Dietary Sodium Restriction and Vascular Dysfunction with Aging

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DIETARY SODIUM RESTRICTION AND VASCULAR DYSFUNCTION WITH AGING

by

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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
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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

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Dr. Douglas R. Seals

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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.

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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±8 mmol/day) vs. normal sodium (153±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 (BH₄) bioavailability (FMDBA no longer improved with oral BH₄) and reduced oxidative stress (FMDBA 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 BH₄ 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!!!
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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.


**Abstracts**


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.


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>140 mmHg) (74). 50% of adults ≥60 years of age and 75% of adults ≥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 ≥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 (BH₄), an essential cofactor for NO synthesis by eNOS, converting BH₄ to its inactive form, BH₂. The consequent reduction in bioavailability of BH₄ 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 BH₄ (98). Thus, in Chapter V, I also tested the hypothesis that the improvements in EDD with DSR are mediated by increased NO bioavailability, increased BH₄ 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 BH₄ bioavailability, and reduced oxidative stress
CHAPTER III

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(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,
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 \( \text{N}^\text{G} - \text{monomethyl-L-arginine (L-NMMA).} \)
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 BH₄ 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 BH4 (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

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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≥0.24) and was not related to sodium intake in the overall group (P≥0.29). 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: \[\text{fasting glucose (mg/dl) x fasting insulin } \mu \text{U/L}}]/405 \] (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 $P \geq 0.11$). 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 $P \geq 0.16$).

### Table 1: Subject Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Normal Na+</th>
<th>Low Na+</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (m/f)</td>
<td>13 (9/4)</td>
<td>12 (7/5)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>60±2</td>
<td>62±1</td>
<td>0.44</td>
</tr>
<tr>
<td>Body Mass (kg)</td>
<td>84±3</td>
<td>80±7</td>
<td>0.61</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.1±0.7</td>
<td>26.3±1.3</td>
<td>0.22</td>
</tr>
<tr>
<td>Waist:Hip Ratio</td>
<td>0.94±0.03</td>
<td>0.87±0.03</td>
<td>0.11</td>
</tr>
<tr>
<td>Total Body Fat (%)</td>
<td>34±2</td>
<td>34±2</td>
<td>0.90</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>138±2</td>
<td>138±1</td>
<td>0.93</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>81±2</td>
<td>79±2</td>
<td>0.68</td>
</tr>
<tr>
<td>Total Cholesterol (mg dL⁻¹)</td>
<td>197±7</td>
<td>214±9</td>
<td>0.16</td>
</tr>
<tr>
<td>LDL-C (mg dL⁻¹)</td>
<td>121±7</td>
<td>135±9</td>
<td>0.19</td>
</tr>
<tr>
<td>HDL-C (mg dL⁻¹)</td>
<td>50±4</td>
<td>50±4</td>
<td>0.92</td>
</tr>
<tr>
<td>VLDL-C (mg dL⁻¹)</td>
<td>26±3</td>
<td>28±5</td>
<td>0.71</td>
</tr>
<tr>
<td>Triglycerides (mg dL⁻¹)</td>
<td>128±17</td>
<td>142±25</td>
<td>0.62</td>
</tr>
<tr>
<td>Glucose (mg dL⁻¹)</td>
<td>95±3</td>
<td>95±3</td>
<td>0.91</td>
</tr>
<tr>
<td>Insulin (µU/L)</td>
<td>7.2±1.6</td>
<td>8.3±1.9</td>
<td>0.66</td>
</tr>
<tr>
<td>HOMA</td>
<td>1.76±0.46</td>
<td>2.08±0.58</td>
<td>0.67</td>
</tr>
<tr>
<td>Physical Activity (MET hrs wk⁻¹)</td>
<td>78±23</td>
<td>42±19</td>
<td>0.24</td>
</tr>
<tr>
<td>Peak VO₂ (ml kg⁻¹ min⁻¹)</td>
<td>26±1</td>
<td>25±1</td>
<td>0.82</td>
</tr>
</tbody>
</table>

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; VO2, volume of oxygen consumption

**Table 2: Circulating Humoral Factors**

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

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>0.53). Total energy intake tended to be lower in the low sodium group (P=0.07).
Table 3: Subject Diet Composition

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

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% (mmΔ) to 52% (%Δ) 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 (%Δ r=-0.53, P<0.01; mmΔ 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. 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. 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 (mm\(\Delta\) or %\(\Delta\)), 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 %\(\Delta\) 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 \textit{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

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²Institute of Biomedical and Clinical Sciences, University of Exeter, Exeter, United Kingdom
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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 (BH₄) bioavailability and reducing oxidative stress.

Methods and Results: EDD, measured by brachial artery flow-mediated dilation (FMDₓ) and forearm blood flow to acetylcholine (FBFₐCh), was 71% and 53% (peak FBFₐCh) higher following 4 weeks of a low sodium (70±8 mmol/day) vs. normal sodium (153±7 mmol/day) diet (p<0.05; randomized, cross-over design; 9M/5F; 61±2 yrs), whereas endothelium-independent dilation (brachial artery dilation to nitroglycerin and FBF to sodium nitroprusside) did not differ between conditions (p≥0.50). Compared to normal sodium, low sodium increased NO bioavailability (significant reduction in FBFₐCh with co-infusion of an endothelial nitric oxide synthase inhibitor), increased BH₄ bioavailability (FMDₓ no longer improved with oral BH₄) and reduced oxidative stress (FMDₓ and FBFₐCh no longer improved with ascorbic acid infusion; reduced endothelial cell nitrotyrosine abundance) (p≤0.06). 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 BH₄ 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 ($\text{FMD}_{\text{BA}}$) or as the forearm blood flow response to acetylcholine ($\text{FBF}_{\text{ACH}}$), 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 ($\text{BH}_4$) (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 BH4 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 (<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 ≥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 x (serum creatinine)\(^{-1.154}\) x age\(^{-0.203}\) x (0.742 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\textsubscript{BA}) (occlusion cuff on the upper forearm), peak shear rate during FMD\textsubscript{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 (BH₄) modulation of EDD was determined by measuring FMDₑₐₓ ~3 hrs after oral administration of BH₄ or placebo tablets, when BH₄ reaches its maximal plasma concentration, as described previously (52, 101) (n=12 low sodium / n=13 normal sodium). Because the key comparison was the BH₄-placebo difference in FMDₑₐₓ during low vs. normal dietary sodium intake states, the order of the placebo (first) and BH₄ (second) sessions was standardized (blinded to subject). Standardizing the order, under these particular conditions, eliminated the measurement variability that would be introduced by randomization. BH₄ is presently only available for oral administration, thus was used to assess modulation of FMDₑₐₓ but not local FBFₐ₅₇. Endothelium-independent dilation to sublingual NTG was also assessed during BH₄ and placebo sessions (n=14 low sodium / n=13 normal sodium, placebo; n=8 low sodium / n=6 normal sodium, BH₄).

The influence of oxidative stress on FMDₑₐₓ on each of these days (BH₄ 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, BH₄).

**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, BH4 (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

<table>
<thead>
<tr>
<th>Baseline Characteristics</th>
<th>Baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (m/f)</td>
<td>14 (9/5)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>61±2</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>29±3</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>88±3</td>
</tr>
<tr>
<td>Waist:Hip (ratio)</td>
<td>0.87±0.03</td>
</tr>
<tr>
<td>Medication Use</td>
<td></td>
</tr>
<tr>
<td>Hypothyroidism drugs</td>
<td>(n=3)</td>
</tr>
<tr>
<td>ACE-inhibitor</td>
<td>(n=1)</td>
</tr>
<tr>
<td>Ca²⁺ channel blocker</td>
<td>(n=1)</td>
</tr>
<tr>
<td>Diuretic</td>
<td>(n=1)</td>
</tr>
<tr>
<td>Anti-depressant</td>
<td>(n=1)</td>
</tr>
</tbody>
</table>

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

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

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

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

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

Data are mean ± S.E. LDL, low-density lipoprotein; ecSOD, extracellular superoxide dismutase.
**Dietary Sodium Restriction Improved FMD\textsubscript{BA} without Changing Endothelium-Independent Dilation to Sublingual NTG.** FMD\textsubscript{BA} ($\%\Delta$) 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\textsubscript{BA} in 12 of the 14 subjects. Panel C displays a significant inverse relation between sodium excretion and FMD\textsubscript{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\textsubscript{4} administration ($p>0.21$; 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\textsubscript{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\textsubscript{BA} ($r=-0.05$, $p=0.86$). In contrast to FMD\textsubscript{BA}, endothelium-independent dilation to sublingual NTG was unchanged by dietary sodium restriction (Figure 2, panel D; $\%\Delta$; mm$\Delta$ not shown; $p>0.50$).
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>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>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 FBFACh (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 FBFACh (not shown). However, arterial endothelial cell protein expression of eNOS and PeNOS were unchanged across sodium conditions (Figures 4C and 4D; p>0.41).
**Figure 4.** Forearm blood flow to increasing doses of acetylcholine (ACh) at baseline (gray line; ACh only) and during co-infusion of  NG 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 BH₄ Bioavailability and Decreased Oxidative Stress. FMDBA 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 BH₄ improved FMDBA under the normal sodium (66% increase) but not low sodium condition (Figure 5A; p<0.01 for repeated measures of saline vs. BH₄), without altering SBP during either sodium condition (p>0.40). Improvements in FMDBA were similar when ascorbic acid and BH₄ were co-administered (Figure 5A; p<0.01 for repeated measures of saline vs. ascorbic acid + BH₄). These results persisted when covarying for SBP (p<0.01 for all three FMDBA comparisons above). Endothelium-independent dilation to NTG did not change with ascorbic acid + BH₄ during either sodium condition (p>0.40; not shown). Co-infusion of ascorbic acid also tended to improve FBFₐCh (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 FBFₐCh (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. 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 (BH4) (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
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 BH4 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 BH₄ bioavailability and reduced oxidative stress. Importantly, the reduction in EDD with advancing age is mediated in part by decreased NO (44, 118) and BH₄ (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 demonstrated improvements in BH₄ bioavailability are contributing to the increase in NO. Due to limitations in the delivery of BH₄ (presently only available orally, thus cannot be used during assessment of FBFₐCh) 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 BH₄ 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 pressure (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 intriguing, 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 BH4 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 reductions in SBP, including increased NO and
BH₄ 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


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


