-196, 2001.

91. **Meneton P, Jeunemaitre X, de Wardener HE, and MacGregor GA.** Links between dietary salt intake, renal salt handling, blood pressure, and cardiovascular diseases. Physiol Rev 85: 679-715, 2005.

92. **Miyoshi A, Suzuki H, Fujiwara M, Masai M, and Iwasaki T.** Impairment of endothelial function in salt-sensitive hypertension in humans. Am J Hypertens 10: 1083-1090, 1997.

93. **Monahan KD, Dinenno FA, Tanaka H, Clevenger CM, DeSouza CA, and Seals DR.** Regular aerobic exercise modulates age-associated declines in cardiovagal baroreflex sensitivity in healthy men. J Physiol 529 Pt 1: 263-271, 2000.

94. **Moncada S.** The L-arginine: nitric oxide pathway, cellular transduction and immunological roles. Adv Second Messenger Phosphoprotein Res 28: 97-99, 1993.

95. **Moreau KL, Gavin KM, Plum AE, and Seals DR.** Ascorbic acid selectively improves large elastic artery compliance in postmenopausal women. Hypertension 45: 1107-1112, 2005.

96. **Muller-Delp JM, Spier SA, Ramsey MW, and Delp MD.** Aging impairs endothelium-dependent vasodilation in rat skeletal muscle arterioles. Am J Physiol Heart Circ Physiol 283: H1662-1672, 2002.

97. **Nurkiewicz TR and Boegehold MA.** High salt intake reduces endothelium-dependent dilation of mouse arterioles via superoxide anion generated from nitric oxide synthase. Am J Physiol Regul Integr Comp Physiol 292: R1550-1556, 2007.

98. **Nurkiewicz TR, Wu G, Li P, and Boegehold MA.** Decreased arteriolar tetrahydrobiopterin is linked to superoxide generation from nitric oxide synthase in mice fed high salt. Microcirculation 17: 147-157, 2010.

99. **Pierce GL, Beske SD, Lawson BR, Southall KL, Benay FJ, Donato AJ, and Seals DR.** Weight loss alone improves conduit and resistance artery endothelial function in young and older overweight/obese adults. Hypertension 52: 1-8, 2008.

100. **Pierce GL, Eskurza I, Walker AE, Fay TN, and Seals DR.** Sex specific effects of habitual aerobic exercise on brachial artery flow-mediated dilation in middle-aged and older adults. Clin Sci (Lond), in press, 2010.

101. **Pierce GL, Jablonski KL, Walker AE, Seibert SM, Black SM, Sharmac S, Devan AE, and Seals DR.** Reduced tetrahydrobiopterin bioactivity contributes to decreased carotid artery compliance and higher carotid artery systolic and pulse pressure with aging in healthy adults. Am J Physiol Heart and Circ Physiol, In revision.

102. **Pierce GL, Lesniewski LA, Lawson BR, Beske SD, and Seals DR.** Nuclear factor-kappa B activation contributes to vascular endothelial dysfunction via oxidative stress in overweight/obese middle-aged and older humans. Circulation 119: 1284-1292, 2009.

103. **Pimenta E, Gaddam KK, Oparil S, Aban I, Husain S, Dell'Italia LJ, and Calhoun DA.** Effects of dietary sodium reduction on blood pressure in subjects with resistant hypertension: results from a randomized trial. Hypertension 54: 475-481, 2009.

104. **Pohl U, Holtz J, Busse R, and Bassenge E.** Crucial role of endothelium in the vasodilator response to increased flow in vivo. Hypertension 8: 37-44, 1986.

105. **Reiter CD, Teng RJ, and Beckman JS.** Superoxide reacts with nitric oxide to nitrate tyrosine at physiological pH via peroxynitrite. J Biol Chem 275: 32460-32466, 2000.

106. **Rippe C, Lesniewski L, Connell M, LaRocca T, Donato A, and Seals D.** Short-term calorie restriction reverses vascular endothelial dysfunction in old mice by increasing nitric oxide and reducing oxidative stress. Aging Cell 9: 304-312, 2010.

107. **Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, Carnethon MR, Dai S, de Simone G, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Greenlund KJ, Hailpern SM, Heit JA, Ho PM, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, McDermott MM, Meigs JB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Rosamond WD, Sorlie PD, Stafford RS, Turan TS, Turner MB, Wong P, N.D. , and Wylie-Rosett J.** Heart disease and stroke statistics--2011 update: a report from the American Heart Association. Circulation 123, 2011.

108. **Rosamond W, Flegal K, Friday G, Furie K, Go A, Greenlund K, Haase N, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G, O'Donnell CJ, Roger V, Rumsfeld J, Sorlie P, Steinberger J, Thom T, Wasserthiel-Smoller S, and Hong Y.** Heart disease and stroke statistics--2007 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 115: e69-171, 2007.

109. **Sacks FM, Svetkey LP, Vollmer WM, Appel LJ, Bray GA, Harsha D, Obarzanek E, Conlin PR, Miller ER, 3rd, Simons-Morton DG, Karanja N, and Lin PH.** Effects on blood pressure of reduced dietary sodium and the Dietary Approaches to Stop Hypertension (DASH) diet. DASH-Sodium Collaborative Research Group. N Engl J Med 344: 3-10, 2001.

110. **Safar ME, Thuilliez C, Richard V, and Benetos A.** Pressure-independent contribution of sodium to large artery structure and function in hypertension. Cardiovasc Res 46: 269-276, 2000.

111. **Schmidt K, Werner ER, Mayer B, Wachter H, and Kukovetz WR.** Tetrahydrobiopterin-dependent formation of endothelium-derived relaxing factor (nitric oxide) in aortic endothelial cells. Biochem J 281 (Pt 2): 297-300, 1992.

112. **Seals DR, Desouza CA, Donato AJ, and Tanaka H.** Habitual exercise and arterial aging. J Appl Physiol 105: 1323-1332, 2008.

113. **Seals DR, Jablonski KL, and Donato AJ.** Aging and vascular endothelial function in humans. Clin Sci (Lond) 120: 357-375, 2011.

114. **Seals DR, Tanaka H, Clevenger CM, Monahan KD, Reiling MJ, Hiatt WR, Davy KP, and DeSouza CA.** Blood pressure reductions with exercise and sodium restriction in postmenopausal women with elevated systolic pressure: role of arterial stiffness. J Am Coll Cardiol 38: 506-513, 2001.

115. **Simon G.** Experimental evidence for blood pressure-independent vascular effects of high sodium diet. Am J Hypertens 16: 1074-1078, 2003.

116. **Sindler AL, Delp MD, Reyes R, Wu G, and Muller-Delp JM.** Effects of ageing and exercise training on eNOS uncoupling in skeletal muscle resistance arterioles. J Physiol 587: 3885-3897, 2009.

117. **Smith-Spangler CM, Juusola JL, Enns EA, Owens DK, and Garber AM.** Population strategies to decrease sodium intake and the burden of cardiovascular disease: a cost-effectiveness analysis. Ann Intern Med 152: 481-487, W170-483, 2010.

118. **Taddei S, Virdis A, Ghiadoni L, Salvetti G, Bernini G, Magagna A, and Salvetti A.** Age-related reduction of NO availability and oxidative stress in humans. Hypertension 38: 274-279, 2001.

119. **Taddei S, Virdis A, Mattei P, Ghiadoni L, Fasolo CB, Sudano I, and Salvetti A.** Hypertension causes premature aging of endothelial function in humans. Hypertension 29: 736-743, 1997.

120. **Taddei S, Virdis A, Mattei P, Ghiadoni L, Gennari A, Fasolo CB, Sudano I, and Salvetti A.** Aging and endothelial function in normotensive subjects and patients with essential hypertension. Circulation 91: 1981-1987, 1995.

121. **Takase B, Uehata A, Akima T, Nagai T, Nishioka T, Hamabe A, Satomura K, Ohsuzu F, and Kurita A.** Endothelium-dependent flow-mediated vasodilation in coronary and brachial arteries in suspected coronary artery disease. Am J Cardiol 82: 1535-1539, A1537-1538, 1998.

122. **Tatchum-Talom R and Martin DS.** Tempol improves vascular function in the mesenteric vascular bed of senescent rats. Can J Physiol Pharmacol 82: 200-207, 2004.

123. **Tzemos N, Lim PO, Wong S, Struthers AD, and MacDonald TM.** Adverse cardiovascular effects of acute salt loading in young normotensive individuals. Hypertension 51: 1525-1530, 2008.

124. **U.S. Department of Agriculture ARS.** Total nutrient intakes: Percent reporting and mean amounts of selected vitamins and minerals from food and dietary supplements, by gender and age, What We Eat in America, NHANES 2007-2008. Available: www.ars.usda.gov/ba/bhnrc/fsrg., 2011.

125. **Umesawa M, Iso H, Date C, Yamamoto A, Toyoshima H, Watanabe Y, Kikuchi S, Koizumi A, Kondo T, Inaba Y, Tanabe N, and Tamakoshi A.** Relations between dietary sodium and potassium intakes and mortality from cardiovascular disease: the Japan Collaborative Cohort Study for Evaluation of Cancer Risks. Am J Clin Nutr 88: 195-202, 2008.

126. **Ungvari Z, Orosz Z, Labinskyy N, Rivera A, Xiangmin Z, Smith K, and Csiszar A.** Increased mitochondrial H2O2 production promotes endothelial NF-kappaB activation in aged rat arteries. Am J Physiol Heart Circ Physiol 293: H37-47, 2007.

127. **van der Loo B, Labugger R, Skepper JN, Bachschmid M, Kilo J, Powell JM, Palacios-Callender M, Erusalimsky JD, Quaschning T, Malinski T, Gygi D, Ullrich V, and**

Luscher TF. Enhanced peroxynitrite formation is associated with vascular aging. J Exp Med 192: 1731-1744, 2000.

128. **Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, and Pritchard KA, Jr.** Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. Proc Natl Acad Sci U S A 95: 9220-9225, 1998.

129. **Weinberger MH.** Salt sensitivity of blood pressure in humans. Hypertension 27: 481-490, 1996.

130. **Widlansky ME, Gokce N, Keaney JF, Jr., and Vita JA.** The clinical implications of endothelial dysfunction. J Am Coll Cardiol 42: 1149-1160, 2003.

131. **Wilkinson IB and Webb DJ.** Venous occlusion plethysmography in cardiovascular research: methodology and clinical applications. Br J Clin Pharmacol 52: 631-646, 2001.

132. **Woodman CR, Price EM, and Laughlin MH.** Aging induces muscle-specific impairment of endothelium-dependent dilation in skeletal muscle feed arteries. J Appl Physiol 93: 1685-1690, 2002.

133. **Wright JD, Wang CY, Kennedy-Stephenson J, and Ervin RB.** Dietary intake of ten key nutrients for public health, United States: 1999-2000. Adv Data: 1-4, 2003.

134. **Yang YM, Huang A, Kaley G, and Sun D.** eNOS uncoupling and endothelial dysfunction in aged vessels. Am J Physiol Heart Circ Physiol 297: H1829-1836, 2009.

135. **Yeboah J, Crouse JR, Hsu FC, Burke GL, and Herrington DM.** Brachial flow-mediated dilation predicts incident cardiovascular events in older adults: the Cardiovascular Health Study. Circulation 115: 2390-2397, 2007.

136. **Yu HC, Burrell LM, Black MJ, Wu LL, Dilley RJ, Cooper ME, and Johnston CI.** Salt induces myocardial and renal fibrosis in normotensive and hypertensive rats. Circulation 98: 2621-2628, 1998.

137. **Zeiher AM, Drexler H, Wollschlager H, and Just H.** Modulation of coronary vasomotor tone in humans. Progressive endothelial dysfunction with different early stages of coronary atherosclerosis. Circulation 83: 391-401, 1991.

138. **Zemel MB and Sowers JR.** Salt sensitivity and systemic hypertension in the elderly. Am J Cardiol 61: 7H-12H, 1988.

139. **Zhu J, Drenjancevic-Peric I, McEwen S, Friesema J, Schulta D, Yu M, Roman RJ, and Lombard JH.** Role of superoxide and angiotensin II suppression in salt-induced changes in endothelial Ca2+ signaling and NO production in rat aorta. Am J Physiol Heart Circ Physiol 291: H929-938, 2006.

140. **Zhu J, Huang T, and Lombard JH.** Effect of high-salt diet on vascular relaxation and oxidative stress in mesenteric resistance arteries. J Vasc Res 44: 382-390, 2007.

141. **Zhu J, Mori T, Huang T, and Lombard JH.** Effect of high-salt diet on NO release and superoxide production in rat aorta. Am J Physiol Heart Circ Physiol 286: H575-583, 2004.