VASOPROTECTIVE MECHANISMS OF DAIRY-BASED FOODS: A DIETARY INTERVENTION STRATEGY FOR REDUCING CARDIOVASCULAR DISEASE RISK

A Dissertation in
Kinesiology
by
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ABSTRACT

Endothelial dysfunction is an early and common pathophysiological pathway in the progression of cardiovascular disease. Endothelial function can be improved with non-pharmacological lifestyle-related interventions, including dietary modifications. Increased total dairy consumption is associated with improved measures of vascular health, which may be attributed to the antioxidant and anti-inflammatory properties of dairy-based proteins. However, some dairy foods, specifically cheese, are also high in sodium. High dietary sodium intake is an independent predictor of cardiovascular mortality and is associated with impaired vascular function. It is unclear whether cheese, despite its high sodium content, has beneficial effects on vascular health. The overarching aim of this dissertation is to examine the acute and short-term effects of dairy milk and cheese on vascular function in middle-aged and older adults. Utilizing skin-sensitive methodologies, this dissertation comprises a series of studies that 1) examine the mechanistic and functional effects of dairy milk and cheese on microvascular endothelium-dependent vasodilation and 2) identify the mechanisms by which dairy cheese may protect against sodium-induced endothelial dysfunction. In the first set of studies, we hypothesized that NO-dependent vasodilation, an index of microvascular endothelial function, would be greater following acute dairy milk consumption compared to a non-dairy beverage (rice milk). Contrary to our hypothesis, NO bioavailability was greater following acute rice milk ingestion compared to dairy milk ingestion, which appeared to be mediated by a greater insulin response following rice milk ingestion. We also hypothesized that 1) acute dairy cheese consumption would improve NO-dependent vasodilation compared to ingestion of an equal amount of dietary sodium from non-dairy sources and 2) this improvement would be mediated by a reduction in oxidative stress. Indeed, we found that acute ingestion of sodium in the form of natural cheddar cheese protected against the impairment in NO bioavailability that followed ingestion of sodium from non-dairy sources (pretzels and soy cheese). Further, this protective effect of dairy on sodium-induced
endothelial dysfunction was mediated by a reduction in ascorbate-sensitive oxidants. In a follow-up study, we investigated the effects of short-term controlled feeding of a high-cheese diet on sodium-induced endothelial dysfunction. We hypothesized that a high sodium (5500 mg vs 1500 mg) diet would impair endothelium-dependent vasodilation but that a high cheese diet would preserve endothelium-dependent vasodilation that is otherwise impaired by high dietary sodium. We found that the high cheese diet protected against sodium-induced oxidative stress by reducing the accumulation of superoxide radicals, specifically those derived from NADPH oxidase. Collectively, these findings suggest that antioxidant properties of dairy-based nutrients protect against sodium-induced endothelial dysfunction and that inclusion of cheese into the diet may be an effective dietary intervention to mitigate age-related increases in cardiovascular disease risk.
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**Chapter 3**


**Chapter 4**


**Appendix A**

INTRODUCTION

Background and Significance

Cardiovascular disease (CVD) is the leading cause of global mortality accounting for 31% of all deaths\(^1\). The annual health care burden of the management of CVD is greater than $860 billion dollars and is projected to increase as our population ages reaching $1044 billion dollars by 2030\(^1\). Approximately 80% of CVDs can be prevented by adopting a healthy lifestyle and maintaining low risk factor levels\(^1\). As such, identification of modifiable and non-pharmacological lifestyle factors, including dietary interventions, are becoming increasingly important as a first line of defense for the prevention of CVD. Poor diet is a significant risk factor related to disease burden and accounts for nearly 15% of CVD mortality\(^1\). Poor diet is also one of the health metrics with the greatest potential for improvement in the US\(^1\). Dairy consumption is associated with decreased cardiovascular risk, yet 85% of individuals over the age of 1 do not meet the recommended intake of 3 daily servings of dairy set forth by the Dietary Guidelines for Americans\(^2\). Moreover, dairy intake declines with age dropping to approximately 1.5 servings per day in adults over the age of 50\(^2\). High dietary sodium, on the other hand, is associated with increased cardiovascular mortality\(^3,4\). The average American dietary sodium intake is well above the recommended intake with 90% of Americans having a sodium intake greater than the recommended upper limit\(^2\). Some dairy products, cheese in particular, are often high in sodium, and thus increasing dairy intake in the form of cheese may consequently increase dietary sodium consumption. It is unclear whether cheese, while contributing to dietary sodium intake, mitigates CVD risk.
**Dairy**

Total dairy intake is associated with various measures of improved vascular health including decreased blood pressure\(^5,6\) and reduced arterial stiffness\(^5-7\). While chronic dairy consumption is associated with lower blood pressure, it is becoming increasingly clear that dairy-based foods have vasoprotective properties and cardiovascular benefits that are independent of any blood pressure-reducing effects. The mechanisms underlying these beneficial effects on vascular health and function are likely multifactorial and include previously demonstrated angiotensin-converting enzyme (ACE)-inhibitory\(^8\), anti-inflammatory\(^9,10\), and antioxidant\(^11,12\) properties of dairy proteins.

**Dietary Sodium**

Individuals with low sodium intake (<2300 mg/d) are at a 32% lower CVD risk than those with high sodium intake (3600-4800 mg/d)\(^1\). Current dietary guidelines recommend limiting daily sodium intake to 2300 mg for healthy adults and further reducing sodium intake to 1500 mg per day for at-risk populations, including individuals with elevated blood pressure\(^2,13\). The average American sodium consumption is 3,440 mg per day, which far exceeds the recommended upper intake limit\(^2\). High dietary sodium consumption is associated with elevations in blood pressure and cardiovascular mortality\(^1,3,4\). However, it is becoming increasingly apparent that high sodium intake has detrimental effects on vessel health independent of changes in blood pressure\(^14,15\).

Controlled studies demonstrate vascular impairments with high sodium consumption and, conversely, improvements in vascular function with dietary sodium restriction (<1200 mg)\(^14,16\). Upon examination of the mechanisms mediating these alterations in vascular function, accumulating evidence suggests a role of oxidative stress in sodium-induced vascular dysfunction\(^15\).
Age-associated Endothelial Dysfunction

Endothelial dysfunction is one of the earliest complications of CVD and is characterized by a reduction in endothelium-dependent vasodilation, augmented vasoconstriction, and structural remodeling of the microvessels. Endothelial dysfunction is evident early in the progression of atherosclerosis and is an important target for the management of CVD risk. Therefore, identifying factors that contribute to endothelial dysfunction, including dietary factors, is clinically relevant.

Age-associated endothelial dysfunction occurs even in the absence of other traditional risk factors. Endothelial dysfunction that occurs with primary aging is mediated by a loss of nitric oxide (NO) signaling and increases in oxidative stress. Reactive oxygen species (ROS), such as superoxide, reduce NO bioavailability by interacting directly with NO to form peroxynitrite (ONOO\(^-\)). Peroxynitrite readily oxidizes BH4, an essential cofactor for endothelial nitric oxide synthase (eNOS). Under conditions of reduced cofactor or substrate bioavailability, eNOS “uncouples” and the electrons flowing to the substrate L-arginine are diverted to oxygen, further potentiating the formation of superoxide (Fig. 1-1).
Figure 1-1: A schematic of the putative mechanisms mediating reductions in nitric oxide (NO) bioavailability and endothelium-dependent dilation in the cutaneous microvasculature of aging adults.

Inflammation is tightly linked to oxidative stress and contributes to endothelial dysfunction in older adults. Putative inflammatory mechanisms by which NO bioavailability is reduced in aging are through an upregulation of arginase and/or inducible nitric oxide synthase (iNOS), two vascular targets of inflammatory mediators. Arginase is the last enzyme of the urea cycle and competes with eNOS for the common substrate L-arginine in the synthesis of NO\textsuperscript{24-26}. Arginase is upregulated in several pathologies characterized by endothelial dysfunction including aging, hypertension, and dyslipidemia\textsuperscript{24-28}. In clinical populations, arginase-inhibition or L-arginine administration restores endothelium-dependent dilation through increased NO signaling\textsuperscript{24-26}. In
addition to increased arginase activity or expression, upregulation of iNOS reduces NO production. iNOS produces high concentrations of NO, which in a pro-oxidant environment reacts with ROS to form superoxide and peroxynitrite. Additionally, iNOS activates arginase through S-nitrosylation, further reducing eNOS-mediated production of NO²⁹, ³⁰. Collectively, these signaling pathways are potential mechanisms by which dairy and sodium consumption may improve or impair endothelial function, respectively.

Skin as a Model Circulation

The cutaneous circulation is an accessible and representative vascular bed that allows for the non-invasive investigation of mechanisms mediating systemic microvascular dysfunction in humans. Deficits in endothelial function in the skin parallel deficits in other vascular beds including coronary³¹, ³², renal³³, retinal³⁴ and skeletal muscle³⁴ circulations, which supports the idea that endothelial dysfunction is a systemic process in pathology. Impaired microvascular responses in the skin are associated with increased cardiovascular risk³⁵, ³⁶ and are predictive of the presence of coronary artery disease³⁷. Additionally, attenuation of pharmacologically-induced cutaneous vasodilation is significantly correlated with impaired conduit arterial flow-mediated dilation³⁸, a measure that is predictive of future CVD³⁹, ⁴⁰. Moreover, cutaneous microvascular dysfunction is evident early in disease progression⁴¹ and can be detected prior to changes in conduit vascular function and clinical manifestations of pathology. Thus, the cutaneous circulation is not only a representative vascular bed for identifying mechanisms of systemic vascular dysfunction in clinical populations but also for examining the effects of systemic treatments²⁵, ⁴², including nutritional interventions⁴³-⁴⁵, on those mechanisms.

Skin-Specific Methodologies for Assessing Endothelial Dysfunction

Several skin-specific methodologies can be utilized to examine endothelial function in the microcirculation and involve both physiological stimuli (e.g., local heating) and/or
pharmacological stimuli (e.g., acetylcholine infusion) to induce endothelium-dependent vasodilation.

**Local Heating**

An increase or decrease in temperature elicits cutaneous vasodilation and vasoconstriction, respectively. Local increases in temperature can be applied to the skin to induce cutaneous vasodilation. Local skin heating is characterized by a biphasic response that consists of an initial peak, which is mediated by local sensory nerves acting through an axon-reflex, followed by a brief nadir and a sustained plateau. The plateau phase of the local heating response is approximately 60% mediated by NO that is produced by the endothelial isoform of nitric oxide synthase, eNOS. The remaining portion of the local heating plateau is NO-independent and is primarily mediated by endothelium-derived hyperpolarizing factors (EDHFs). EDHFs are a class of vasodilators that act through hyperpolarization of the vascular smooth muscle. Most EDHFs stimulate calcium-activated potassium channels, which are found on both endothelial cell and vascular smooth muscle membranes. Hyperpolarization of the endothelium spreads to the vascular smooth muscle through myoendothelial gap junctions, thereby contributing to the vasodilatory response. Likely candidates for EDHFs involved in the vasodilator response to local heating include epoxyeicosatrienoic acids (EETs), H2O2 and lipoxygenase derivatives.

Local heating is a well-accepted clinical tool for evaluating microvascular function. Alterations in skin vascular reactivity to local heating are observed prior to clinical manifestations of disease. Attenuation of the NO-mediated component of the local heating plateau has been demonstrated in several clinical populations including chronic kidney disease, polycystic ovary syndrome, postural tachycardia syndrome, aging, hypercholesterolemia, and hypertension. In fact, we recently demonstrated that NO-dependent vasodilation was reduced in adults with psoriasis, an inflammatory disease that is associated with an increase in
cardiovascular disease risk (Appendix A). Additionally, the vascular response to local heating can be used to assess the effectiveness of interventions that target signaling pathways in the cutaneous microvasculature. Interventions aimed at increasing NO bioavailability, including antioxidant and tetrahydrobiopterin (BH) administration, restore the NO contribution to the local heating vasodilatory response in aging and vascular pathologies.

Acetylcholine-Induced Vasodilation

In addition to local increases in temperature, administration of vasoactive pharmacological agents can be used to examine endothelial function and mechanisms of vascular control. Acetylcholine-induced dilation is endothelium-dependent and mediated by NO and cyclooxygenase-derived prostanoids. Acetylcholine administration has been utilized to assess NO-dependent vasodilation in the cutaneous endothelium. In addition to quantifying NO bioavailability, blood flow responses to graded infusions of acetylcholine can be pharmacologically modelled to assess endothelium sensitivity to vasodilatory stimuli. Altered acetylcholine-induced vasodilation has been demonstrated in the cutaneous microvasculature of aged adults and clinical populations including hypertension, highlighting its utility in detecting endothelial dysfunction in vascular pathology.

Summary

The studies that comprise this dissertation were conducted to determine the separate and combined effects of sodium and dairy-based nutrients on microvascular function in middle-aged and older adults after acute (single-meal) and short-term (8-day) milk and cheese consumption. The first of four studies examined the acute effects of fluid milk consumption on cutaneous microvascular function in normotensive to pre-hypertensive, middle-aged to older adults. The second study examined the relative effects of acute sodium ingestion in cheese and non-dairy sodium ingestion on cutaneous microvascular function also in normotensive to pre-hypertensive
middle-aged to older adults. The third and fourth studies were follow-up studies to the second study and 1) investigated the vasoprotective effects of short-term (8-day) cheese intake on microvascular dysfunction induced by dose-dependent increases in dietary sodium (1500 mg vs 5500 mg) and 2) identified the mechanisms by which cheese protects against dietary sodium-induced oxidative stress.

**Specific Aims & Hypotheses**

**Specific Aim 1.** The purpose of the study “Acute Dairy Milk Ingestion Does Not Improve Nitric Oxide-Dependent Vasodilation in the Cutaneous Microcirculation” was to determine the impact of 2 and 4 servings of acute fluid milk consumption on cutaneous microvascular function in middle-aged to older adults.

**Hypothesis 1a:** Acute fluid milk consumption will increase NO-dependent vasodilation in the cutaneous microcirculation in a dose-dependent manner compared to a non-dairy placebo (rice milk).

**Hypothesis 1b:** Local ascorbate administration will improve NO-dependent vasodilation following acute rice milk, but not dairy milk, consumption.

**Hypothesis 1c:** Local arginase or iNOS inhibition will improve NO-dependent vasodilation following acute rice milk, but not dairy milk, consumption.

**Specific Aim 2.** The purpose of the study “Dairy Cheese Consumption Ameliorates Single-Meal Sodium-Induced Cutaneous Microvascular Dysfunction by Reducing Ascorbate-Sensitive Oxidants in Healthy Older Adults” was to examine the relative effects of acute sodium ingestion
in cheese (2 and 4 servings) and non-dairy sodium ingestion on microvascular function in middle-aged to older adults.

**Hypothesis 2a:** Acute cheese consumption will improve NO-dependent vasodilation in the cutaneous microcirculation compared to both a sodium-matched placebo (pretzels) and a sodium-matched, non-dairy cheese placebo (soy cheese) in a dose-dependent fashion.

**Hypothesis 2b:** Local ascorbate administration will improve NO-dependent vasodilation following acute soy cheese or pretzel ingestion, but not following acute dairy cheese ingestion.

**Hypothesis 2c:** Local arginase or iNOS inhibition will improve NO-dependent vasodilation following acute soy cheese or pretzel ingestion, but not following acute dairy cheese ingestion.

**Specific Aim 3.** The purpose of the study “Controlled Feeding of an 8-day High Dairy Cheese Diet Prevents Sodium-Induced Endothelial Dysfunction in the Cutaneous Microcirculation through Reductions in NADPH Oxidase-Derived Reactive Oxygen Species” was to 1) characterize the vasoprotective effects of cheese on microvascular dysfunction induced by dose-dependent increases in dietary sodium in middle-aged and older adults and 2) identify the mechanisms by which cheese protects against dietary sodium-induced oxidative stress.

**Hypothesis 3a:** Endothelium-dependent vasodilatory responses to physiological (local heating) and pharmacological (acetylcholine infusion) stimuli will be impaired by a high-sodium diet (5500 mg) compared to a low-sodium diet (1500 mg).

**Hypothesis 3b:** Controlled feeding of a high-cheese diet (4 servings/d x 8 d) will preserve endothelium-dependent vasodilatory responses to physiological (local heating) and
pharmacological (acetylcholine infusion) stimuli that are otherwise impaired by a high-sodium diet (5500 mg vs 1500 mg sodium).

**Hypothesis 3c:** Localized mechanism-specific antioxidant treatments (apocynin, tempol, and ascorbate) will augment sodium-induced endothelial dysfunction following a high-sodium diet alone, but not following a high-sodium diet containing dairy cheese.
Current guidelines recommend lifestyle modifications, including physical activity and nutritional approaches (e.g., reduced salt intake), as strategies to treat and prevent cardiovascular disease (CVD). Many foods, including dairy, contain bioactive components that are able to modulate physiological functions. Dietary intake of macronutrients (e.g., protein and fiber) and micronutrients (e.g., magnesium, calcium, and potassium) are associated with improved measures of cardiovascular health and reductions in blood pressure. Consequently, the contribution of dietary components to CVD risk has been and continues to be an important topic of investigation. Given the accumulating evidence for improved cardiovascular health, increased dairy consumption may be a potential strategy to mitigate age-associated increases in CVD risk in middle-aged and older adults.

Cardioprotective Benefits of Dairy Consumption

Some of the earliest evidence for cardioprotective actions of dairy emerged from the Dietary Approaches to Stop Hypertension (DASH) study, a multi-center randomized controlled trial that was conducted to examine the effects of dietary patterns on blood pressure. Participants were randomly assigned to one of the following sodium-matched (3000 mg/d) 8-week dietary interventions: 1) a control diet that was typical of the average American diet 2) a high fruit and vegetable (F&V) diet or 3) a combination diet (F&V + low-fat dairy). The combination diet contained 2.7 servings of dairy per day, which is nearly equivalent to the recommended intake of 3 servings per day and is almost twice as much as the average consumption of 1.5 servings per day. Not surprisingly, the combination diet reduced SBP and DBP by 5.5 mmHg and 3.0 mmHg more than the control diet. Interestingly, the combination diet reduced SBP and DBP by 2.7
mmHg and 1.9 mmHg more than the F&V diet, a diet that was matched for micronutrient content but lacked dairy products. These findings suggested that inclusion of dairy into the diet may be an effective nutritional strategy to prevent age-related increases in blood pressure and vascular dysfunction. However, the DASH study investigated the effects of dietary patterns, not the effects of specific foods or nutrients. As such, the individual effects of dairy foods per se were not measured.

Since the completion of the DASH study, several epidemiological studies have examined the relation between total dairy intake and vascular health and have reported an inverse association between dairy consumption and CVD risk\textsuperscript{73-80}, blood pressure\textsuperscript{6, 7, 81-83}, and arterial stiffness\textsuperscript{6, 7}. Dairy comprises a variety of foods (e.g., milk, cheese, and yogurt) that differ in their nutrient profile, and their effects on CVD risk factors (e.g., cholesterol, blood pressure, etc.). In a recent systematic review, examination of specific dairy food types indicated that fluid dairy products (milk and yogurt) were associated with an 8% reduction in elevated blood pressure risk\textsuperscript{84}, which is in agreement with other meta-analyses reporting a significant association between milk consumption and improved cardiovascular outcomes\textsuperscript{74, 75, 77}. Large-scale population-based studies also show a significant inverse relation between cheese consumption and the risk for CVD\textsuperscript{79, 80, 85, 86}, CHD\textsuperscript{73, 87} and stroke\textsuperscript{73, 85, 88}, which is of particular interest given its high sodium content and suggests that dairy-based nutrients may protect against deleterious effects of other unfavorable dietary components such as sodium. Taken together, a growing body of evidence suggests that macro- and/or micronutrients in dairy-based foods have beneficial effects on vascular health and cardiovascular outcomes\textsuperscript{71} and warrants controlled intervention studies to investigate how individual dairy foods affect vascular function and to identify the specific mechanisms underlying the cardioprotective effects of dairy.
Bioactive Components of Dairy-Based Foods

Micronutrients
Consumption of micronutrients commonly found in dairy foods, specifically calcium and potassium, reduces blood pressure and CVD-related morbidity. In a recent prospective study, low-fat dairy intake was associated with a reduced risk of hypertension in middle-aged and older women but this association was attenuated when adjusted for calcium intake, which supports a role for calcium in the hypotensive effects of dairy. Other studies contradict the findings of Wang et al. and provide support for an inverse relation between dairy intake and hypertension risk, independent of calcium intake. In fact, Djousse et al. reported that 1) the inverse association between dairy intake and prevalent hypertension was even slightly stronger when adjusted for dietary calcium and 2) the inverse association between calcium intake and hypertension was abolished after adjustment for dairy intake, which indicates that the dairy-hypertension association was not mediated through dietary calcium. Among specific dairy foods, Patterson et al. found that cheese intake was inversely associated with MI risk and when adjusted for calcium, the association was attenuated but cheese intake still tended to be inversely related to MI risk, suggesting that this beneficial effect of cheese is likely mediated by both calcium and non-calcium components of dairy. Taken together, the association between blood pressure and dairy intake appears to be stronger than that between blood pressure and dietary calcium, meaning that components in dairy foods other than calcium likely contribute to the hypotensive effects of dairy. Overall, few studies have attempted to separate the cardioprotective effects of micronutrients in dairy foods from those of other dairy-based macronutrients. Additionally, while it appears that calcium may partially contribute to the hypotensive effects of dairy, the role of calcium, if any, in the blood pressure-independent vascular benefits of dairy remains unclear.
Similar to dietary calcium, dietary potassium is also associated with reduced CVD risk\textsuperscript{95, 96}. While the direct effect of potassium intake on vascular function remains equivocal, several studies have shown blood pressure-independent benefits of potassium intake on endothelial function, cell stiffness and NO release\textsuperscript{97-99}. In controlled dietary studies, Gijsbers \textit{et al.} and Blanch \textit{et al.} demonstrated improvements in basal and post-prandial endothelium-dependent dilation, respectively, with high potassium intake\textsuperscript{98, 100}. As such, high dietary potassium is becoming increasingly recognized as a dietary factor associated with improved vascular health and cardiovascular risk\textsuperscript{101, 102} and may contribute to the vascular benefits associated with high dairy intake.

\textbf{Dairy Proteins}

Dairy-based proteins are largely comprised of whey- and casein-derived bioactive peptides that are capable of modulating physiological functions. Eighty percent of bovine milk protein is derived from casein and includes caseinophosphopeptides, casoxins, and the lactotripeptides Ile-Pro-Pro (IPP) and Val-Pro-Pro (VPP)\textsuperscript{103}. The remaining 20% of bovine milk protein is derived from whey and consists of β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulins, glycomacropeptide, lactoferrin and lactoperoxidase\textsuperscript{104}. Several of these milk-derived proteins have been shown to shift the vascular phenotype to a pro-dilator state through ACE-inhibitory, anti-inflammatory, and antioxidant properties (\textbf{Fig. 2-1}).
Figure 2-1: Diagram of the proposed mechanisms by which macronutrients in dairy foods may improve endothelial function, which include antioxidant, anti-inflammatory, and angiotensin-converting enzyme properties of dairy-based proteins.

ACE-inhibitory properties

Angiotensin converting enzyme (ACE) is a key enzyme in the renin-angiotensin system and plays an important role in vascular function and blood pressure control\textsuperscript{105}. ACE catalyzes the conversion of angiotensin I to the potent vasoconstrictor angiotensin II, which increases peripheral vascular resistance and stimulates aldosterone release. ACE also inactivates bradykinin, a protein that regulates blood pressure by increasing the synthesis of prostaglandins and nitric oxide, consequently causing vasoconstriction and increasing peripheral vascular
ACE is present throughout the body including the vascular endothelium. Tryptic digestion of milk protein produces numerous peptides with potent ACE inhibitory activity. Interactions between ACE catalytic sites and VPP and IPP, two of the most extensively studied lactotripeptides, have been documented. In a spontaneously hypertensive rat model, 16 weeks of VPP and IPP-containing fermented milk consumption decreased angiotensin II production through inhibition of ACE. Similarly, in hypertensive humans, VPP and IPP consumption significantly reduced the circulating blood angiotensin II to angiotensin I ratio, indicative of ACE-inhibitory actions of lactotripeptides. By inhibiting ACE, milk-derived peptides may also reduce angiotensin II-induced activation of NADPH oxidase and subsequent production of reactive oxygen species, further increasing NO bioavailability.

In addition to its conversion to angiotensin II, angiotensin I can be converted to angiotensin-(1-7) by ACE2. Angiotensin-(1-7) stimulates endothelium-dependent vasodilation through the Mas receptor and opposes the pressor effect of the AT1 receptor. Ehlers et al. showed that incubation with IPP augmented angiotensin-(1-7)-induced dilation and potentiated the dilatory response to bradykinin, which suggests a role of the angiotensin-(1-7)-Mas axis in the modulation of vascular function by lactotripeptides.

To date, the majority of studies demonstrating ACE-inhibitory properties of dairy-based peptides have used isolated lactotripeptides or fermented milk products. While ACE-inhibitory peptides have been identified in dairy products, including milk and cheese, the in vivo ACE-inhibitory activity of dairy proteins in natural foods merits further investigation.

**Anti-inflammatory properties**

Dietary patterns, including high dairy consumption, modify inflammation. Inflammation is present in many chronic diseases and contributes to the progression of CVD. Cross-sectional
studies indicate that dairy intake is associated with reduced measures of inflammation\(^9,10\). In vitro, treatment of endothelial cells with milk-derived proteins reduces the expression of pro-inflammatory mediators, including TNF\(\alpha\), IL-8, and the adhesion molecules VCAM-1, ICAM-1 and E-selectin\(^{123,124}\). Further, modulation of the inflammatory phenotype appears to be mediated by inhibition of the NF\(\kappa\)B pathway as treatment of endothelial cells with a milk-derived hydrolysate significantly reduced activation of the pro-inflammatory transcription factor NF\(\kappa\)B \(^{123}\). Several studies have translated these findings and investigated the in vivo anti-inflammatory effects of dairy proteins in models of obesity and metabolic syndrome, two clinical conditions characterized by heightened inflammation. A 3-week high-dairy intervention in obese mice reduced adipose expression of IL-6, TNF\(\alpha\), and MCP-1 and plasma TNF\(\alpha\) and IL-6 concentrations relative to a control diet\(^{125}\). In obese humans, acute consumption of casein and whey protein reduced post-prandial MCP-1 and CCL5/RANTES, two inflammatory biomarkers that are predictive of atherosclerosis\(^{126}\). Similarly, following a chronic high-dairy intervention, inflammatory biomarkers, including IL-6, TNF\(\alpha\), and MCP-1, decreased by 21-35\% in adults with metabolic syndrome\(^{127}\) and obesity\(^{128}\), independent of changes in adiposity. In adults with hypertension, another condition associated with inflammation, Hirota et al. observed a greater reduction in TNF\(\alpha\) concentrations following 1-week supplementation of casein hydrolysate compared to a placebo control\(^{129}\). Additional studies have provided evidence for anti-inflammatory properties of milk-based peptides in postmenopausal women\(^{130}\) and COPD patients\(^{131}\), in which reductions in pro-inflammatory IL-6 and TNF\(\alpha\) concentrations and increases in anti-inflammatory IL-10 concentrations were reported. Cumulatively, a large body of literature supports a beneficial modulation of circulating inflammatory markers in response to dairy protein consumption in a variety of populations.
Antioxidant properties

Reactive oxygen species (ROS) impact cell signaling in the vasculature and contribute to the manifestation of endothelial dysfunction and the development of many chronic diseases including CVD and atherosclerosis\textsuperscript{132}. An individual’s antioxidant capacity is determined by both dietary ingestion and endogenous synthesis of antioxidants. Milk-derived peptides function as radical scavengers\textsuperscript{11, 12}, act as lipid peroxidation inhibitors\textsuperscript{133}, and increase the expression of antioxidant enzymes\textsuperscript{134, 135}. In mice, a high-dairy diet significantly reduced ROS production relative to a control diet\textsuperscript{125}. Further, the high-dairy diet decreased ROS production more than a high-calcium diet, demonstrating additional non-calcium antioxidant components of dairy, which may be milk-based proteins. Power-Grant et al. examined the antioxidant properties of a milk-based protein matrix following simulated \textit{in vitro} digestion and acute \textit{in vivo} digestion in healthy middle aged to older women\textsuperscript{136}. The protein matrix demonstrated potent antioxidant capacity with increasing radical scavenging activity after simulated digestion. Additionally, plasma antioxidant capacity increased following consumption of the protein matrix in 50-70 year old women, suggesting that dairy-based proteins may protect against age-related alterations in ROS production and antioxidant capacity. Interestingly, the participants who had the lowest basal antioxidant concentration showed the highest post-prandial response. Antioxidant capacity peaked at 90 minutes post-ingestion, the time at which peak intestinal concentrations of bioactive peptides are recovered following milk peptide ingestion\textsuperscript{137}, and remained elevated throughout the 180-minute postprandial period. In line with these acute antioxidant effects, chronic dairy intake reduced the oxidative stress biomarkers MDA and oxLDL in adults with metabolic syndrome\textsuperscript{127} and similarly decreased MDA and 8-isoprostane-F\textsubscript{2α} in obese individuals\textsuperscript{128}, independent of changes in adiposity.

In a limited number of studies examining specific antioxidant systems, cellular incubation with whey\textsuperscript{134} or casein\textsuperscript{135} protein hydrolysates increased the activity of catalase, an enzyme responsible
for converting hydrogen peroxide to oxygen and water. In mice, a 3-week high-dairy diet reduced the expression of NADPH oxidase, an enzyme that produces superoxide radicals, relative to a control diet.\textsuperscript{25} Taken together, the ROS scavenging properties of milk-derived peptides are well-documented; however, additional research is needed to examine the \textit{in vivo} antioxidant effects of dairy on vascular function and identify the specific antioxidant mechanisms and enzymes altered by dairy foods.

\textbf{Dairy Intake and Vascular Function}

\textit{Vascular Responses to Acute Dairy Ingestion}

Accumulating evidence from both animal and human studies supports actions of dairy-based macronutrients in improving measures of vascular health.\textsuperscript{5, 138, 139, 140} Acute responses to dietary interventions contribute to alterations in endothelial function. Aberrant postprandial hyperglycemia (PPH) responses are predictive of cardiovascular mortality.\textsuperscript{141} It has been postulated that, although transient, PPH-mediated endothelial dysfunction contributes to the development of CVD, even in healthy normoglycemic individuals. PPH impairs vascular endothelial function primarily through an oxidative stress-mediated reduction in NO.\textsuperscript{142} Interestingly, low-fat milk ingestion has been shown to prevent the postprandial reduction in endothelium-dependent dilation (measured via flow-mediated dilation) that otherwise follows ingestion of an isocaloric volume of non-dairy milk by limiting perturbations in oxidant activity and NO bioavailability.\textsuperscript{143} This protective effect on PPH-induced vascular function occurred in the absence of any change in postprandial blood pressure, demonstrating acute blood pressure-independent vasoprotective properties of low-fat dairy milk. Whey-derived peptide supplementation has also been shown to improve reactive hyperemia and increase total plasma nitrite/nitrates during a 2-hour post-ingestion period, suggesting that, acutely, milk-based proteins improve resistance and conduit vascular endothelial responses in NO-dependent and -independent...
manner’s Future studies are needed to further characterize the effects of dairy on postprandial responses and their impact on long-term vascular health.

**Vascular Responses to Chronic Dairy Ingestion**

Compelling evidence in animal models suggests that chronic ingestion of milk-derived proteins improves endothelium-dependent vasodilation. Six-week casein hydrolysate supplementation in spontaneously hypertensive rats (SHRs) improved aortic and mesenteric acetylcholine-induced relaxation, increased aortic eNOS expression, and reduced phenylephrine-induced vasoconstriction. Similarly, Yamaguchi et al. reported increased mRNA expression of eNOS in SHRs following 5-day VPP and IPP supplementation. In addition, Sipola et al. demonstrated that administration of α-lactorphin and β-lactorphin, two milk-based tetrapeptides, augmented acetylcholine-induced vasorelaxation in mesenteric arteries of SHRs, an effect that was abolished by the non-specific NOS inhibitor L-NAME. These findings suggest that the improvements in vascular function are mediated by enhanced NO release from the endothelium, which is in agreement with similar studies using IPP and VPP supplementation. β-lactorphin application also augmented SNP-induced vasorelaxation, demonstrating greater sensitivity of the vascular smooth muscle to NO; however, dairy protein-mediated improvements in endothelium-independent vessel function is not a universal finding.

In humans, partially-controlled feeding (i.e., participants were provided with dairy products and replacement control products that were matched for energy, sodium, and saturated fat) of 3 servings of dairy per day for 4 weeks enhanced endothelial function, as assessed by reactive hyperemia, despite a higher sodium intake and lower fruit and vegetable consumption in the dairy phase. Machin et al. performed a similar 4-week intervention study with a crossover between a high dairy (+4 servings/d of non-fat dairy) and no dairy (+4 servings/d of fruit products and no dairy) diet and observed improvements in arterial stiffness and brachial artery endothelium-
dependent dilation following the high dairy phase in middle-aged and older adults with elevated blood pressure\textsuperscript{150}. Additionally, decrements were observed with removal of dairy in the no dairy phase, indicating that baseline dairy consumption was providing vasoprotective benefits. A strength of these human studies is the use of whole dairy products to demonstrate the effects of dairy-based nutrients as they occur naturally in foods; however, completely controlled dietary interventions are needed to fully assess the impact of dairy foods on vascular function in humans.

In the aforementioned studies, concurrent changes in blood pressure occurred with dairy-protein supplementation making it difficult to isolate the direct effects and underlying mechanisms of dairy on vascular health and function. While an abundance of research supports hypotensive effects of dairy\textsuperscript{145, 151-153}, the current literature points towards additional blood pressure-independent benefits (e.g., improvements in vascular function, oxidative stress, and inflammation) that more fully account for the cardioprotective effects of dairy. In a placebo-controlled, double-blind crossover study in mild hypertensive men, 7 days of casein hydrolysate ingestion improved reactive hyperemia independent of changes in blood pressure\textsuperscript{129}. Furthermore, in individuals that presented endothelial dysfunction during non-dairy control conditions, a 5-week low-fat dairy intervention similarly augmented reactive hyperemia compared to a non-dairy phase with no concurrent change in blood pressure, which suggests that high dairy intake may be a particularly effective strategy to improve vascular function in individuals at greater CVD risk\textsuperscript{154}.

To elucidate the underlying signaling pathways, Hirota \textit{et al.} investigated the effects of VPP and IPP on endothelial function and NO production in cultured endothelial cells and isolated arterial vessels\textsuperscript{148}. VPP and IPP-induced vasorelaxation was significantly attenuated with NOS-inhibition, K+ channel inhibition, and bradykinin B2 receptor inhibition. Further, vasorelaxation was not observed when the endothelium was removed, suggesting that VPP and IPP-induced
vasorelaxation is mediated by actions on the endothelium and not directly on the vascular smooth muscle. Additionally, the individual amino acids that comprise these tripeptides failed to induce vasorelaxation, indicating that the intact peptides are responsible for the observed vascular improvements. Together, these data suggest that a potential mechanism by which milk-derived peptides improve endothelial-dependent dilation is through an increase in NO and EDHFs.

Most human studies to date lacked appropriate controls for dairy food components or did not adequately control for the displacement of other dietary components that the dairy products replaced. Additional controlled intervention studies are therefore needed to identify the bioactive compounds responsible for the effects of dairy-based food consumption on vascular health. Further, studies that have examined the bioactive properties of whey and casein have primarily utilized isolated peptides and enzymatic hydrolysates. The bioavailability of vasoactive peptides depends on the degree of hydrolysis, digestion, absorption, metabolism, resistance to gastric peptidases, and excretion of the ingested proteins. Whether the \textit{in vitro} functionalities of milk-based proteins translate \textit{in vivo} remains unclear. Thus, the bioactivity of milk-based proteins when consumed in the form of whole dairy foods and the potential for dairy foods to protect against vascular dysfunction characterized by oxidative stress and inflammation warrants further investigation.

\textbf{Dietary Sodium and Vascular Health}

High sodium intake is associated with adverse cardiovascular outcomes and cardiovascular mortality\textsuperscript{3, 4, 155}, whereas low sodium intake is associated with a reduced risk of cardiovascular events\textsuperscript{156}. The remainder of this literature review highlights the research aimed at understanding the deleterious effects of high dietary sodium on vascular health. In conjunction with the DASH Study, the DASH-Sodium trial examined the effects of three sodium levels (50, 100 and 150
mmol/d) on blood pressure in individuals consuming either the DASH diet or the control diet\textsuperscript{157}. The high sodium level matched the average American sodium consumption while the intermediate and lower sodium levels reflected the recommended upper limit for healthy adults\textsuperscript{2} and at-risk individuals (e.g., hypertensive adults)\textsuperscript{13}, respectively. Reductions in dietary sodium intake decreased both DBP and SBP in all subgroups with greater reductions observed in the control diet. Interestingly, the blood pressure-reducing effect of the DASH diet relative to the control diet was the greatest at the higher sodium level compared to the lower sodium levels, suggesting that the cardioprotective actions of dairy in the DASH diet may be particularly effective under high dietary sodium conditions. Further, the improvements in blood pressure from sodium intake reduction were greater with increasing age, suggesting that lowering salt consumption or limiting the negative vascular effects of sodium (e.g., increasing dairy consumption) may be a useful strategy to prevent age-related increases in CVD.

Some individuals are salt-sensitive meaning that their blood pressure increases with high salt intake and decreases with salt restriction. Salt-sensitivity is predictive of future hypertension and is associated with increased cardiovascular mortality\textsuperscript{158-160,161-166}. However, it is now recognized that high dietary salt is associated with an increased risk of CVD \textit{independent of blood pressure}\textsuperscript{167}. Indeed, sodium restriction is associated with a reduced risk of cardiovascular events despite minimal changes in blood pressure ($\Delta$SBP <2 mmHg, $\Delta$DBP <1 mmHg)\textsuperscript{156}, indicating that high dietary sodium may impair vascular function in normotensive, salt-resistant individuals. Blood pressure-independent alterations in the microvasculature with high salt consumption include impaired endothelial function\textsuperscript{14, 15, 168-171}, anatomical loss of microvessels\textsuperscript{172}, augmented responses to sympathetic stimuli\textsuperscript{173}, and increased arterial stiffness\textsuperscript{174-176}. Such vascular impairments are present in resistance vessels of several vascular beds including skeletal muscle\textsuperscript{177}, cerebral\textsuperscript{179}, and mesenteric\textsuperscript{180, 181} circulations during high salt consumption.
**Dietary Sodium-Induced Vascular Dysfunction**

Animal studies provide strong evidence that sodium intake adversely affects endothelial function and NO bioavailability. In the spinotrapezius muscle of salt-resistant rats fed a high-salt diet (HS) for 4 weeks, the vasodilatory response to acetylcholine was significantly attenuated\textsuperscript{168}. Further, NOS-inhibition constricted arterioles in HS rats but not low salt-fed rats (LS), demonstrating a loss of basal NO and its influence on resting vascular tone. No differences in the response to SNP were observed with salt loading, indicating no alteration in vascular smooth muscle responsiveness to an endothelium-independent source of NO. These authors also observed a reduction in endothelial-dependent dilation in response to an increase in shear stress in HS rats, which was due to suppressed NO activity\textsuperscript{182}. In agreement with these studies, impaired vasodilatory responses to acetylcholine and the prostaglandin I\textsubscript{2} receptor agonist iloprost, but not to SNP, were observed during a 3 day high salt diet with no elevation in mean arterial pressure\textsuperscript{179}.

In humans, similar impairments in conduit arterial and microvascular function are observed with high dietary sodium intake. Utilizing an eNOS-dependent vasodilatory stimulus (i.e., local skin heating) and pharmacological inhibition of NOS, DuPont \textit{et al.} examined the direct effects of hypertonic saline (3\% NaCl) on endothelium-dependent dilation in the cutaneous microcirculation\textsuperscript{183}. Compared to lactated Ringer’s and normal saline (0.9\% NaCl), hypertonic saline attenuated the initial peak, total vasodilation, and NO component of the local heating response, demonstrating adverse effects of sodium on the microvasculature. One week of dietary sodium loading similarly attenuated the full vasodilatory response and NO contribution to local skin heating in salt-resistant individuals\textsuperscript{15}.

Brachial artery FMD is predictive of adverse cardiovascular events\textsuperscript{17,184}. FMD is a noninvasive method to measure peripheral endothelial function that is largely NO-dependent and is correlated
with endothelial function in the coronary circulation\textsuperscript{185,186}. Acutely, deficits in FMD (≤ 30 minutes post-ingestion) are observed following a high-sodium meal (65 mmol) in the absence of any change in blood pressure\textsuperscript{187}. Following two weeks of dietary sodium restriction (50 mmol vs 150 mmol), FMD was improved in overweight men and women\textsuperscript{188}. Although reductions in blood pressure were observed, there was no correlation between the changes in FMD and changes in blood pressure, again suggesting that a blood pressure-independent mechanism may mediate the improvement in endothelial function with low-salt intake. In healthy salt-resistant participants (<5 mmHg ΔMAP between the low and high sodium phases), controlled feeding of a high-sodium diet (300-350 mmol/d) for 7 days significantly impaired FMD\textsuperscript{14}. Similar sodium-induced impairments in endothelial function are observed in populations characterized by increased CVD risk, including older adults and individuals with elevated SBP\textsuperscript{16,189}. It is important to note that although the sodium content of the high salt diet in some of these studies is far greater than the average American intake, in many instances the experimental sodium loads closely approximate what many individuals consume and clearly demonstrates blood pressure-independent impairments in vascular function induced by high dietary sodium (i.e., that which vastly exceeds the need for normal physiological function).

**Mechanisms of Sodium-Induced Endothelial Dysfunction**

Under healthy conditions, several antioxidant systems exist to control the balance between ROS production and elimination, and protect against increases in oxidative stress generated by normal metabolic processes. Mitochondria are significant sources of ROS as electrons can be diverted to oxygen at several points along the electron transport chain\textsuperscript{190}. NADPH oxidase and xanthine oxidase are two enzymes that produce ROS in the vasculature. More specifically, NADPH oxidases are a family of enzymes that transfer electrons from NADPH to oxygen, leading to the production of superoxide. Xanthine oxidase catalyzes the conversion of hypoxanthine to uric acid, generating superoxide and hydrogen peroxide as byproducts. Additionally, uncoupled eNOS
can generate superoxide, which occurs when electrons from the enzyme’s heme core are donated to molecular oxygen instead of L-arginine. Together, NADPH oxidase, xanthine oxidase and uncoupled eNOS represent potential sources of excess ROS production under pathological conditions. Increases in ROS can also occur through reductions in endogenous antioxidants including the enzymes superoxide dismutase (SOD) and catalase\(^{190}\). SOD enzymes convert superoxide radicals \((O_2^\cdot^-)\) into less detrimental oxygen species, specifically hydrogen peroxide \((H_2O_2)\) or molecular oxygen \((O_2)\). Hydrogen peroxide can be subsequently converted to water and oxygen by the enzyme catalase. Thus, SOD and catalase provide important antioxidant defense mechanisms against increases in ROS production.
Figure 2-2: Schematic of the putative sources of reactive oxygen species and the signaling pathways in the peripheral vasculature that may contribute to reductions in nitric oxide bioavailability and endothelium-dependent dilation in vascular pathology.

In many pathologies associated with endothelial dysfunction, dysregulation of antioxidant systems leads to an exaggerated increase in ROS, via a reduction in antioxidant capacity and/or excess oxidant production, which has the potential to disrupt vascular homeostasis through protein modifications and altered cellular signaling (Fig. 2-2). An emerging body of literature points towards a role for an increase in ROS in impaired endothelial function induced by high dietary sodium intake. In the microcirculation of HS rats, acetylcholine-induced dilation is blunted and concurrently microvessel oxidant activity is increased, suggesting that increased ROS generation contributes to the attenuated endothelium-dependent vasodilation by inactivating...
endothelium-derived NO. Again, no differences in the response to SNP were detected, which suggests that the likely site of ROS generation is not the vascular smooth muscle, but rather, the vascular endothelium.

Additional evidence suggests the involvement of NADPH oxidase, xanthine oxidase, and/or NOS uncoupling as sources of superoxide production in response to a high salt diet. Inhibition of NADPH oxidase and/or xanthine oxidase normalizes resting arteriolar wall oxidant activity and NO bioavailability, indicating that enhanced NADPH oxidase and xanthine oxidase activity may contribute to the loss of basal NO concentrations. Inhibition of NADPH oxidase also restores methacholine-induced vasodilation and NO bioavailability, demonstrating a potential role for NADPH oxidase-derived superoxide in sodium-induced impairments in endothelium-dependent dilation. In the strapezius muscle of HS mice, elevated acetylcholine-induced arteriolar wall oxidant activity is significantly reduced in the presence of a NOS inhibitor. These findings suggest that uncoupled NOS may be a source of sodium-induced superoxide production impairing endothelium-dependent dilation. BH₄ is an essential cofactor for eNOS and a reduction in BH₄ bioavailability is one of the primary mechanisms by which eNOS uncoupling can occur. In spinotrapezius muscle arterioles of HS mice, arteriolar BH₄ concentrations are significantly decreased while superoxide concentrations are increased compared to low salt fed mice. Further, administration of a superoxide scavenger augmented BH₄ concentrations and restored total vasodilation and the NO component of the acetylcholine-induced vasodilatory response in HS mice. Because BH₂, an inactive oxidation product of BH₄, is elevated in HS mice, it is likely that the reduction in BH₄ concentration is due to increased BH₄ oxidation. The most likely reactive species candidate is peroxynitrite, the reaction product of NO and superoxide and potent oxidizer of BH₄.
In addition to increases in ROS production, high dietary sodium may impair the function of antioxidant systems that under normal conditions act to degrade ROS. Downregulation of superoxide dismutase has been demonstrated in the spinotrapezius muscle microvessels, cerebral arteries, and aorta of HS rats. Indeed, administration of a SOD mimic and catalase normalizes microvessel oxidant activity and acetylcholine-induced dilation in the microvasculature of HS rats. Consistent with these findings, impaired methacholine-induced NO release in HS rats can be restored with the SOD mimic tempol. Additionally, a 4-week high salt diet attenuated intermediate calcium-sensitive potassium channel-mediated hyperpolarization of the endothelial cell membrane, an important step for eNOS activation, in response to the endothelium-dependent agonist acetylcholine. However, treatment with the SOD mimic tempol abolished this impairment, suggesting that high salt-induced alterations in antioxidant defense mechanisms may not only contribute to the direct scavenging of NO but also impede signaling pathways required for the production of NO.

Further investigation suggests that angiotensin II suppression may be responsible for the sodium-mediated impairment of SOD. High dietary sodium intake is normally accompanied by reductions in circulating angiotensin II concentrations, a response that serves to increase sodium excretion and restore normal sodium balance. Blunted acetylcholine-induced dilation and reduced expression of SOD in HS rats can be restored with a sub-pressor dose of angiotensin II, suggesting that suppression of angiotensin II plays a role in the downregulation of SOD and resulting oxidative stress. Zhu et al. similarly found that agonist-induced increases in intracellular calcium concentrations and NO bioavailability were attenuated in aortic endothelial cells of rats on a HS diet, which can be attributed to angiotensin II suppression and increased superoxide production, as chronic tempol administration or low-dose angiotensin II infusion prevented the reduction in NO release and impaired calcium signaling. Although these observations may seem counterintuitive given that angiotensin II stimulates superoxide
generation through AT1 receptors, these findings suggest that a threshold level of circulating angiotensin II may be necessary for normal SOD expression and activity. However, the contribution, if any, of sodium-induced suppression of angiotensin II on oxidative stress-mediated endothelial dysfunction has not been examined in humans.

In agreement with the aforementioned animal studies, it is apparent that increases in oxidative stress are responsible, at least in part, for the impairments in endothelial function observed with high dietary sodium intake in humans. Jablonski et al. found that dietary sodium restriction augments acetylcholine-induced endothelium-dependent dilation by reducing oxidative stress and increasing BH₄ and NO bioavailability, as BH₄ and/or ascorbic acid (nonspecific antioxidant) administration abolished the differences in dilation observed between the two sodium conditions. SOD activity was also elevated during low-sodium intake. In the cutaneous microcirculation, local ascorbic acid administration restores the local heating plateau through an increase in NO-dependent vasodilation, demonstrating an oxidative stress-mediated suppression of endothelium-dependent dilation in response to high salt intake. Collectively, the current literature indicates that an increase in ROS production via NADPH oxidase, xanthine oxidase and/or NOS uncoupling, and a loss of antioxidant defense mechanisms, including downregulation of SOD, likely contribute to sodium-induced endothelial dysfunction. However, additional human studies are needed to identify the specific enzymatic source(s) and mechanisms of sodium-induced oxidative stress.
**Figure 2-3:** Schematic of the putative signaling pathways that may contribute to the production of reactive oxygen species and subsequent reduction in nitric oxide bioavailability in the peripheral vasculature induced by high dietary sodium intake. Reprinted with permission from Boeghehold MA. The Effect of High Salt Intake on Endothelial Function: Reduced Vascular Nitric Oxide in the Absence of Hypertension. *Journal of Vascular Research*. 2013;50:458-6. Copyright © 2013 Karger Publishers, Basel, Switzerland.
Summary

Given what is currently known about the effects of both dairy and sodium consumption on vascular health and cardiovascular disease risk, it is unclear what effect sodium intake in the form of cheese has on microvascular endothelial function. Epidemiological data demonstrate a significant inverse correlation between sodium intake and vascular health but a positive correlation between cheese intake and vascular health. Thus, non-sodium dairy-based nutrients may counteract the detrimental effects of sodium on vascular function. As such, sodium intake in the form of cheese may not confer the same cardiovascular disease risk as sodium from non-dairy sources, a finding that would have important implications for the development of dietary guidelines and health policy. The chapters of this dissertation will examine the effects of fluid dairy (dairy-based nutrients in the presence of low sodium) and cheese (dairy-based nutrients in the presence of high sodium) on microvascular function in healthy middle-aged and older adults.
ACUTE DAIRY MILK INGESTION DOES NOT IMPROVE NITRIC OXIDE-DEPENDENT VASODILATION IN THE CUTANEOUS MICROCIRCULATION

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality in the United States and is responsible for ~17% of national healthcare expenditures. As such, effective non-pharmacological prevention strategies and early identification of modifiable lifestyle risk factors (e.g., dietary habits) are essential to limit the growing burden of CVD. Bioactive micro- and macronutrients in particular may have a preventative and protective effect against disease.

Chronic dairy consumption is associated with attenuated age-related increases in blood pressure and improved cardiovascular outcomes. Increased dairy intake has positive effects on blood pressure and measures of conduit-vessel function including pulse wave velocity, and arterial stiffness. However, little is known about the mechanisms by which dairy consumption may improve vessel function specifically at the level of the microcirculation.

The putative mechanisms mediating improvements in vascular function induced by dairy intake are likely complex and may involve the synergistic effects of milk proteins and elemental components. In vitro data indicate that bioactive peptides derived from the two primary milk proteins, whey and casein, exhibit angiotensin converting enzyme (ACE) inhibitor properties and direct antioxidant and radical scavenging activity. In humans chronic consumption of these peptides (1-10 weeks) decrease measures of systemic inflammation (IL-6, MCP-1, and TNFα), and the mineral composition in dairy (calcium, potassium, and magnesium), moderately reduces blood pressure. The common vascular signaling pathway that links each of these purported mechanisms (angiotensin II inhibition, antioxidant properties, anti-inflammatory, etc.) is through increasing nitric oxide (NO) bioavailability. NO is a potent...
vasoprotective agent produced by the vascular endothelium and is essential for vessel health and function. Reduced NO bioavailability is prevalent in all cases of cardiovascular dysfunction, and proceeds the onset of clinical detectable cardiovascular disease\textsuperscript{217-219}.

The cutaneous circulation is an accessible and representative circulation for the \textit{in vivo} study of mechanisms mediating vascular function and dysfunction in humans\textsuperscript{220-222}. Deficits in cutaneous function are highly correlated with measures of vessel dysfunction in the coronary and renal circulations\textsuperscript{223, 224}. Moreover, altered cutaneous microvascular function is evident prior to long-term changes in blood pressure or presentation of clinical symptoms\textsuperscript{225}. As such, the cutaneous vascular bed has utility for the \textit{in vivo} examination of molecular mechanisms by which intervention strategies may affect vessel function in humans.

Given the epidemiological evidence that increased dairy consumption reduces CVD risk across the lifespan\textsuperscript{226, 227} and the evidence that the mechanisms associated with this decrease converge on the NO pathway, the aim of this study was to determine the mechanistic effect of acute milk consumption on cutaneous microvascular function in middle-aged adults. We hypothesized that acute dairy milk consumption (2 and 4 servings) would increase NO-dependent vasodilation in a dose-dependent manner compared to a non-dairy rice milk control.

**METHODS**

**Subjects**

All protocols were approved by the Institutional Review Board at The Pennsylvania State University and complied with the guidelines in the \textit{Declaration of Helsinki}. All participants voluntarily provided written and verbal consent prior to the experiment. Eleven subjects (61±2 years; 5 men, 6 women) participated in the study. Prior to participation, subjects underwent a medical screening that included a 12-lead electrocardiogram, fasting blood chemistry, and
physical examination. Subjects also completed a 24 h ambulatory blood pressure monitoring while enrolled in the study. Inclusion criteria required a daily dairy intake of less than 2 servings. Daily dairy intake was assessed with a modified food frequency questionnaire specific to dairy consumption. Subjects had a 2-day wash-in period of no dairy consumption and abstained from alcoholic and caffeinated beverages for 12 h, vigorous physical activity for 24 h, and food for 8 h prior to each experiment. All subjects were non-smokers, non-diabetic, non-obese (body mass index $< 30 \text{ kg m}^{-2}$), and were not on any prescription medications that may alter vascular function (e.g. statins, antidepressants, antihypertensives, etc.). Women taking hormone replacement therapy were excluded from the study.

**Experimental Protocol**

On four separate visits, subjects ingested 473 mL (2 servings) of 1% dairy milk (Giant Eagle), 946 mL (4 servings) of 1% dairy milk, 473 mL of rice milk (Nature’s Promise Original Enriched), or 946 mL of rice milk with a minimum of 1 week between visits. A subset (n=5) of subjects also ingested 473 mL of water on an additional visit. The treatment order was randomly assigned for each subject. The caloric, macronutrient, and micronutrient content of each treatment are displayed in Table 3-1.
Table 3-1: The micronutrient and macronutrient content of 2 and 4 servings of 1% milk and rice milk.

<table>
<thead>
<tr>
<th></th>
<th>Per 2 dairy serving equivalents</th>
<th>Per 4 dairy serving equivalents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1% milk</td>
<td>Rice milk</td>
</tr>
<tr>
<td>Serving size (mL)</td>
<td>473</td>
<td>473</td>
</tr>
<tr>
<td>Calories (kcal)</td>
<td>220</td>
<td>200</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Saturated Fat (g)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Carbohydrates (g)</td>
<td>24</td>
<td>46</td>
</tr>
<tr>
<td>Sugars (g)</td>
<td>24</td>
<td>30</td>
</tr>
<tr>
<td>Fiber (g)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>260</td>
<td>180</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>600</td>
<td>600</td>
</tr>
<tr>
<td>Potassium (mg)</td>
<td>740</td>
<td>0</td>
</tr>
</tbody>
</table>

All experiments were performed in a thermoneutral environment with subjects in a semi-supine position. Using a sterile technique, one intradermal microdialysis fiber (10 mm, 30kDa membrane limit, MD 2000; Bioanalytical Systems) was placed in the ventral forearm skin. Ice was applied to the forearm for 5 minutes to anesthetize the skin prior to fiber placement. A 25-gauge needle was inserted into the skin with entry and exit points 2-3 cm apart. The fiber was threaded through the needle which was subsequently removed leaving the semipermeable portion of the fiber remaining under the skin. Red blood cell flux (RBF), an index of skin blood flow, was measured by a laser-Doppler flowmetry probe placed in a local heating unit (MoorLab, Temperature Monitor SH02; More Instruments) directly over the microdialysis membrane. Brachial artery blood pressure was recorded at 5-min intervals (Cardiocap; GE Healthcare) throughout the protocol.
Localized microdialysis pharmaceutical perfusates were dissolved in lactated Ringer’s just before use, microfiltered (Acrodisc; Pall, Ann Arbor, MI), and covered in foil to prevent light degradation. Prior to baseline data collection, the fiber was perfused (2 µL/min) with lactated Ringer’s for 60-90 minutes to allow the skin to recover from any trauma caused by the insertion of the microdialysis fiber. Subjects ingested the selected milk treatment after 30 minutes of hyperemia. This time point for consumption was chosen so that the local heating plateau would occur 60-90 minutes post-treatment, which is the time period after milk peptide ingestion when peak intestinal concentrations of bioactive peptides are recovered\textsuperscript{137}.

Subjects were instrumented with an intravenous catheter after fiber placement for the collection of blood samples. A fasted blood sample was taken before treatment administration. Blood samples were then taken every 30 minutes post-treatment until the completion of the study. The blood samples were collected in EDTA treated tubes, which were subsequently refrigerated and centrifuged. The plasma samples were stored at -80°C until future use. Plasma insulin concentrations were measured at baseline and at 90 minutes post-ingestion using a commercially available ELISA (Mercodia, Upsala, Sweden) according to the manufacturer’s instructions. Samples were analyzed in duplicate with an average CV <10%.

Baseline measurements were collected for 20 minutes at a local skin temperature of 33°C. After a stable baseline period, the local skin temperature was increased to 42°C at a rate of 0.5°C every 5 seconds. Once a 10-min plateau was reached (~40 min), 15 mM N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME; Calbiochem, Billerica, MA), a non-specific NOS inhibitor, perfused the site at a rate of 4 µL/min to quantify NO-dependent vasodilation\textsuperscript{228-230}. After 10 minutes of stable red blood cell flux measurements (~45 min), maximal vasodilation was induced by perfusing the fiber with 28 mM sodium nitroprusside (SNP; USP, Rockville, MD) and increasing local skin temperature
to 43°C (30 min). Work in our laboratory and others has demonstrated that this protocol is highly specific to endothelial nitric oxide synthase production and allows the direct quantification of functional NO-dependent vasodilation in the cutaneous microcirculation.

**Data Acquisition & Statistical Analysis**

Data were collected with Windaq (Windaq; Dataq Instruments) at a frequency of 40 Hz. Cutaneous vascular conductance (CVC) was calculated as RBF divided by mean arterial pressure (MAP). Data were normalized to a percent of maximum CVC. CVC data were averaged over a stable 5-min period at baseline, the local heating plateau, the L-NAME plateau and maximum vasodilation. A three-way repeated measures ANOVA was used to detect within-subject effects of dietary treatment and serving size on the phases of the local heating response. There was no main effect of serving size on functional vascular measures, thus the data for the 473 mL and 946 mL servings were combined and a nested two-way repeated measures ANOVA was performed to detect differences between dietary treatment on the parameters of the local heating response (SAS; Version 9.4). Bonferroni post-hoc corrections were performed to account for multiple comparisons when necessary. Significance was accepted using α=0.05. Unless otherwise indicated, all values are presented as mean ± SEM.

**RESULTS**

Subject characteristics are displayed in Table 3-2.
Table 3-2: Subject characteristics (n=11; 5 male, 6 female).

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong> (years)</td>
<td>61</td>
<td>2</td>
</tr>
<tr>
<td><strong>BMI</strong> (kg m(^{-2}))</td>
<td>26.1</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>DBP</strong> (mmHg)</td>
<td>74</td>
<td>2</td>
</tr>
<tr>
<td><strong>SBP</strong> (mmHg)</td>
<td>125</td>
<td>5</td>
</tr>
<tr>
<td><strong>HDL</strong> (mg dl(^{-1}))</td>
<td>61</td>
<td>7</td>
</tr>
<tr>
<td><strong>LDL</strong> (mg dl(^{-1}))</td>
<td>123</td>
<td>11</td>
</tr>
<tr>
<td><strong>Total Cholesterol</strong> (mg dl(^{-1}))</td>
<td>195</td>
<td>12</td>
</tr>
<tr>
<td><strong>Fasting Glucose</strong> (mg dl(^{-1}))</td>
<td>96</td>
<td>4</td>
</tr>
<tr>
<td><strong>HbA1c</strong> (%)</td>
<td>5.6</td>
<td>0.1</td>
</tr>
</tbody>
</table>

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure

**Figure 3-1** depicts an original record of the response to skin local heating.

**Figure 3-1**: Representative tracing of the local heating response in one subject. ↓, Decrease in skin blood flow with nitric oxide synthase inhibition. \(\text{N}^\text{G}\)-nitro-l-arginine methyl ester.
There were no differences in the local heating plateau between the dairy milk and rice milk treatments (Fig. 3-2).

**Figure 3-2:** (a) Local heating plateau and (b) % nitric oxide (NO)-dependent dilation following dairy milk or rice beverage ingestion. * $P=0.004$ difference v. dairy milk. ———, Response in five subjects who completed a water (fasted) trial, included for reference. CVC, cutaneous vascular conductance.

However, the %NO-dependent vasodilation was attenuated following dairy milk ingestion compared to the %NO-dependent vasodilation following rice milk ingestion (R: 49±5, D: 55±5 %CVC max; p<0.01).
To determine whether the insulin responses contributed to the differences in NO-dependent vasodilation between milk treatments, plasma insulin concentrations were measured using the plasma samples from the 90-minute time point, which coincided with the timing of NO quantification during the local heating protocol. The plasma insulin concentration following dairy milk consumption was lower compared to rice milk consumption for both the 2 and 4 servings (2D: 84±10, 2R: 205±20 pmol/L; p<0.001, 4D: 161±37, 4R: 311±45 pmol/L; p<0.001). The lower plasma insulin concentrations following the dairy milk treatments were associated with decreased NO-dependent vasodilation (Fig. 3-3).

Figure 3-3: Plasma insulin response and corresponding % nitric oxide (NO)-dependent dilation 90 min following consumption of two or four servings of dairy milk or rice beverage. The inset depicts the %NO-dependent vasodilation for the dairy milk and rice beverage separated by dose (two servings: 473 ml and four servings: 946 ml). □, Response in five subjects who completed a water (fasted) trial, included for reference.
DISCUSSION

The principle finding of this study was that NO-dependent vasodilation was attenuated following acute dairy milk consumption compared to rice milk. Despite the local heating plateau being similar between dairy and rice milk, when NO-dependent vasodilation was directly quantified it was reduced following dairy milk consumption. Plasma insulin concentrations were also lower following dairy milk ingestion and were associated with decreased NO-dependent vasodilation during local skin heating. Contrary to our hypothesis, these data suggest that acute dairy milk consumption does not augment NO-dependent vasodilation in the cutaneous microcirculation.

The macronutrient content of dairy milk and rice milk differ substantially, which may explain differences observed in the present study. Dairy milk has a low glycemic index compared to rice milk, and was associated with a smaller insulin plasma response. Insulin induces vasodilation in the cutaneous microcirculation through a NO-dependent mechanism. In the present study plasma insulin concentrations were lower following the dairy milk treatments relative to their respective isovolumetric rice milk treatments. Moreover, the lower plasma insulin concentrations following the dairy milk treatments were associated with reduced NO-dependent vasodilation.

Dairy milk proteins have a demonstrated positive benefit on vascular function in both animal and human models. For example, milk peptide supplementation in hypertensive rat models show improvements in endothelial-dependent vasodilation and endothelial nitric oxide synthase (eNOS) expression. In humans, vascular function, measured by flow-mediated vasodilation in the brachial artery and reactive hyperemia, are improved after milk peptide ingestion. Reasons for the discrepancy between these findings and the present study include differences in the form of the milk proteins and the acute nature of the intervention.
studies examining the effects of milk proteins on vascular function have used isolated milk protein hydrolysate \(^{129, 238-241}\) instead of milk or other dairy products. Isolated milk peptides and dairy milk may differ in their resistance to peptidases during digestion and ability to transport across the intestinal wall in an active form. Additionally, many of these studies have been chronic interventions (≥ 1 week) \(^{129, 238-240}\), which may be required to observe differences in vascular function. Ballard et al. conducted a study examining acute low-fat milk consumption on vascular function and found that, unlike rice milk ingestion, dairy milk ingestion maintained endothelial function by reducing the postprandial hyperglycemia \(^{242}\). Differences from the present study include measurement of conduit arterial function, quantified by brachial artery flow-mediated dilation, instead of microvascular function. Moreover, the study by Ballard et al. was conducted on individuals with metabolic syndrome, which may also explain the differences between the two studies.

It is important to note that in the present study the overall vasodilation response to local heating was not different between dairy and rice milk treatments, or different compared to the fasted (water) trial. There is a great deal of redundancy in the mechanisms contributing to the cutaneous vasodilator response to local heat \(^{243-246}\), and our data do not suggest that acute milk ingestion reduces vascular function. Rather, because there was no change of the local heating plateau, the acute exposure to higher insulin concentrations may have modulated the NO contribution to the total vasodilator response following rice milk ingestion. One of the strengths of the current study is that we directly quantified functional NO-dependent vasodilation using an eNOS-dependent stimulus \(^{228}\). In doing this we were able to dissect out the amount of vasodilation due to NO with our specific dietary treatments. This is an initial first step in determining the direct acute effects of dairy on vascular function. The possibility remains that chronic dairy consumption improves endothelial function over a longer time course.
Limitations

Intestinal recovery of milk peptides occurs within 60 to 90 minutes of consumption \(^{137}\) and acute milk consumption has been shown to alter conduit arterial function within 90 minutes post-ingestion \(^{242}\). A chronic milk intervention may be appropriate to elucidate the effects of dairy intake on microvascular function, as it would allow for sufficient time for the milk peptides to act on the peripheral vasculature and would not be masked by an acute insulin response.

Because the proposed mechanisms by which dairy consumption may affect blood vessel function converge on the NO pathway, we focused primarily on NO-dependent mechanisms of microvascular function. However, it is possible that NO-independent mechanisms mediate improvements in vascular function observed with milk peptide consumption. Evidence for contribution from other mechanisms has been documented by increases in resistance vessel blood flow in response to reactive hyperemia, a measure that is largely independent of NO, after milk peptide consumption \(^{239}\).

We chose to recruit healthy middle-aged individuals for this study because (1) our lab has previously shown a moderate age-related deficit in NO-dependent vasodilation in this population \(^{228}\), and (2) this subject group represents a population that would most likely benefit from lifestyle modifications for the prevention of CVD. Other research in this area has focused on populations with overt cardiovascular disease \(^{149, 247}\). As such, it is possible that milk consumption has a more pronounced acute treatment effect on endothelial function in individuals with established vascular disease.

Summary

In summary, the local heating-induced vasodilatory response following dairy milk consumption was not different than the response following rice milk consumption. Contrary to our hypothesis,
NO-dependent vasodilation was decreased after dairy milk ingestion compared to rice milk ingestion. This finding may be associated with a lower acute insulin response following dairy milk intake. Although dairy milk consumption did not acutely increase NO bioavailability, chronic dairy milk consumption may improve or protect endothelial function given the potential ACE-inhibitory and anti-oxidant properties as well as the long-term reductions in blood pressure previously observed 90, 91, 93, 202, 203.

ACKNOWLEDGMENTS
The authors would like to express their gratitude for the assistance of Jane Pierzga, Susan Slimak, Dr. Jody Greaney, Dr. Jessica Kutz and Dan Craighead. Author Contributions: L.M.A, W.L.K, A.E.S designed research; B.K.A., A.E.S conducted research; A.E.S. analyzed data; B.K.A wrote the paper. All authors read and approved the final manuscript.

FUNDING
This research was supported by Dairy Management Inc.

CONFLICT OF INTEREST
None.
Chapter 4

DAIRY CHEESE CONSUMPTION AMELIORATES SINGLE-MEAL SODIUM-INDUCED CUTANEOUS MICROVASCULAR DYSFUNCTION BY REDUCING ASCORBATE-SENSITIVE OXIDANTS IN HEALTHY OLDER ADULTS

INTRODUCTION
Cardiovascular disease (CVD) is the leading cause of mortality in developed nations, with 40% of all deaths in the United States attributable to CVD. The annual health care burden of the treatment and management of CVD is greater than $656 billion and projected to increase as the population ages. As such, identification of modifiable risk factors and non-pharmacological interventions are important for CVD prevention. Increasing dairy intake is an emerging lifestyle factor that is associated with a decreased CVD risk. Long term dairy consumption is associated with lower blood pressure in healthy, aged individuals, but its cardioprotective activities are also mediated independent of its blood pressure-lowering effect. One putative mechanism through which dairy consumption may benefit vascular function is through the antioxidant properties of dairy peptides. Administration of dairy peptides in animal models reduces markers of inflammation and attenuates measures of oxidative stress, including total antioxidant capacity, suggesting that these mechanisms may decrease lifetime risk of cardiovascular morbidity and mortality.

A high dietary sodium intake is independently associated with elevations in arterial blood pressure as well as increased cardiovascular morbidity and mortality. Animal studies of the vascular effects of high dietary sodium implicate endothelium-derived oxidative stress, particularly the production of superoxide, in reduced NO bioavailability and endothelial dysfunction. Similarly, human studies demonstrate that sodium restriction (≤1.5...
g/day) reverses age-associated endothelial dysfunction by increasing NO-dependent vasodilation. Similarly, non-invasive measures of conduit artery endothelial function show that low dietary sodium intake is associated with enhanced flow-mediated vasodilation in middle aged and older adults. In contrast to sodium restriction, even short-term increases in dietary sodium (7 days) impair flow-mediated vasodilation in conduit arteries of otherwise healthy young adults; and even a single high-salt meal can significantly suppress brachial artery flow-mediated dilation within 30 minutes in healthy young adults.

The human cutaneous circulation is an accessible vascular bed for examining mechanisms of microvascular dysfunction in vivo. There is a significant relation between microvascular dysfunction measured in the skin and that measured invasively in the coronary and renal circulations, and intervention-induced improvements in vascular function are detectible in the cutaneous circulation prior to improvements in clinical outcomes. Importantly, dietary sodium-induced impairments in endothelial function are detectable in the cutaneous microvasculature of otherwise healthy adults, independent of changes in blood pressure or blood chemistry. These mechanistic in vivo human studies further demonstrate that even short term (7 day) increases in dietary sodium impair endothelial function and reduce NO bioavailability via an increase in oxidative stress.

Increased consumption of dairy products in the form natural cheese may inadvertently increase dietary sodium intake. Consequently, increasing dairy consumption, particularly in the form of cheese, may paradoxically hinder adherence to dietary sodium recommendations, while still mitigating CVD risk. It is currently unknown whether vasoprotective activities of dairy, provided as natural cheeses, protect against sodium-induced impairments in the vasculature. Therefore, we sought to examine the protective role of macronutrients in natural dairy-cheese against acute dietary sodium induced microvascular dysfunction. We hypothesized that acute natural dairy-
cheese ingestion would improve NO-dependent vasodilation compared to an equal dietary sodium intake from non-dairy sources. Further, we hypothesized that this effect would be mediated by a reduction in ascorbate-sensitive oxidants.

METHODS

Subjects: All protocols were approved by the Institutional Review Board at The Pennsylvania State University and complied with the guidelines in the Declaration of Helsinki. All participants voluntarily provided written and verbal consent prior to the experiment. Fourteen subjects (61±2 years; 8 men, 6 women) participated in the study. Prior to participation, subjects underwent a medical screening that included a 12-lead electrocardiogram, fasting blood chemistry, and physical examination. Subjects also completed a 24 hour ambulatory blood pressure monitoring while enrolled in the study. Inclusion criteria required a daily dairy intake of less than 2 servings. Daily dairy intake was assessed with a modified food frequency questionnaire specific to dairy consumption. Subjects had a 2-day wash-in period where they did not consume any dairy. Experimental visits were separated by at least 3 days, to ensure the 2-day low-dairy wash-in before each visit. There were no recommendations regarding sodium intake during the wash-in. Subjects abstained from alcoholic and caffeinated beverages for 12h, vigorous physical activity for 24h, and food for 8h prior to each experiment. All subjects were non-smokers, non-diabetic, non-obese (body mass index < 30 kg m\(^{-2}\)), and were not taking prescription medications that may alter vascular function (e.g. statins, antidepressants, antihypertensives, dietary supplements, aspirin, etc.). Women taking any form of hormone replacement therapy were excluded from the study. Subject characteristics are presented in Table 4-1.
Table 4-1. Human subject characteristics. Mean ± SEM

<p>| | |</p>
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</tr>
<tr>
<td>Age (years)</td>
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</tr>
<tr>
<td>BMI (kg m(^{-2}))</td>
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<td>SBP (mmHg)</td>
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<tr>
<td>DBP (mmHg)</td>
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<td>Total Cholesterol (mg dL(^{-1}))</td>
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<tr>
<td>HDL (mg dL(^{-1}))</td>
<td>59 ± 4</td>
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<tr>
<td>LDL (mg dL(^{-1}))</td>
<td>128 ± 6</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.7 ± 0.1</td>
</tr>
</tbody>
</table>

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, serum high density lipoprotein; LDL, serum low density lipoprotein; HbA1c, hemoglobin A1c.

Experimental Protocol: Figure 4-1 presents a schematic representation of the experimental protocol. On five separate visits, subjects arrived at the laboratory following an overnight (≥8 hours) fast. Each experimental visit spanned approximately 4 hours. Two intradermal microdialysis fibers (10mm, 20kDa cutoff membrane, MD 2000; Bioanalytical Systems, West Lafayette, IN) were placed into the dermal layer of the ventral left forearm for the local delivery of pharmacological agents\(^{268}\). Pharmacological agents were mixed just prior to use, dissolved in lactated Ringer’s solution, sterilized using syringe microfilters (Acrodisc; Pall, Ann Arbor, MI), and wrapped in foil to prevent degradation due to light exposure. Microdialysis sites were randomly assigned to receive either 10mM ascorbic acid (Sigma, St. Louis, MO) for local delivery of the non-specific antioxidant \(^{267, 269}\); or lactated Ringer’s solution to serve as control.
Site-specific pharmacological solutions were perfused through the microdialysis fibers at a rate of 2µL/min (Bee Hive controller and Baby Bee microinfusion pumps; Bioanalytical Systems).

Figure 4-1: Schematic representation of the protocol. Subjects entered the laboratory after fasting, had two intradermal microdialysis fibres placed, a fasted blood draw and then ingested the treatment diet. Following the resolution of hyperaemia, subjects were instrumented and skin blood flow data were collected at baseline, throughout local heating and during maximal vasodilation. The entire protocol lasted approximately 4 h. Each arrow represents a microdialysis site. l-NAME, N\(^{G}\)-nitro-l-arginine; SNP, sodium nitroprusside; VD, vasodilation.

Following microdialysis fiber placement, subjects were instrumented with an intravenous catheter for blood collection. A fasted sample was collected before dietary treatment administration. Blood samples were then collected every 30 minutes post-treatment until the completion of the study. Whole blood samples were collected in EDTA treated tubes containing o-phenanthroline, p-hydroxy-mercuribenzoic acid, and pepstatin (Wake Forest University, Winston-Salem, NC). Whole blood samples were centrifuged, and plasma samples were frozen and stored at -80°C until future use. Plasma sodium was measured at baseline and at 90 minutes post-ingestion using an electrolyte analyzer (ProLyte, Diamond Diagnostics, Holliston, MA). Plasma angiotensin II concentrations were measured at baseline and at 90 minutes post-ingestion using a commercially
available ELISA (Abcam, Cambridge, UK) according to the manufacturer’s instructions. Samples were analyzed in duplicate with an average CV <10%.

Thirty minutes after microdialysis fibers were placed, subjects consumed either 85g cheddar dairy cheese (560mg Na), 85g soy cheese (560mg Na), 65g pretzels (560mg Na), 170g dairy cheese (1120mg Na), or 130g pretzels (1120mg Na) in randomized order. The treatment order was randomly assigned for each subject using a random number generator and was administered by the investigators. One subject did not consume soy cheese due to palatability issues. Our initial study design included a 170g soy cheese treatment (1120mg Na) however, several subjects refused this treatment due to palatability and we excluded it from further testing. The caloric, macronutrient, and sodium content of each dietary treatment is displayed in Table 4-2. The 30 minute time point for consumption was chosen such that the local heating plateau for our eNOS-dependent vascular stimulus would occur 60-90 minutes post-treatment, corresponding with the time period after milk peptide ingestion when peak intestinal concentrations of bioactive peptides are recovered.

Table 4-2. Sodium, calories, and macronutrient content of dietary treatments.

<table>
<thead>
<tr>
<th></th>
<th>85g cheddar cheese</th>
<th>85g soy cheese</th>
<th>65g pretzel</th>
<th>170g cheddar cheese</th>
<th>130g pretzel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (mg)</td>
<td>560</td>
<td>560</td>
<td>560</td>
<td>1120</td>
<td>1120</td>
</tr>
<tr>
<td>Calories (kcal)</td>
<td>360</td>
<td>210</td>
<td>255</td>
<td>720</td>
<td>509</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>28</td>
<td>21</td>
<td>0</td>
<td>56</td>
<td>0</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>0</td>
<td>6</td>
<td>56</td>
<td>0</td>
<td>112</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>20</td>
<td>3</td>
<td>5</td>
<td>40</td>
<td>10</td>
</tr>
</tbody>
</table>
Sixty to 90 minutes were allowed for hyperemia associated with fiber placement to resolve before baseline data were collected, followed by a standard local heating protocol to induce endothelial nitric oxide synthase (eNOS)-dependent vasodilation as previously described \(^{229, 268}\). After \(\sim 30-40\) min of local heating, when skin blood flow reached an established plateau, 20mM \(\text{N}^\text{G}\)-nitro-L-arginine (L-NAME; Calbiochem, San Diego, CA) was perfused at a rate of 4\(\mu\)L/min to quantify NO-dependent vasodilation at all sites \(^{232, 271}\). After infusion of L-NAME and subsequent stabilization of a post-L-NAME plateau in skin blood flow, 28mM sodium nitroprusside (Nitropress; Abbott Laboratories, Chicago, IL) was perfused and local temperature increased to 43\(^\circ\)C to elicit maximal dilation (CVC\(_{\text{max}}\)) \(^{229, 272}\). Work in our laboratory and others has demonstrated that this protocol is highly specific to eNOS production of NO and allows the direct quantification of functional NO-dependent vasodilation in the cutaneous microcirculation \(^{228, 231, 232}\).

Cutaneous red blood cell flux was continually measured directly over each microdialysis site with an integrated laser-Doppler flowmetry probe placed in a local heating unit (Moor Instruments SHO2). Mean arterial pressure (MAP) was measured at the brachial artery throughout the protocol using an automated blood pressure monitor (CardioCap, GE). Cutaneous vascular conductance (CVC) was calculated as red blood cell flux divided by MAP and expressed as a percent of site-specific maximal vasodilation (%CVC\(_{\text{max}}\)) \(^{229, 273}\).

**Data Acquisition & Statistical Analysis:** Sample size was determined \emph{a priori} by power analysis (\(p=0.8, \alpha =0.05\)). Data were collected with Windaq (Windaq; Dataq Instruments) at a frequency of 40 Hz. A three-way (dietary treatment*local treatment*subject) repeated-measures mixed-model ANOVA was used to detect dietary treatment and local treatment differences in local heating plateau, NO-dependent vasodilation, and maximal CVC (version 9.1.3; SAS, Cary,
A two-way (dietary treatment*subject) repeated measures ANOVA was used to detect dietary treatment differences in plasma sodium and angiotensin II. Bonferroni post-hoc corrections were performed to account for multiple comparisons when necessary. Significance was accepted at $\alpha=0.05$. All values are presented as mean $\pm$ SEM.

RESULTS

There was no difference in baseline or maximal (28mM SNP, 43ºC) CVC between microdialysis sites or across treatments.

Figure 4-2 shows original data records of the skin blood flow response normalized to maximal cutaneous vascular conductance ($\%CVC_{max}$) during local heating in the control and ascorbate-treated microdialysis sites of one subject following non-dairy dietary sodium (pretzel, 1120mg Na) and dairy-cheese sodium (cheddar cheese, 1120mg Na) consumption. The percent decrease with NOS inhibition (L-NAME) following each treatment is indicated.
Figure 4-2: Representative tracing of skin blood flow ($\%CVC_{\text{max}}$) during local heating in the control and ascorbate-treated microdialysis sites of one subject following non-dairy dietary sodium (a; pretzel, 1120 mg Na) and dairy cheese sodium (b; Cheddar cheese, 1120 mg Na) consumption. The difference between the local heating plateau and the post-$N^G$-nitro-l-arginine methyl ester (l-NAME) plateau indicates the vasodilation attributed to the production of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS) ($\%$NO-dependent dilation). CVC, cutaneous vascular conductance.

Figure 4-3 illustrates the total vasodilatory response to local heating (local heating plateau, $\%CVC_{\text{max}}$) in Ringers (control) and ascorbate perfused microdialysis sites following each dietary
treatment. There was no difference in the local heating plateau between sites or among dietary treatments (all \(p>0.05\)).

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**Figure 4-3**: Values are means (\(n\ 14\)), with their standard errors of vasodilation response (\(%CVC_{\text{max}}\)) to local heating in Ringer’s solution (○, control) and ascorbate-perfused (□ antioxidant) microdialysis sites following each dietary treatment. CVC, cutaneous vascular conductance.

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**Figure 4-4** shows the percent NO-dependent vasodilation during local heating in Ringers (control) and ascorbate perfused microdialysis sites following each dietary treatment. NO-dependent vasodilation was greater following 560mg Na contained in dairy cheese (57±3 %) compared to 560mg Na in soy cheese (42±3%; \(p=0.002\)) or 560mg Na in pretzels (43±4%);
p=0.004). NO-dependent vasodilation was also greater following 1120mg Na contained in dairy cheese (55±5 %) compared to 1120mg Na in pretzels (46±5%; p=0.04). Local ascorbate perfusion augmented NO-dependent vasodilation compared to the control microdialysis site following Na ingestion in soy cheese (control: 42±3 vs. ascorbate: 54±3%) (p=0.01) and pretzel treatments with 560mg Na (control: 43±4 vs. ascorbate: 56±5%; p=0.006) and 1120mg Na (control: 46±5 vs. ascorbate: 56±3%; p=0.02). Local ascorbate perfusion did not augment NO-dependent vasodilation compared to the control microdialysis site following 560mg Na ingestion (control: 57±3 vs. ascorbate: 60±3%; p=0.6) and 1120mg Na ingestion (control: 55±5 vs. ascorbate: 54±3%; p=0.7) in dairy cheese.
Figure 4-4: Values are means (n 14), with their standard errors of %NO-dependent vasodilation response to local heating in Ringer’s solution (●, control) and ascorbate-perfused (□, antioxidant) microdialysis sites following each dietary treatment. *P<0.05 compared with dairy products control within sodium ingestion. †P<0.05 compared with Ringer’s site within dietary treatment. NO, nitric oxide.

There were no differences in plasma sodium or angiotensin II concentrations at baseline, or 90 minutes post ingestion, among any of the dietary treatments (sodium: p = 0.1 main effect of treatment, p = 0.2 main effect of time) (angiotensin II: p=0.6 main effect of treatment, p=0.8 main effect of time).
DISCUSSION

To our knowledge, this is the first study to directly examine the potential protective effect of cheddar cheese on dietary sodium-induced microvascular endothelial dysfunction. Our data demonstrate that acute (single-meal) dairy cheese consumption is protective against sodium-induced impairments in NO-dependent vasodilation in the microcirculation. Further, acute localized administration of the non-specific antioxidant ascorbate normalized NO-dependent vasodilation following non-dairy sodium ingestion, but had no effect on NO-dependent vasodilation after ingesting natural cheese, suggesting that non-sodium components of natural cheese protect against acute sodium-induced endothelial dysfunction in the microvasculature of healthy older adults. The primary finding of this study is that the presence of macronutrients in dairy-based natural cheese ameliorates acute sodium-induced reductions in NO-dependent vasodilation by reducing ascorbate-sensitive oxidants. These data suggest that paradoxically increasing dietary sodium by increasing cheese consumption may not confer the same CVD risk as dietary sodium consumption in the absence of dairy.

A high dietary sodium consumption is independently associated with elevated arterial blood pressure $^{259}$ as well as increased cardiovascular morbidity and mortality $^{260}$. Individual sodium excretion resulting from increased dietary intake of sodium $>$5.8 g/day is strongly associated with increased systolic and diastolic pressures of 10-11 mmHg and 6 mmHg, respectively. High quality meta-analyses indicate that reducing sodium intake reduces blood pressure and the risk of stroke and fatal coronary heart disease $^{274}$.

Animal studies of the vascular effects of high dietary sodium implicate endothelium-derived oxidative stress, particularly the production of superoxide, in reduced NO bioavailability and endothelial dysfunction $^{179, 180, 261-263}$. Similarly, human studies demonstrate that sodium restriction ($\leq$1.5 g/day) reverses age-associated endothelial dysfunction by increasing NO-
dependent vasodilation and decreasing superoxide dismutase expression. Even short term increases in dietary sodium (7 days) induce microvascular dysfunction measured in the cutaneous circulation of healthy young adults, an impairment that is mediated by increases in oxidant stress and occurs independent of changes in blood pressure. Similar to our current findings, Dickinson et al demonstrated that a single high-salt meal significantly suppresses brachial flow-mediated dilation 30 and 60 minutes after ingestion in healthy adults. Collectively, the animal and human literature agree that elevated dietary sodium consumption impairs endothelial function and reduces NO bioavailability via increased oxidant stress mechanisms, even in instances where patients do not have overt CVD. Our data add to this body of literature suggesting a single high sodium meal/snack from non-dairy sources acutely reduces NO-dependent vasodilation in healthy middle-aged adults.

The positive impact of chronic dairy consumption on cardiovascular health has been demonstrated in many population-based studies. Increased total dairy intake is associated with improvements in global measures of vascular health and function including blood pressure, pulse wave velocity, arterial compliance, and arterial stiffness. The precise mechanism(s) by which dairy may confer cardiovascular benefits are currently unclear but include angiotensin converting enzyme (ACE) inhibition, protection and enhancement of bioavailable NO, and anti-inflammatory and anti-oxidant properties of dairy proteins and micronutrients. Because no single and specific mechanism has been definitively shown to account for the beneficial effects of increased dairy intake on vascular function, it is likely that lifetime increases in dietary dairy consumption confer cardiovascular benefits through several of these putative mechanisms. However, given the specific role of oxidant stress in dietary sodium-induced vessel dysfunction we focused our investigation on the antioxidant properties of dairy proteins. In support of this global hypothesis, our data suggest that the antioxidant properties of dairy peptides may play a primary role in the protection against dietary sodium induced
reductions in NO bioavailability. Furthermore, we did not observe changes in circulating angiotensin II in response to our acute dietary treatments.

In the current study, we did not observe a reduction in the total vasodilator response to local heating of the skin, but rather an attenuation in the direct functional quantification of NO-mediated vasodilation with non-dairy sodium ingestion. These findings agree with earlier work performed in our laboratory that suggests that middle-aged adults maintain total vasodilator responsiveness to local heating but have reduced eNOS-mediated vasodilation compared to young adults. The current data suggest that a secondary NO-independent pathway is upregulated to compensate for the decrease in eNOS function following acute sodium consumption. However, endothelial derived NO is synthesized ubiquitously throughout the vasculature and plays a crucial anti-atherogenic vasoprotective role. Given the putative role of NO in vascular health and vessel function, as well as possible age-associated reductions in other NO-independent endothelial pathways (prostaglandins, endothelium-derived hyperpolarizing factors, etc.), interventions that target the production and protection of NO at the endothelium are clinically relevant strategies to preserve vascular health and reduce CVD risk across the lifespan.

This study examined acute (single meal) vascular responses to dietary sodium with and without dairy. As expected, we did not observe a time or treatment-dependent change in plasma sodium concentrations. Plasma sodium concentration is tightly regulated, and our findings are consistent with other dietary studies in which high dietary sodium is associated with vessel dysfunction independent of changes in plasma sodium concentrations. We selected our 2 sodium doses based on 2 and 4 servings of natural cheddar cheese. Interestingly, we did not observe a dose-response relation between dietary sodium from non-dairy sources and attenuated NO-dependent dilation. This may have occurred because our doses were relatively close together, or because our
doses were high enough that we were at or near a ceiling effect. The dose-response relation between acute dietary sodium and endothelial dysfunction was not an experimental endpoint in this study, however further work in this area is warranted.

Our test foods were specifically matched for dietary sodium, with the soy cheese comparison included to account for potential differences with fat content and gastric emptying. There was a difference in protein content between cheddar cheese and soy cheese meals in this study. As such, we cannot rule-out the possibility that protein from non-dairy sources may similarly affect NO-dependent dilation. Furthermore, our study design did not account for differences in fat or carbohydrate composition between treatments, a factor that may influence the inflammatory milieu of the vascular endothelium. However, given the documented antioxidant properties of dairy peptide hydrolysates and the beneficial effect of dairy peptide ingestion on vascular endothelial function in humans, our findings fit within the broader hypothesis that specific antioxidant properties of hydrolyzed dairy peptides play a role in preserved or maintained vascular function. Collectively, our data strongly suggest that the macronutrients in dairy protect against acute sodium-induced endothelial dysfunction and that this protective effect is mediated by antioxidant properties of milk proteins. It is still unknown if this vasoprotection is maintained over longer periods of elevated dietary sodium intake. Furthermore, it is unclear if this effect is dependent on sodium being directly incorporated in the dairy (e.g. natural cheese) or if the same benefits would occur if the diet contained high dairy and high sodium separately (e.g. pretzels and milk). Further studies of the protective antioxidant mechanisms of dairy proteins, as well as their chronic role in vasoprotection against high dietary sodium are warranted.

**Summary.** Overall, the current study suggests that the macronutrients in natural cheeses ameliorate sodium-induced vessel dysfunction following a high sodium meal, through antioxidant mechanisms. Increased dairy consumption is associated with decreased risk of
cardiovascular morbidity and mortality, and our data suggest that the antioxidant properties of dairy likely contribute to this association by protecting against dietary sodium-induced impairments in vascular function. Consequently, increasing dietary dairy intake may represent a modifiable and non-pharmacological lifestyle factor that can increase vascular health and function through the production and protection of bioavailable NO.

ACKNOWLEDGEMENTS
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AUTHOR CONTRIBUTIONS
AES, designed research, conducted research, analyzed data and performed statistical analysis, wrote paper, had primary responsibility for final content; BKA, conducted research, analyzed data, wrote paper; WLK, designed research, wrote paper; LMA, designed research, analyzed data, wrote paper, had primary responsibility for final content. All authors read and approved the final version of the manuscript. All laboratory work was conducted at PSU.

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CONFLICT OF INTEREST
None.
CONTROLLED FEEDING OF AN 8-DAY HIGH DAIRY CHEESE DIET PREVENTS SODIUM-INDUCED ENDOTHELIAL DYSFUNCTION IN THE CUTANEOUS MICROCIRCULATION THROUGH REDUCTIONS IN NADPH OXIDASE-DERIVED REACTIVE OXYGEN SPECIES

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality in the United States, accounting for roughly 40% of all deaths\(^1\). As such, identification of modifiable risk factors, including dietary patterns, are becoming increasingly important for the management and prevention of CVD. Increasing dairy intake is an emerging lifestyle factor that is associated with reduced CVD risk\(^73-80\), yet the large majority of Americans over the age of 50 do not meet the recommended intake of 3 servings of dairy per day set forth by the US dietary guidelines\(^2\). Limiting sodium intake is another modifiable dietary factor that is associated with reduced CVD risk\(^1,3,4\). Since some dairy products, cheese in particular, are high in sodium, increasing dairy intake in the form of cheese may inadvertently increase dietary sodium consumption. However, it is currently unknown whether dairy cheese consumption mitigates CVD risk despite its contribution to increased dietary sodium intake.

Some individuals are salt-sensitive, meaning that their blood pressure rises with high dietary sodium intake and decreases with dietary sodium restriction. Salt-sensitivity is associated with an increased risk of hypertension and cardiovascular mortality\(^160\). However, an abundance of research indicates that high dietary sodium ingestion impairs conduit arterial and microvascular function independent of its effects on blood pressure\(^14,15\). In normotensive, salt-resistant adults, endothelium-dependent dilation, assessed by brachial artery flow-mediated dilation (FMD), is significantly reduced following controlled feeding of a high sodium diet\(^14\). Similar blood pressure-independent impairments in endothelial function are observed in the cutaneous
microcirculation of salt-resistant adults as dietary sodium loading attenuates local skin heating-induced vasodilation through a reduction in the vasoprotective dilator nitric oxide (NO)\textsuperscript{15}. An emerging body of literature points towards a role for oxidative stress in sodium-induced endothelial dysfunction\textsuperscript{281, 282}. Known contributors to sodium-induced oxidative stress in animal models include NADPH and xanthine oxidases\textsuperscript{169, 180}, uncoupled nitric oxide synthase (NOS)\textsuperscript{170, 189, 192}, and reduced superoxide dismutase (SOD) activity \textsuperscript{196, 261}. Indeed, sodium-mediated impairments in endothelium-dependent dilation are abolished with administration of NADPH and xanthine oxidase inhibitors, an SOD mimetic, or the essential NOS cofactor tetrahydrobiopterin (BH\textsubscript{4}) in high salt-fed rodents. In line with these animal studies, sodium restriction in middle-aged to older adults improves the vasodilatory response to intrabrachial artery infusion of the endothelium-dependent agonist acetylcholine through reductions in ascorbate-sensitive oxidants, demonstrating that limiting sodium intake protects vascular function through antioxidant mechanisms\textsuperscript{189}. Similarly, local administration of the nonspecific antioxidant ascorbic acid restores endothelium-dependent dilation in the cutaneous microvasculature through an increase in NO-dependent vasodilation, providing further evidence that the impairment in endothelial function in response to high salt intake is mediated by oxidative stress mechanisms. However, specific enzymatic sources of sodium-induced oxidative stress have not been extensively investigated in humans.

Chronic dairy consumption is associated with improved measures of vascular health including lower blood pressure and reduced arterial stiffness\textsuperscript{5-7}. Separate from their hypotensive effects, dairy foods have been shown to protect vascular endothelial function, which is likely mediated by multiple mechanisms including previously demonstrated antioxidant properties of dairy-based macronutrients\textsuperscript{11, 12}. Milk-derived peptides function as radical scavengers\textsuperscript{11, 12}, act as lipid peroxidation inhibitors\textsuperscript{133}, and augment the production of antioxidant enzymes\textsuperscript{134, 135}. In animal models, diets high in dairy reduce NADPH oxidase expression\textsuperscript{125} and, in humans, acute ingestion
of milk-derived proteins increases plasma antioxidant capacity\textsuperscript{136}. Collectively, these studies suggest that dairy proteins may preserve endothelial function through scavenging of reactive oxygen species and merit further investigation of the specific antioxidant mechanisms by which dairy may mitigate increases in oxidative stress.

Recent data from our lab demonstrate that acute single-meal cheese consumption protects against sodium-induced impairments in NO bioavailability\textsuperscript{283}. Furthermore, we found that acute localized administration of ascorbic acid normalized NO-dependent vasodilation following sodium ingestion from non-dairy sources but did not improve NO-dependent vasodilation following dairy cheese ingestion. These previous findings suggest that non-sodium components of dairy cheese protect against acute sodium-induced endothelial dysfunction through antioxidant mechanisms. However, the chronic vasoprotective role of dairy cheese against dietary sodium-induced vascular dysfunction is unclear. Thus, the purpose of this study was to 1) characterize the vasoprotective effects of controlled feeding of an 8-day high cheese diet on sodium-induced endothelial dysfunction and 2) identify the specific antioxidant mechanisms by which dairy cheese protects against sodium-induced oxidative stress. We hypothesized that a high sodium (5500 mg vs 1500 mg) diet will impair endothelium-dependent vasodilation but that a high cheese diet (6 oz/d) will preserve endothelial function that is otherwise impaired by high dietary sodium. We also hypothesized that a high cheese diet will protect against sodium-induced oxidative stress by reducing the accumulation of superoxide radicals caused by high dietary sodium. To mechanistically examine the vasoprotective role of dairy cheese against sodium-induced impairments in endothelial function, we directly quantified NO-dependent vasodilation via local skin heating and measured cutaneous vascular responsiveness to the endothelium-dependent agonist acetylcholine during high and low sodium diets that were either devoid of dairy or contained 4 daily servings of cheese.
METHODS

Subjects

All protocols were approved by the Institutional Review Board at The Pennsylvania State University and complied with the guidelines in the Declaration of Helsinki. All participants voluntarily provided written and verbal consent prior to enrollment. Nine subjects (65±3 years; 4 men, 5 women) participated in the study. Prior to participation, subjects underwent a medical screening that included a medical history, resting blood pressure assessment, fasting blood chemistry and physical examination. Subjects were 55-75 years old and had an office seated SBP between 120 and 140 mmHg, and DBP between 70 and 90 mmHg. Subjects were non-smokers and were not taking dietary supplements or prescription medications that may alter vascular function (e.g. antidepressants, antihypertensives, or statins). Subjects were screened for any known cardiovascular, neurological, metabolic, or renal diseases. Women taking hormone replacement therapy were excluded from the study. Subject characteristics are presented in Table 5-1.
Table 5-1: Baseline subject characteristics

<table>
<thead>
<tr>
<th>Sex (M,F)</th>
<th>(4,5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>65±3</td>
</tr>
<tr>
<td>BMI (kg m^2)</td>
<td>27.7 ± 1.0</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>124 ± 1</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>81 ± 1</td>
</tr>
<tr>
<td>Total Cholesterol (mg dL⁻¹)</td>
<td>196 ± 7</td>
</tr>
<tr>
<td>HDL (mg dL⁻¹)</td>
<td>60 ± 7</td>
</tr>
<tr>
<td>LDL (mg dL⁻¹)</td>
<td>114 ± 7</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.5 ± 0.1</td>
</tr>
</tbody>
</table>

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, serum high density lipoprotein; LDL, serum low density lipoprotein; HbA1c, hemoglobin A1c.

Dietary Protocol

Subjects were enrolled in four separate 8-day controlled feeding dietary interventions in a randomized, crossover study design. All food and beverages were prepared by a registered dietician. The four dietary periods were as follows: 1) low sodium (1500 mg) diet devoid of dairy products (LNa), 2) low sodium diet (1500 mg) containing 6 ounces of cheese per day (LNaC), 3) high sodium (5500 mg) diet devoid of dairy products (HNa), and 4) high sodium diet (5500 mg) containing 6 ounces of cheese per day (HNaC). All four diets were matched for total energy and macronutrients. Subjects were permitted to use sodium-free seasonings and drink coffee or tea with minimal amounts of non-dairy, sodium-free creamer while on the diets. At the time of
enrollment, participants met with a registered dietician to identify their eucaloric energy requirements and were placed accordingly into one of three caloric levels (1900, 2300, and 2700 kcal/d). The macronutrient composition of the dietary interventions providing 2300 kcal/d is presented in Table 5-2. A comprehensive nutrient profile of each diet is provided in Appendix B.

Table 5-2: Macronutrient composition and sodium content of each dietary intervention

<table>
<thead>
<tr>
<th></th>
<th>LNa</th>
<th>LNaC</th>
<th>HNa</th>
<th>HNaC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories (kcal)</td>
<td>2335</td>
<td>2348</td>
<td>2320</td>
<td>2289</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>84.2 (31.4%)</td>
<td>91.0 (34.0%)</td>
<td>81.7 (31.1%)</td>
<td>82.5 (31.8%)</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>288.9 (49.1%)</td>
<td>258.4 (46.4%)</td>
<td>288.4 (49.3%)</td>
<td>272.0 (47.1%)</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>114.0 (19.4%)</td>
<td>113.1 (19.6%)</td>
<td>112.8 (19.5%)</td>
<td>117.5 (21.0%)</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>1514</td>
<td>1475</td>
<td>5477</td>
<td>5496</td>
</tr>
<tr>
<td>Potassium (mg)</td>
<td>3255</td>
<td>2454</td>
<td>3165</td>
<td>2433</td>
</tr>
</tbody>
</table>

Dietary treatment periods were separated by a minimum 1-week washout period. A schematic representation of the experimental protocol for each dietary period is presented in Figure 5-1.
Figure 5-1: Schematic of the timeline for each dietary intervention.

Twenty-four Hour Urine & Blood Pressure

On day 7 of each dietary intervention, participants collected a 24 h urine sample which was analyzed for urinary sodium and potassium (SmartLyte® Electrolyte Analyzer, Diamond Diagnostics, Holliston, MA), urine osmolality (3320 Micro-Osmometer, Advanced Instruments, Norwood, MA), urine specific gravity, and total volume. On day 7, subjects also underwent 24 h ambulatory blood pressure monitoring (Ambulo 2400, Tiba Medical, Portland, OR) which was used to identify subjects with salt-sensitive blood pressure. During the 24 h period, blood pressure was measured every 20 min during waking hours and every hour during sleeping hours. Subjects that exhibited more than a 10 mmHg increase in 24 h mean arterial pressure from the low sodium diet to the high sodium diet were classified as salt-sensitive.

Blood Analyses

Blood draws were performed on day 8 of each dietary treatment period. Hemoglobin and hematocrit were analyzed from whole blood (Quest Diagnostics, Pittsburg, PA).
Assessment of Micro- and Macrovascular Function

On the mornings of day 8 and 9, cutaneous vasodilator responsiveness to exogenous acetylcholine and local heat were assessed (Figs. 5-2A and 2B). The acetylcholine dose response and local heating protocols were performed on the mornings of day 8 and day 9 in a randomized order. On the day of the experiments, subjects reported to the laboratory fasting and remained fasting until completion of the experiment. Subjects abstained from vigorous physical activity for 24 h prior to each experiment.

Figure 5-2: Protocol schematics of the (A) acetylcholine dose response and (B) local heating experiments during which cutaneous red blood cell flux was measured for assessment of microvascular endothelial function.
Acetylcholine-Induced Vasodilation

Experiments were conducted in a thermoneutral environment with subjects in a semi-supine position. Using sterile technique, five intradermal microdialysis fibers (10 mm, 55 kDa cutoff membrane; CMA, Holliston, MA) were placed in the dermal layer of the ventral forearm. Ice was applied to the forearm for 5 minutes to anesthetize the skin prior to fiber placement. A 23-gauge needle was inserted into the skin with entry and exit points ~2 cm apart. The microdialysis fiber was threaded through the lumen of the needle. The needle was subsequently removed leaving the semipermeable membrane remaining under the skin. Red blood cell flux, an index of skin blood flow, was measured directly over each microdialysis membrane by a laser-Doppler flowmetry probe placed in a local heating unit (MoorLab, Temperature Monitor SH02; More Instruments) which was set to a thermoneutral temperature of 33°C unless noted otherwise. Brachial blood pressure was recorded (Cardiocal; GE Healthcare) every 5 minutes during the protocol.

All pharmaceutical perfusates were dissolved in lactated Ringer’s solution just before use, microfiltered (Acrodisc; Pall, Ann Arbor, MI), and covered in foil to prevent degradation from light exposure. Prior to baseline data collection, 60-90 minutes were allowed for any hyperemia caused by the insertion of the fibers to resolve. During the hyperemia resolution period, the fibers were randomly assigned and perfused (2 μL/min; Bee Give controller and Baby Bee microinfusion pumps; Bioanalytical Systems, West Lafayette, IN) with 1) lactated Ringer’s to serve as control 2) 15 mM N^G^-nitro-L-arginine methyl ester (L-NAME; Calbiochem, EMD Millipore, Billerica, MA) to inhibit nitric oxide synthase 3) 10 mM L-ascorbate (Sigma, St. Louis, MO) to serve as a non-specific antioxidant 4) 100 μM apocynin (Tocris Bioscience, Bristol, UK and Sigma, St. Louis, MO) to inhibit NADPH oxidase or 5) 10 μM tempol (Sigma, St. Louis, MO) to serve as a SOD mimetic.
Following 20 minutes of baseline data collection, ascending concentrations of acetylcholine (ACh; 10^{-12} to 10^{-1} M; USP, Rockville, MD) alone or in combination with 15 mM LNAME, 10 mM L-ascorbate, 100 μM apocynin, or 10 μM temprool were perfused through the microdialysis fibers. Following completion of the acetylcholine dose response, 28 mM sodium nitroprusside (SNP; USP, Rockville, MD) was perfused at all sites and local skin temperature was increased to 43°C to induce maximal vasodilation^{20, 53, 290}.

**Local Heating**

Four intradermal microdialysis fibers were placed in the dermal layer of the ventral forearm as described above. Following fiber placement, the fibers were randomly assigned and perfused with lactated Ringer’s, 10 mM L-ascorbate, 100 μM apocynin, or 10 μM temprool. After a stable baseline period, local skin temperature was increased to 42°C at a rate of 0.5°C every 5 seconds. Once a 10-min plateau was reached, all fibers were perfused with 15 mM LNAME at a rate of 4 μL/min to quantify NO-dependent vasodilation^{42, 53, 59}. After 10 minutes of stable red blood cell flux measurements, maximal vasodilation was induced as described above (28 mM SNP, local heating 43°C).

**Flow-Mediated Dilation and Nitroglycerin-Induced Dilation**

On day 8 of each dietary intervention, brachial artery flow-mediated dilation (FMD) and nitroglycerin (NTG)-induced dilation (NID) were performed to assess endothelium-dependent and -independent dilation, respectively. Experiments were conducted in a thermoneutral environment with subjects in a supine position. After 10 min of quiet rest, FMD was assessed with high-frequency ultrasound. A blood pressure cuff was placed on the right forearm, distal to the ultrasound probe. Longitudinal images of the brachial artery ~5-10 cm above the elbow were obtained with ultrasound imaging (Acuson Aspen 128XP with a 10-mHz linear array transducer). Images were obtained during a 1 min baseline period, 5 min of arterial occlusion via cuff inflation.
at 250 mmHg (Hokanson), and 2 min of reactive hyperemia. Blood velocity was recorded during 10-15 seconds of baseline and immediately following cuff release for calculation of reactive hyperemia.

The subjects underwent a 10-min resting period between the FMD and NID protocols. To assess endothelium-independent dilation, ultrasound images were recorded during a 1-min baseline period and 5 minutes following NTG administration (sublingual; 0.4 mg).

**Data Acquisition & Statistical Analysis**

*Local Heating and Acetylcholine-Induced Vasodilation* Data were collected with Windaq (Windaq; Dataq Instruments) at a frequency of 40 Hz. Cutaneous vascular conductance (CVC) was calculated as red blood cell flux divided by mean arterial pressure. Data were normalized to a percent of maximum CVC. Acetylcholine-dose concentrations were log-transformed and CVC was expressed as a percentage of maximum. A two-way repeated measures ANOVA was used to compare vasodilation responses to acetylcholine administration. Pharmacological curve modeling was performed using a four-parameter nonlinear regression with variable slope to detect differences in vascular sensitivity (logEC$_{50}$) to acetylcholine (Prism v7.01, GraphPad, San Diego, CA). During local heating, CVC data were averaged over a stable 10-min period at baseline, the local heating plateau, the L-NAME plateau and maximum vasodilation. NO-dependent vasodilation was calculated as the difference between CVC at the local heating plateau and CVC at the post-LNAME plateau. A two-way repeated measures ANOVA was used to detect differences across dietary treatments on the parameters of the local heating response (baseline, local heating plateau, LNAME plateau) (SAS; Version 9.4).

*FMD and NTG* Automated edge detection software (Brachial Analyzer) was used to measure brachial artery diameter continuously throughout the protocol. Baseline diameter was an average of all images obtained during the 1-min baseline period. Peak artery diameter was the largest
diameter obtained during the 2-min reactive hyperemia and the 5-min post-NTG period for calculation of FMD and NID, respectively. FMD and NID were calculated as a percentage change from baseline diameter. Blood flow velocity and velocity time integral were measured using duplex pulsed Doppler. Arterial blood flow was calculated as velocity time integral × heart rate × arterial cross sectional area where brachial artery cross sectional area was calculated as $\pi \times (\frac{1}{2} \times \text{baseline artery diameter})^2$. Reactive hyperemia was expressed as a percent change in blood flow following cuff release and was calculated as \[ \frac{(\text{hyperemic flow volume} - \text{baseline flow volume})}{\text{baseline flow volume}} \times 100 \]. A mixed-model ANOVA (SAS; Version 9.4) was used to detect differences across dietary treatments on FMD, NID, and hemodynamic parameters of the FMD response.

Tukey’s post-hoc corrections were performed to account for multiple comparisons when necessary. Significance was accepted using $\alpha=0.05$. Unless otherwise indicated, all values are presented as mean ± SEM.

**RESULTS**

**Hemodynamic and Biochemical Parameters**

Mean hemodynamic and biochemical parameters during each dietary intervention are displayed in Table 5-3.
Table 5-3: Hemodynamic and biochemical parameters during each dietary intervention.

<table>
<thead>
<tr>
<th></th>
<th>LNa</th>
<th>LNaC</th>
<th>HNa</th>
<th>HNaC</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h SBP (mmHg)</td>
<td>124.1 ± 2.0</td>
<td>123.2 ± 3.4</td>
<td>127.0 ± 3.9</td>
<td>126.2 ± 4.0</td>
</tr>
<tr>
<td>24 h DBP (mmHg)</td>
<td>77.4 ± 1.4</td>
<td>76.4 ± 1.5</td>
<td>78.8 ± 1.6</td>
<td>77.6 ± 1.6</td>
</tr>
<tr>
<td>24 h MAP (mmHg) *</td>
<td>93.0 ±1.1</td>
<td>92.0 ± 1.7</td>
<td>94.9 ± 2.0</td>
<td>93.8 ± 2.2</td>
</tr>
<tr>
<td>24 h Na⁺ Excretion (mmol/24h)</td>
<td>69.8 ± 9.6</td>
<td>59.7 ± 6.2</td>
<td>253.8 ± 22.3</td>
<td>215.1 ± 20.8</td>
</tr>
<tr>
<td>24 h K⁺ Excretion (mmol/24h)</td>
<td>34.2 ± 3.9</td>
<td>31.8 ± 2.7</td>
<td>40.9 ± 3.2</td>
<td>37.7 ± 3.0</td>
</tr>
<tr>
<td>Urine Osmolality (mOsm kg H₂O⁻¹)</td>
<td>403 ± 75</td>
<td>420 ± 49</td>
<td>502 ± 70</td>
<td>521 ± 64</td>
</tr>
<tr>
<td>Urine Specific Gravity</td>
<td>1.012 ± 0.002</td>
<td>1.012 ± 0.002</td>
<td>1.021 ± 0.009</td>
<td>1.014 ± 0.002</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>14.0 ± 0.3</td>
<td>14.0 ± 0.3</td>
<td>13.4 ± 0.3</td>
<td>13.3 ± 0.3</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>42.4 ± 0.9</td>
<td>41.4 ± 0.9</td>
<td>39.8 ± 0.8</td>
<td>39.5 ± 0.7</td>
</tr>
</tbody>
</table>

*Salt-sensitive subjects (≥ 10 mmHg Δ MAP between LNa and HNa diets) were excluded.

Salt-sensitive subjects (> 10 mmHg change in mean arterial pressure from LNa to HNa) were excluded from analysis. Thus, 24 h systolic, diastolic, and mean arterial blood pressure were not different across diets (p>0.05). As expected, urinary sodium excretion was significantly higher following HNa and HNaC compared to LNa and LNaC (LNa: 63.0 ± 7.8 vs LNaC: 59.7 ± 6.2 vs HNa: 253.8 ± 22.3 vs HNaC: 215.1 ± 20.8 mmol/24h; p<0.05), providing support for the appropriate classification of the included subjects as salt-resistant. No significant differences in urinary potassium excretion, urine osmolality, urine specific gravity, hematocrit, or hemoglobin were observed across diets (all p>0.05).

**Acetylcholine-Induced Vasodilation**

Cutaneous vascular responsiveness to acetylcholine was significantly attenuated during HNa compared to LNa (Fig 5-3A; LNa: -4.70 ± 0.20 M vs HNa: -2.45 ± 0.95 M logEC<sub>50</sub>: p = 0.0003). Conversely, vascular sensitivity to acetylcholine during LNaC or HNaC was not different from...
LNa (LNa: -4.70 ± 0.20 M vs LNaC: -5.29 ± 0.27 M vs HNaC: -4.36 ± 0.69 M logEC$_{50}$; p > 0.05).

**Figure 5-3**: Cutaneous vascular conductance (%max) in response to (A) perfusion of exogenous acetylcholine (ACh) alone and (B) during concurrent perfusion of the nitric oxide synthase (NOS) inhibitor, LNAME. LNa, low sodium diet; LNaC, low sodium diet containing cheese; HNa, high sodium diet; HNaC, high sodium diet containing cheese.

* p<0.05 HNa vs. LNa; † p<0.05 HNa vs. LNaC; # p<0.05 HNa vs. HNaC

Further, no differences in vasodilatory responsiveness to acetylcholine during concurrent perfusion of LNAME were observed between HNa and LNa or HNaC (Fig 5-3B; HNa + LNAME: -2.71 ± 0.98 M vs LNa + LNAME: -3.53 ± 0.56 M vs HNaC + LNAME: -4.62 ± 0.78 M logEC$_{50}$; p > 0.05), suggesting that high dietary sodium impairs endothelium-dependent dilation through a decrease in NO-dependent vasodilation and that inclusion of cheese in a high
sodium diet restores endothelial function, at least in part, through an increase in NO bioavailability.

Local ascorbate or apocynin (Fig 5-4; HNa: -2.45 ± 0.95 M vs. HNa + Apocynin: -5.02 ± 0.43 M logEC\textsubscript{50}; p=0.02, HNa: -2.45 ± 0.95 M vs. HNa + Ascorbate: -4.66 ± 0.21 M logEC\textsubscript{50}; p=0.0004), but not tempol (HNa: -2.45 ± 0.95 M vs. HNa + Tempol: -4.26 ± 0.59 M logEC\textsubscript{50}; p=0.10), administration significantly improved vascular sensitivity to acetylcholine during HNa to the level observed during HNaC. Local administration of ascorbate, tempol, or apocynin did not further improve vascular responsiveness to acetylcholine during LNa, LNaC, or HNaC (data not shown; all p>0.05).
Figure 5-4: Cutaneous vascular conductance (%max) in response to perfusion of exogenous acetylcholine (ACh) alone during HNa and HNaC and with concurrent perfusion of ascorbate (nonspecific antioxidant), apocynin (NADPH oxidase inhibition), or tempol (superoxide dismutase mimetic) during HNa. HNa, high sodium diet; HNaC, high sodium diet containing cheese. * p<0.05 HNa vs. HNaC; † p<0.05 HNa vs. HNa + Apocynin; # p<0.05 HNa vs. HNa + Tempol; ‡ p<0.05 HNa vs. HNa + Ascorbate

Local Skin Heating

Baseline %CVCmax was not different across diets. The total vasodilatory response to local heat (Fig. 5-5A; all p>0.05) and the NO contribution to the response was not different across diets (Fig. 5-5B; all p>0.05) or microdialysis treatments (data not shown; all p>0.05). Maximum vasodilation (CVC) was also not different across diets (data not shown; all p>0.05).
Figure 5-5: (A) Local heating plateau and (B) % nitric oxide (NO)-dependent vasodilation during LNa, LNaC, HNa, and HNaC. LNa, low sodium diet; LNaC, low sodium diet containing cheese; HNa, high sodium diet; HNaC, high sodium diet containing cheese.

Flow-Mediated Dilation and Nitroglycerin-Induced Dilation

Endothelium-dependent dilation measured via brachial artery FMD appears to be reduced during HNa, but not during HNaC, compared to LNa (Fig. 5-6A; LNa: 4.9 ± 1.8% vs HNa: 3.3 ± 1.3%; p=0.095, LNa: 4.9 ± 1.8% vs HNaC: 4.9 ± 2.5%; p=0.71). Endothelium-independent dilation measured via NTG-induced dilation was not different across diets (Fig. 5-6B; p>0.05).
Figure 5-6: (A) Flow-mediated dilation (FMD) and (B) nitroglycerin (NTG)-induced dilation during LNa, LNaC, HNa, and HNaC. LNa, low sodium diet; LNaC, low sodium diet containing cheese; HNa, high sodium diet; HNaC, high sodium diet containing cheese.

Brachial artery diameter, average blood velocity, and blood flow volume before and after cuff occlusion did not differ across diets (Table 5-4; all p>0.05). As such, reactive hyperemia was not different across diets.
Table 5-4. Hemodynamic parameters of the FMD response during each diet.

<table>
<thead>
<tr>
<th></th>
<th>LNa</th>
<th>LNaC</th>
<th>HNa</th>
<th>HNaC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline Blood Flow (ml/min)</td>
<td>117.0±13.9</td>
<td>124.9±20.1</td>
<td>112.2±14.5</td>
<td>126.0±21.9</td>
</tr>
<tr>
<td>Baseline Blood Velocity (cm/s)</td>
<td>15.2±0.45</td>
<td>18.3±3.4</td>
<td>13.3±1.3</td>
<td>15.9±1.3</td>
</tr>
<tr>
<td>Baseline Artery Diameter (mm)</td>
<td>4.67±0.32</td>
<td>4.43±0.24</td>
<td>4.90±0.32</td>
<td>4.66±0.23</td>
</tr>
<tr>
<td>Hyperemic Blood Flow (ml/min)</td>
<td>927.7±86.4</td>
<td>893.3±94.3</td>
<td>923.8±98.0</td>
<td>898.0±94.8</td>
</tr>
<tr>
<td>Hyperemic Blood Velocity (cm/s)</td>
<td>84.5±9.6</td>
<td>93.9±10.9</td>
<td>79.6±8.0</td>
<td>83.8±5.6</td>
</tr>
<tr>
<td>Hyperemic Artery Diameter (mm)</td>
<td>4.85±0.33</td>
<td>4.62±0.24</td>
<td>5.04±0.32</td>
<td>4.75±0.23</td>
</tr>
<tr>
<td>Reactive Hyperemia (%)</td>
<td>725.3±64.2</td>
<td>686.5±81.0</td>
<td>773±103.9</td>
<td>686.6±71.9</td>
</tr>
</tbody>
</table>

DISCUSSION

The results of the present study demonstrate that high dietary sodium ingestion impairs cutaneous vascular responsiveness to acetylcholine through reductions in NO bioavailability in the cutaneous microcirculation of salt-resistant, middle-aged to older adults. Importantly, inclusion of cheese into a high sodium diet prevented the reduction in vascular sensitivity to acetylcholine otherwise observed with high dietary sodium ingestion, which may be mediated by an increase in NO-dependent vasodilation. These differences in cutaneous microvascular endothelium-dependent vasodilation were similarly observed in the conduit arterial macrocirculation as brachial artery FMD, but not NTG-induced dilation, was reduced during the high sodium diet but not during the high sodium diet containing cheese. Further, in the cutaneous microvasculature, NADPH oxidase inhibition or local ascorbate administration restored vasodilatory responsiveness to acetylcholine during the high sodium diet but not during the low sodium diets or the high sodium diet containing cheese. These findings suggest that increases in oxidative stress, specifically NADPH oxidase-derived reactive oxygen species, contribute to sodium-induced...
endothelial dysfunction and that inclusion of 4 daily servings of dairy cheese into a high sodium diet protects against sodium-induced NADPH oxidase-derived oxidative stress.

FMD is a noninvasive method to measure peripheral endothelial function that is largely NO-dependent and is predictive of future cardiovascular disease. Several studies have demonstrated an impairment in FMD with high dietary sodium intake. In line with these studies, the forearm blood flow response to intrabrachial artery infusion of acetylcholine is impaired with moderate sodium intake compared to low sodium intake in healthy middle-aged adults. The results of the present study support these findings and further demonstrate that inclusion of a dairy source of sodium, namely cheese, into a diet prevents the reduction in brachial artery endothelium-dependent dilation and acetylcholine-induced cutaneous vasodilation that is otherwise observed with high dietary sodium intake.

Although we observed a sodium-induced impairment in endothelium-dependent vasodilation in response to acetylcholine, we unexpectedly did not observe differences in total vasodilation or NO-dependent vasodilation during local skin heating between the low and high sodium diets. In contrast, Greaney et al. observed a reduction in the vasodilatory response and NO contribution to local heating during controlled feeding of a high sodium diet. However, the sodium load in that study was much lower in the low sodium diet (~500 mg) and significantly higher in the high sodium diet (~8000 mg) than in the current study. Although we expected to see an impairment with a sodium load of 5500 mg (vs 1500 mg), it is possible that a threshold amount of sodium exists at which impairments in NO signaling occur in the cutaneous microvasculature.

Additionally, we did not find an improvement in NO-dependent vasodilation during local skin heating with the inclusion of cheese into a high sodium diet. This finding may be at least partially explained by the difference in the amount of potassium across diets. The LNaC and HNaC diets had a lower potassium content compared to the LNa and HNa diets (~3200 mg in HNa and LNa...
vs 2400 mg in HNaC and LNaC). Dietary potassium intake improves measures of NO bioavailability and may partially mitigate sodium-induced endothelial dysfunction\textsuperscript{100, 291, 292}. In fact, the ratio of dietary sodium-to-potassium intake is becoming increasingly recognized as a predictor of CVD, even more so than dietary sodium consumption alone\textsuperscript{101, 102}. Thus, a higher potassium content in the non-cheese diets may have counteracted some of the deleterious effects of sodium on NO-dependent vasodilation, making it difficult to detect an effect from including cheese in the high sodium diet.

A mounting body of literature suggests that increases in oxidative stress mediate impairments in endothelial function induced by high dietary sodium intake. In animal models, inhibition of NADPH oxidase restores cholinergic-mediated NO production and vascular relaxation in animals fed a high salt diet\textsuperscript{180, 191}. Additionally, in the skeletal muscle circulation of high salt-fed mice, arteriolar oxidant activity is augmented and concentrations of tetrahydrobiopterin (BH\textsubscript{4}) are reduced compared to low salt-fed mice\textsuperscript{192, 262}. BH\textsubscript{4} is an essential cofactor for the function of endothelial NOS\textsuperscript{62}. Reductions in cofactor bioavailability can lead to ‘uncoupling’ of NOS by which electrons from the enzyme’s heme core are donated to molecular oxygen instead of the substrate L-arginine, thus producing superoxide. In humans, Jablonski et al. demonstrated that dietary sodium restriction augments acetylcholine-induced brachial artery endothelium-dependent dilation and that co-infusion of ascorbate or BH\textsubscript{4} supplementation abolishes the differences in endothelial function between the low and high sodium conditions\textsuperscript{189}. In agreement with the aforementioned studies, we observed improvements in acetylcholine-induced vasodilation with local administration of ascorbate or apocynin, an inhibitor of NADPH oxidase, during HNa but not LNa, suggesting that NADPH oxidase and potentially NOS uncoupling contribute to sodium-induced increases in oxidative stress.
Improvement in endothelial function with NADPH oxidase blockade or ascorbate administration during high sodium intake indicates that increases in superoxide production likely contribute to sodium-induced endothelial dysfunction. As such, we would also expect to observe a similar improvement with administration of SOD, an enzyme that scavenges superoxide. Downregulation of SOD has been demonstrated in the spinotrapezius muscle microvessels, cerebral arteries, and aorta of high salt-fed rats. In addition, impaired cholinergic-mediated NO release and hyperpolarization of the endothelial cell membrane, an important step for eNOS activation, in high salt-fed rats can be restored with tempol administration, suggesting that reductions in SOD expression and/or activity contribute to impaired endothelium-dependent dilation and NO signaling under high dietary sodium conditions. Indeed, Jablonski et al. found an upregulation of SOD activity during dietary sodium restriction in middle-aged and older adults. Although we observed an improvement with local tempol administration during HNa, it did not reach statistical significance. The concentration of tempol used in the present study (10 μM) has been shown to be effective in other pathologies characterized by increased superoxide production; however, it may not have been a sufficient dose to scavenge superoxide in the current sodium-induced pro-oxidant environment.

In controlled studies examining the vasoprotective effects of dairy consumption, ingestion of dairy or milk-derived proteins reduces measures of oxidative stress. In mice, 3 weeks of a high dairy diet reduced NADPH oxidase expression and intracellular ROS concentrations. Similarly, in humans, consumption of isolated dairy proteins or nonfat dairy milk significantly increases plasma antioxidant capacity and reduces markers of oxidative stress including MDA and oxLDL. Consistent with the aforementioned studies, our lab recently provided further evidence for the antioxidant properties of dairy by demonstrating that acute dairy cheese consumption prevents sodium-induced endothelial dysfunction through a reduction of ascorbate-sensitive oxidants. The results from the current study extend these findings and show that short-term...
inclusion of cheese into a high salt diet preserves endothelium-dependent dilation through reductions in NADPH oxidase-derived reactive oxygen species.

**Limitations**

Although there is robust evidence for bioactive properties of milk-derived peptides, we cannot attribute the beneficial vascular effects observed in the present study specifically to the dairy proteins in cheese. The dietary interventions were matched for macronutrients (i.e. total protein, fat, and carbohydrates) but not micronutrients (e.g., calcium, potassium, and magnesium). Thus, we cannot identify the relative contributions of specific nutrient(s) in cheese (e.g., dairy proteins) to the vascular benefits observed in the current study. Future studies are needed to examine the vascular effects of specific nutrients in dairy cheese on sodium-induced microvascular dysfunction.

We did not obtain measures of cholesterol or glucose control following each dietary intervention. Because the diets were only 8 days in duration and were well-matched for energy and macronutrient content, we would not expect cholesterol or insulin resistance to be different across diets. However, we cannot exclude the possibility that positive alterations in cholesterol or glucose homeostasis contributed to the beneficial effects of dairy observed in the current study.

**Summary**

The average dairy consumption remains significantly lower than the recommended intake of 3 servings per day. Further, the average dietary sodium intake in the United States is significantly greater than the recommended upper limit of 2300 mg of sodium per day set forth by the American Heart Association and only a small percentage (~5%) of total sodium consumption is in the form of dairy foods. Thus, although cheese consumption may contribute to sodium intake, our findings suggest that natural dairy cheese prevents sodium-induced blood vessel dysfunction.
through reductions in oxidative stress and that consuming dairy cheese, rather than other non-dairy sources of sodium, may be an effective strategy to reduce CVD risk in salt-resistant, middle-aged and older adults.

ACKNOWLEDGEMENTS
The authors would like to express their gratitude for the assistance of Paul Wagner, Amy Ciccarella, Susan Slimak, and Jane Pierzga.

FUNDING
This research was supported by the National Dairy Council.
Chapter 6

CONCLUSIONS AND FUTURE DIRECTIONS

The series of studies that comprise this dissertation were conducted to examine the protective role of dairy on microvascular function. This dissertation specifically examined 1) the potential antioxidant and anti-inflammatory effects of acute low-fat milk and cheese consumption on endothelial function and 2) the antioxidant effects of short-term cheese ingestion on sodium-induced microvascular dysfunction. Collectively, these studies suggest that the antioxidant properties of dairy-based nutrients, specifically the non-sodium components of dairy cheese, protect against sodium-induced impairments in endothelial function and that inclusion of cheese into the diet may be an effective dietary intervention to mitigate age-related increases in CVD risk. This chapter summarizes the findings of these studies and provides future directions for this line of research.

Acute Dairy Milk Ingestion Does Not Improve Nitric Oxide-Dependent Vasodilation in the Cutaneous Microcirculation

The principle finding of this study was that the vasodilatory response to local skin heating following dairy milk consumption was not different than the response following rice milk consumption. Contrary to our initial hypothesis, NO-dependent vasodilation was reduced after dairy milk ingestion compared to rice milk ingestion, a difference that was likely mediated by a lower acute insulin response following dairy milk consumption.

Implications

While we did not observe an improvement in microvascular function following acute milk consumption compared to a rice milk control, there may be a vasoprotective effect of chronic
dairy milk consumption. Given the large body of research demonstrating beneficial antioxidant and anti-inflammatory properties of milk-based proteins, the chronic benefits of including fluid dairy products (i.e., milk) into the routine diet merits further investigation. Additional chronic, controlled intervention studies are needed to examine these potential long-term effects of milk on vascular health and function.

**Dairy Cheese Consumption Ameliorates Single-Meal Sodium-Induced Cutaneous Microvascular Dysfunction by Reducing Ascorbate-Sensitive Oxidants in Healthy Older Adults**

The results of this study demonstrated that single-meal ingestion of sodium in the form of cheese protects against acute sodium-induced microvascular dysfunction. NO-dependent vasodilation was significantly greater following consumption of dairy-based sodium (i.e., cheese) compared to an equal sodium load from non-dairy sources. Further, our data suggest that the protective effect of dairy cheese on sodium-induced endothelial dysfunction is mediated by antioxidant mechanisms.

**Implications**

The findings of the present study substantiate the current literature suggesting that dairy-based proteins act as antioxidants. Our data extend this literature and demonstrate that the antioxidant properties of dairy-based nutrients may protect against sodium-induced increases in oxidative stress. Taken together, these data suggest that consumption of sodium in the form of cheese may be an appropriate dietary intervention strategy to mitigate age-related increases in vascular dysfunction.
Short-Term Cheese Consumption Reduces Sodium-Induced Endothelial Dysfunction in the Cutaneous Microcirculation of Healthy Older Adults

The principle findings of this study are two-fold. First, we found that high dietary sodium ingestion impairs endothelium-dependent vasodilation through increases in NADPH oxidase-derived reactive oxygen species in salt-resistant, older adults. Secondly, inclusion of cheese into a high sodium diet prevented the reduction in endothelium-dependent dilation observed with high dietary sodium ingestion through reductions in sodium-induced oxidative stress.

Implications

The findings of the current study suggest that increases in oxidative stress, specifically NADPH-derived reactive oxygen species, contribute to sodium-induced endothelial dysfunction and that inclusion of 4 daily servings of dairy cheese into a high sodium diet protects against sodium-induced oxidative stress and endothelial dysfunction through the antioxidant properties of dairy-based nutrients. Thus, although dairy cheese consumption may contribute to dietary sodium intake, these findings suggest that dairy cheese prevents sodium-induced microvascular dysfunction and that replacing non-dairy sources of sodium with dairy cheese in a routine diet may improve blood vessel function in middle-aged and older adults with salt-resistant blood pressure.

Future Directions

1) In the study that comprises Chapter 4 examining the acute effects of cheese intake on microvascular function, we investigated potential anti-inflammatory effects of dairy-based cheese on sodium-induced endothelial dysfunction in addition to the reported antioxidant effects. We specifically investigated the role of iNOS and arginase in the protective role of dairy-based cheese on sodium-induced microvascular dysfunction and found that the improvement in NO bioavailability from sodium ingestion in dairy cheese compared to
sodium ingestion from non-dairy sources was not mediated by arginase- or iNOS-mediated mechanisms. We did not directly examine the role of these inflammatory mediators with short-term consumption of cheese. While changes in these inflammatory mediators were not detected with acute cheese consumption, reductions in inflammation may play a role in the chronic vasoprotective role of dairy.

2) The series of studies in this dissertation did not investigate dietary sodium-mediated vascular responses in salt-sensitive individuals but the effect of dairy-based nutrients in mitigating sodium-induced vascular dysfunction may be different between individuals with salt-sensitive and salt-resistant blood pressure. There is currently a large body of literature that indicates that salt-sensitivity is associated with increased risk of hypertension and cardiovascular mortality\textsuperscript{160}. Given the beneficial effects of dairy on vascular health, both dependent and independent of reductions in blood pressure, the potential improvements in cardiovascular risk with dairy consumption may be even greater in salt-sensitive individuals and warrants further investigation.

3) Another important question regarding dairy and sodium consumption is whether the beneficial effects of consuming sodium in the form of cheese is due to the “packaging” of sodium in cheese or if the same beneficial effects on sodium-induced vascular dysfunction would be observed if dairy products were consumed separately from sodium (e.g., incorporating milk in a diet that is high in non-dairy sodium). Additional controlled feeding studies are needed to further investigate the effects of incorporating low-sodium dairy products in an otherwise high-sodium diet.

4) This dissertation is focused on peripheral vascular alterations with dairy and sodium ingestion. However, there is strong evidence that sodium consumption impairs sympathetic
nervous system function and may therefore may alter neural control of the vasculature\(^{293-296}\). In fact, high dietary sodium exaggerates sympathetic responsiveness and blood pressure variability in normotensive, salt-resistant rats\(^ {293} \). Whether dairy mitigates sodium-induced autonomic dysfunction is unknown and may be an important component of the cardioprotective properties of dairy.

5) The micro- and macronutrient composition differ between and within types of dairy products. In addition to mitigating sodium-induced vascular impairments, dairy may also protect against detrimental effects of other dietary nutrients. The fat content, in particular, varies widely among dairy foods. Low-fat dairy is associated with a lower CVD risk among normotensive individuals\(^ {76} \) and is inversely associated with a lower risk of hypertension in a dose-dependent manner\(^ {90, 297} \). However, inconsistent findings have been documented regarding the effects of high-fat dairy on vascular health with conflicting reports of an association between both increased cardiovascular risk\(^ {88, 298} \) and reduced cardiovascular mortality\(^ {299} \) with high-fat dairy consumption. Cheese is a dairy product that is high in saturated fat. While the impact of saturated fat on cardiovascular health is inconclusive, saturated fat may have different effects on CVD risk depending on the food source. In fact, a reduced CVD risk is associated with dairy saturated fat consumption, driven largely by cheese consumption, whereas an increased CVD risk is associated with saturated fat consumption from non-dairy sources such as meat\(^ {300} \). These findings suggest that dairy-based micronutrients and proteins may also protect against unfavorable effects of saturated fat; however, these potential benefits of dairy on saturated fat-mediated alterations in vascular health have not been extensively explored.
BIBLIOGRAPHY


2. Agriculture USDA HaHSaUSDo. 2015-2020 dietary guidelines for americans. December 2015


35. IJzerman RG, de Jongh RT, Beijk MAM, van Weissenbruch MM, Delemarre-van de Waal HA, Serne EH, Stehouwer CDA. Individuals at increased coronary heart disease risk are characterized by an impaired microvascular function in skin. *Eur J Clin Invest*. 2003;33:536-542


41. Kraemer-Aguiar LG, Laflor CM, Bouskela E. Skin microcirculatory dysfunction is already present in normoglycemic subjects with metabolic syndrome. *Metabolism.* 2008;57:1740-1746


57. Sprung VS, Cuthbertson DJ, Pugh CJA, Daousi C, Atkinson G, Aziz NF, Kemp GJ, Green DJ, Cable NT, Jones H. Nitric oxide-mediated cutaneous microvascular function is impaired in polycystic ovary syndrome but can be improved by exercise training. *J Physiol-London*. 2013;591:1475-1487


81. Pal S, Ellis V. The chronic effects of whey proteins on blood pressure, vascular function, and inflammatory markers in overweight individuals. *Obesity*. 2010;18:1354-1359


89. Sontia B, Touyz RM. Role of magnesium in hypertension. *Archives of biochemistry and biophysics*. 2007;458:33-39


157. Bray GA, Vollmer WM, Sacks FM, Obarzanek E, Svetkey LP, Appel LI, Grp DCR. A further subgroup analysis of the effects of the dash diet and three dietary sodium levels on blood pressure: Results of the dash-sodium trial. Am J Cardiol. 2004;94:222-227


103


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


109. Mullally MM, Meisel H, FitzGerald RJ. Identification of a novel angiotensin-I-
converting enzyme inhibitory peptide corresponding to a tryptic fragment of

110. Rival SG, Boeri CG, Wichers HJ. Caseins and casein hydrolysates. 2.
Antioxidative properties and relevance to lipoxygenase inhibition. *Journal of
agricultural and food chemistry*. 2001;49:295-302

111. Hernandez-Ledesma B, Davalos A, Bartolome B, Amigo L. Preparation of
antioxidant enzymatic hydrolysates from alpha-lactalbumin and beta-
lactoglobulin. Identification of active peptides by hplc-ms/ms. *Journal of
agricultural and food chemistry*. 2005;53:588-593

112. Suetsuna K, Ukeda H, Ochi H. Isolation and characterization of free radical
scavenging activities peptides derived from casein. *The Journal of nutritional
biochemistry*. 2000;11:128-131

113. Stancliffe RA, Thorpe T, Zemel MB. Dairy attenuates oxidative and
inflammatory stress in metabolic syndrome. *The American journal of clinical
nutrition*. 2011;94:422-430

114. Zemel MB, Sun X, Sobhani T, Wilson B. Effects of dairy compared with soy on
oxidative and inflammatory stress in overweight and obese subjects. *The
American journal of clinical nutrition*. 2010;91:16-22

blood pressure of potassium, calcium, and magnesium in women with low

116. Patki PS, Singh J, Gokhale SV, Bulakh PM, Shrotri DS, Patwardhan B. Efficacy
of potassium and magnesium in essential hypertension: A double-blind, placebo

117. Green DJ, Maiorana AJ, Siong JH, Burke V, Erickson M, Minson CT,
Bilsborough W, O'Driscoll G. Impaired skin blood flow response to
environmental heating in chronic heart failure. *European heart journal*.
2006;27:338-343

118. Holowatz LA, Kenney WL. Up-regulation of arginase activity contributes to
attenuated reflex cutaneous vasodilatation in hypertensive humans. *The Journal of
physiology*. 2007;581:863-872

119. Hodges GJ, Nawaz S, Tew GA. Evidence that reduced nitric oxide signal
contributes to cutaneous microvascular dysfunction in peripheral arterial disease.
*Clinical hemorheology and microcirculation*. 2015;59:83-95

120. Abularrage CJ, Sidawy AN, Aidinian G, Singh N, Weiswasser JM, Arora S.
Evaluation of the microcirculation in vascular disease. *Journal of vascular
surgery*. 2005;42:574-581

121. RG JJ, de Jongh RT, Beijk MA, van Weissenbruch MM, Delemarre-van de Waal
HA, Serne EH, Stehouwer CD. Individuals at increased coronary heart disease
risk are characterized by an impaired microvascular function in skin. *European
journal of clinical investigation*. 2003;33:536-542

122. Briasoulis A, Tousoulis D, Androulakis ES, Papageorgiou N, Latsios G,
Stefanadis C. Endothelial dysfunction and atherosclerosis: Focus on novel
therapeutic approaches. *Recent patents on cardiovascular drug discovery*.
2012;7:21-32


245. Fieger SM, Wong BJ. Adenosine receptor inhibition with theophylline attenuates the skin blood flow response to local heating in humans. *Experimental physiology.* 2010;95:946-954


294. Stocker SD, Madden CJ, Sved AF. Excess dietary salt intake alters the excitability of central sympathetic networks. Physiology & behavior. 2010;100:519-524


ENDOTHELIAL FUNCTION IS IMPAIRED IN THE CUTANEOUS MICROCIRCULATION OF ADULTS WITH PSORIASIS THROUGH REDUCTIONS IN NITRIC OXIDE-DEPENDENT VASODILATION

ABSTRACT

Psoriasis is an independent risk factor for cardiovascular disease; however, the underlying mechanisms are not fully understood. Deficits in conduit arterial function are evident in patients with psoriasis, but potential impairments in microcirculatory endothelial function remain unclear. We hypothesized that cutaneous microvascular dysfunction would be detectable in otherwise healthy individuals with psoriasis. Two intradermal microdialysis fibers were placed in (nonlesional) forearm skin of nine patients (3 men and 6 women, 39 ± 5 yr) with moderate (16 ± 2% of body surface area) plaque psoriasis and nine healthy (nonpsoriatic) control subjects (3 men and 6 women, 38 ± 5 yr) for local delivery of 1) lactated Ringer solution (control) and 2) 10 mM L-ascorbate (a nonspecific antioxidant). An index of skin blood flow was measured using laser-Doppler flowmetry during local heating (42°C). Nitric oxide (NO)-dependent vasodilation was directly quantified after perfusion of the nonspecific NO synthase inhibitor N\textsuperscript{G}-nitro-l-arginine methyl ester (15 mM). A third fiber was perfused with increasing concentrations (10\textsuperscript{−10} – 10\textsuperscript{−2} M) of norepinephrine to elicit adrenoreceptor-mediated cutaneous vasoconstriction. NO-dependent vasodilation was attenuated in patients with psoriasis (57 ± 5% and 39 ± 7% maximum cutaneous vascular conductance in control subjects and adults with psoriasis, respectively, \( P < 0.01 \)). L-Ascorbate did not improve NO-dependent vasodilation (\( P > 0.05 \)). There was no group difference in maximal vasoconstriction or microvascular sensitivity to norepinephrine (\( P > 0.05 \)). These data suggest that NO bioavailability is reduced in otherwise healthy individuals with psoriasis, which contributes to systemic microvascular dysfunction.
NEw & NOTEWORTHY In adults with psoriasis, reduced nitric oxide bioavailability mediates impaired endothelium-dependent vasodilation, independent of increases in oxidative stress. Furthermore, the degree of psoriatic symptomology is directly related to greater reductions in nitric oxide-dependent vasodilation.

INTRODUCTION
Psoriasis, a chronic inflammatory disease affecting 2–3% of the population, is an independent risk factor for cardiovascular disease (CVD) (6, 21, 45) and is associated with a 57% increase in cardiovascular mortality after adjustment for major conventional risk factors (45). Patients with psoriasis have an increased risk of hypertension and atherosclerosis compared with age- and sex-matched individuals without psoriasis (7). Although the mechanisms involved in the psoriasis-CVD comorbidity are not fully understood, epidemiological data provide strong support for inflammation as a common underlying mechanism (46).

The pathogenesis of psoriasis is mediated, at least in part, by activation of T helper type 1 and T helper type 17 immune responses (56), resulting in the overproduction of cytokines (e.g., TNF-α, IL-6, and IL-1β) and, ultimately, leading to a systemic proinflammatory milieu (43). One putative mechanism linking inflammation to endothelial dysfunction, the primary initiator of atherosclerosis (18, 39, 41, 71), is loss of the endothelium-derived vasodilator nitric oxide (NO). Importantly, inflammation-mediated reductions in NO bioavailability precede the manifestation of clinical CVD (71). Inflammation is also tightly linked to the generation of oxidative stress, and, in animal models, proinflammatory cytokines reduce vascular production of NO, in part through increases in reactive oxygen species (ROS) (38, 49). In adults with psoriasis, deficits in endothelial function assessed via arterial flow-mediated vasodilation (8, 17, 66) and
echocardiography-derived coronary flow reserve (9, 50) have been documented; however, the precise mechanisms mediating these vascular impairments are unclear.

Coupled with impairments in endothelium-dependent vasodilation, augmented vascular reactivity to vasoconstrictor stimuli is characteristic of microvascular dysfunction and contributes to increased cardiovascular risk (11, 78). Inflammatory cytokines activate several proconstrictor signaling pathways that lead to impaired vascular function (44, 73). Furthermore, excessive adrenoreceptor-mediated vasoconstrictor responsiveness contributes to vascular dysfunction in pathological conditions characterized by systemic inflammation (10). Increased arterial stiffness has been reported in patients with psoriasis (9, 22, 62, 76), and although these studies have provided indirect support for enhanced vasoconstrictor tone in psoriasis, no studies have functionally examined vascular reactivity to vasoconstricotor stimuli in the psoriatic population.

Therefore, the purpose of the present investigation was to mechanistically assess cutaneous microvascular function in nonlesional skin of adults with moderate plaque psoriasis. In psoriasis, skin-related symptoms are the outward manifestation of the systemic disease process (12, 61); therefore, examination of microvascular dysfunction in nonlesional skin of adults with psoriasis is clinically relevant. The cutaneous circulation is a representative vascular bed that allows for the targeted in vivo investigation of the specific signaling mechanisms mediating the regulation of vascular function in healthy adults as well as adults with preclinical CVD (3, 31, 32, 35, 40, 41, 55, 57). In addition, deficits in endothelial function in the skin parallel deficits in coronary (39) and renal (16) circulations, highlighting the clinical utility of examining the cutaneous microcirculation. We hypothesized that 1) NO-dependent vasodilation would be attenuated, 2) NO-mediated vasodilation would be improved by the acute scavenging of ROS with the potent nonspecific antioxidant ascorbate, and 3) adrenoreceptor-mediated vasoconstrictor reactivity would be augmented in nonlesional skin of patients with psoriasis compared with healthy adults.
MATERIALS AND METHODS

Subjects

All protocols were approved by the Institutional Review Board of The Pennsylvania State University and complied with the guidelines in the Declaration of Helsinki. All participants voluntarily provided written and verbal consent before the experiment. Nine adults with psoriasis and nine healthy age- and sex-matched men and women participated in the study. Subjects underwent a medical screening that included a 12-lead electrocardiogram, fasting blood chemistry, and physical examination by a board-certified dermatologist. All patients with psoriasis had ≥5% of body surface area (BSA) involvement but otherwise had normal cholesterol (LDL < 150 mg/dl), blood pressure (systolic blood pressure < 140 mmHg and diastolic blood pressure < 90 mmHg), and blood glucose (HbA1c < 6.0%). One subject had a history of psoriatic arthritis. Subjects were nonsmokers and were not taking systemic medications for psoriasis or any other prescription medications that may alter vascular function (e.g., statins, antidepressants, and antihypertensives). Patients with psoriasis were permitted to use topical steroids (except on the forearms), narrow-band ultraviolet light, or natural sunlight to alleviate symptoms. Subjects abstained from alcoholic and caffeinated beverages for 12 h and vigorous physical activity for 24 h before each experiment.

Experimental Protocols

Experiments were performed in a thermoneutral environment with subjects in a semisupine position. Sterile technique was used to place three intradermal microdialysis fibers (10 mm, 55-kDa membrane limit, CMA, Holliston, MA) in the ventral forearm skin ≥5 cm apart to prevent cross-reactivity between sites. Ice was applied to the forearm for 5 min to anesthetize the skin
before fiber placement. At each site, a 23-gauge needle was inserted into the dermis, with entry and exit points 3 cm apart. The fibers were threaded through the needles, which were subsequently removed, leaving the semipermeable portion of the fiber under the skin. Before data collection, two microdialysis sites were perfused with lactated Ringer solution and one microdialysis site was perfused with l-ascorbate (Sigma, St. Louis, MO) at a rate of 2 µl/min (Bee Hive controller and Baby Bee microinfusion pumps, Bioanalytical Systems) during the 60–90 min that is required to allow the skin to recover from trauma due to placement of the fibers. After the hyperemia period, red blood cell flux, an index of skin blood flow, was measured by laser-Doppler flowmetry probes placed in local heating units (SH02 temperature monitor, Moor Instruments) directly over the microdialysis membranes. Brachial artery blood pressure was recorded at 5-min intervals (Cardiocap, GE Healthcare) throughout the protocol. Pharmaceutical agents were dissolved in lactated Ringer solution and microfiltered (Acrodisc, Pall, Ann Arbor, MI) just before use. Optimal concentrations of the pharmaceutical agents were determined in previous pilot studies. Ascorbic acid (1 mg/ml, Sigma) was dissolved in solution with norepinephrine (NE; Sigma) to act as a preservative and extend the half-life of NE (26, 34, 65); this methodology is consistent with studies previously published by our laboratory (24–26, 42, 63, 64). In pilot studies for NE dose-response protocols (26), the cutaneous adrenergic response was not different between NE-perfused sites with and without ascorbic acid. Therefore, the addition of ascorbic acid to NE likely did not contribute to the responses observed in the present study. Solutions were covered with foil to prevent degradation due to light exposure.

**Protocol 1: endothelial NO synthase-dependent vasodilation**

Two microdialysis fibers were randomly selected for the quantification of NO-dependent vasodilation, one of which was treated with l-ascorbate before NO quantification (Fig. A-1A). Direct local heating of the skin, a physiological endothelial NO synthase (eNOS)-dependent dilator stimulus (14), is a standardized and reliable method to measure NO-dependent dilation and
has been used to examine microvascular function in a variety of pathologies (5, 19, 28, 32, 47, 57, 59). The skin blood flow response to local heating is characterized by an initial sensory nerve-mediated peak in skin blood flow followed by a sustained plateau that is ~70% reliant on NO-dependent mechanisms (14). Baseline measurements were collected for 20 min at a local skin temperature of 33°C. After a stable baseline period, the local skin temperature was increased to 42°C at a rate of 0.5°C every 5 s. Once a 10-min plateau was reached (40 min), 15 mM $N^G$-nitro-l-arginine methyl ester (l-NAME; Calbiochem, Billerica, MA), a nonspecific NOS inhibitor, perfused both sites at a rate of 4 µl/min. After 10 min of stable red blood cell flux measurements (45 min), maximal vasodilation was induced by perfusing the fibers with 28 mM sodium nitroprusside (SNP; USP, Rockville, MD) and increasing skin temperature to 43°C (20 min).

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**Figure A-1.** Protocol schematic of local heating to quantify endothelial nitric oxide synthase-dependent vasodilation (**A**) and the norepinephrine (NE) dose response to examine peripheral adrenergic vasoconstrictor responses (**B**). l-NAME, $N^G$-nitro-l-arginine methyl ester; SNP, sodium nitroprusside.
Protocol 2: peripheral adrenergic sensitivity

The third fiber was selected to deliver nine concentrations of NE for the characterization of cutaneous adrenergic responsiveness to exogenous NE (Fig. A-1B). Baseline measurements were recorded for 20 min with skin temperature clamped at 33°C. After a stable baseline period, the fiber was perfused with increasing concentrations ($10^{-10}$, $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$, $10^{-5}$, $10^{-4}$, $10^{-3}$, and $10^{-2}$ M) of NE for 5 min per dose.

Data Acquisition and Statistical Analysis

Data were collected with WinDaq (DATAQ Instruments) at a frequency of 40 Hz. Cutaneous vascular conductance (CVC) was calculated as red blood cell flux divided by mean arterial pressure. Local heating data were normalized to a percentage of maximum CVC ($CVC_{\text{max}}$). CVC data were averaged over a stable 10-min period at baseline, at the local heating plateau, at the l-NAME plateau, and at maximum vasodilation. NO-dependent vasodilation was calculated as the difference between CVC at the local heating plateau and CVC at the post-l-NAME plateau. Three-way repeated-measures ANOVA was performed to examine differences between groups and microdialysis sites during different phases of local heating (baseline, local heating plateau, and post-l-NAME plateau) (SAS v9.4). Tukey’s post hoc comparisons were performed when warranted. Linear regression analysis was used to assess the relation between NO-dependent vasodilation and percent lesional BSA in the psoriasis group (Prism v7.01, GraphPad, San Diego, CA). Given the additional joint symptomology in psoriatic arthritis, lesional BSA may not fully reflect disease severity in patients with psoriatic arthritis. Thus, the subject with a history of psoriatic arthritis was excluded from this analysis. In protocol 2, NE dose concentrations were logarithmically transformed, and CVC was expressed as a percentage of baseline (baseline CVC = 100%). Data were averaged during a stable baseline period and the last minute of each NE
dose. Pharmacological curve modeling was performed using a four-parameter nonlinear regression with variable slope (74) to detect differences in the sensitivity (logEC\textsubscript{50}) and maximal vasoconstrictor response to NE (Prism v7.01). Significance was accepted at $P < 0.05$. Unless otherwise indicated, all values are means ± SE.

RESULTS

Subject characteristics are shown in Table A-1. The psoriasis and control groups were age and sex matched and had similar blood biochemistry. Blood pressure and anthropometric characteristics were not different between groups.

Table A-1: Subject characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Psoriasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M,F)</td>
<td>(3,6)</td>
<td>(3,6)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>38 ± 5</td>
<td>39 ± 5</td>
</tr>
<tr>
<td>BMI (kg m\textsuperscript{-2})</td>
<td>22.8 ± 1.3</td>
<td>25.8 ± 2.5</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>109 ± 3</td>
<td>118 ± 4</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>70 ± 2</td>
<td>74 ± 3</td>
</tr>
<tr>
<td>HDL (mg dl\textsuperscript{-1})</td>
<td>69 ± 7</td>
<td>54 ± 5</td>
</tr>
<tr>
<td>LDL (mg dl\textsuperscript{-1})</td>
<td>107 ± 13</td>
<td>106 ± 13</td>
</tr>
<tr>
<td>Fasting Glucose (mg dl\textsuperscript{-1})</td>
<td>86 ± 3</td>
<td>87 ± 3</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.4 ± 0.1</td>
<td>5.3 ± 0.1</td>
</tr>
<tr>
<td>BSA (%)</td>
<td>0 ± 0</td>
<td>16 ± 2*</td>
</tr>
</tbody>
</table>

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated Hb. Data are presented as mean ± SEM. * $p<0.001$

Original records of skin blood flow during a local heating protocol in one patient with psoriasis and one control subject are shown in Figure A-2.
Figure A-2. Original recording of cutaneous vasodilator response [percent maximum cutaneous vascular conductance (CVC$_{\text{max}}$)] to local heat in one healthy adult [control (CONT)] and one adult with psoriasis (PSO). Arrows designate decreases in skin blood flow with nitric oxide synthase inhibition. l-NAME, $N_G$-nitro-l-arginine methyl ester.

Baseline percent CVC$_{\text{max}}$ was not different between groups (7 ± 1% and 10 ± 3% CVC$_{\text{max}}$ for control and psoriasis groups, respectively, $P = 0.69$). Total vasodilation in response to local heat (97 ± 1% and 86 ± 5% CVC$_{\text{max}}$ for control and psoriasis groups, respectively, $P = 0.09$) and the NO contribution to the vasodilatory response (57 ± 5% and 39 ± 7% CVC$_{\text{max}}$ for control and psoriasis groups, respectively, $P < 0.01$) were reduced in patients with psoriasis compared with healthy adults (Fig. A-3A). In patients with psoriasis, local antioxidant treatment with ascorbate did not improve the total vasodilatory response to local heat ($P = 0.65$) or the NO-dependent component of the response ($P = 0.51$; Fig. A-3B). Maximal endothelium-independent SNP-
induced vasodilation was not different between groups (1.81 ± 0.21 and 1.87 ± 0.20 flux/mmHg for control and psoriasis groups, respectively, $P > 0.05$).

Figure A-3. A: local heating plateau, post-$N^G$-nitro-l-arginine methyl ester (l-NAME) plateau, and nitric oxide (NO)-dependent vasodilation at the site treated with Ringer solution (Ringer’s) in adults with psoriasis (PSO; $n = 9$) and healthy adults [control (CONT); $n = 9$]. B: local heating plateau and NO-dependent vasodilation at the site treated with Ringer solution and the site treated with ascorbate in adults with psoriasis. $CVC_{\text{max}}$, maximum cutaneous vascular conductance. $*P < 0.05$ vs. CONT.
Percent lesional BSA was significantly correlated with NO-dependent vasodilation in patients with psoriasis ($R^2 = 0.54, P = 0.04$; Fig. A-4).

**Figure A-4.** Correlation between percent lesional body surface area (%BSA) and nitric oxide (NO)-dependent vasodilation in patients with psoriasis ($n = 8$). $\text{CVC}_{\text{max}}$, maximum cutaneous vascular conductance.

Adrenoreceptor-mediated vasoconstrictor reactivity, represented as a percentage of baseline, is shown in **Figure A-5.** Baseline CVC was not different between groups (0.21 ± 0.05 and 0.16 ± 0.04 flux/mmHg for control and psoriasis groups, respectively, $P = 0.47$). There was no difference between groups in cutaneous vascular adrenergic responsiveness, assessed as log$\text{EC}_{50}$ (%$\text{CVC}_{\text{base}}$: −6.30 ± 0.13 and −6.16 ± 0.16 log M for control and psoriasis groups, respectively, $P$
Furthermore, maximal NE-induced vasoconstriction was also not different between patients with psoriasis and healthy adults ($P = 0.42$; Fig. A-5).

![Figure A-5](image.png)

**Figure A-5.** Norepinephrine (NE) dose-response curves, expressed as a percentage of baseline, in adults with psoriasis (PSO; $n = 9$) and healthy adults [control (CONT); $n = 9$]. CVC$_{\text{max}}$, maximum cutaneous vascular conductance.

**DISCUSSION**

In the present study, NO-mediated endothelium-dependent vasodilation in response to local heating, a physiological eNOS-dependent dilator stimulus, was blunted in patients with psoriasis. Furthermore, the degree of psoriatic symptomology, estimated using the percent lesional BSA, was significantly related to reductions in NO-dependent vasodilation in the psoriasis group, suggesting that greater disease severity is associated with larger reductions in NO bioavailability. Contrary to our initial hypothesis, acute antioxidant treatment did not improve NO-dependent vasodilation in the psoriasis group. Finally, cutaneous adrenoreceptor-mediated vasoconstrictor
reactivity was not augmented in the psoriasis group. Collectively, these findings suggest that reductions in NO-dependent signaling mechanisms contribute to cutaneous microvascular endothelial dysfunction in psoriasis.

The impairments in endothelial function and reductions in NO bioavailability observed in the present study agree with findings in animal models used to investigate the vascular effects of inflammatory cytokines central to the pathology of psoriasis. Overexpression of IL-17 in mouse keratinocytes induces psoriasis-like skin inflammation, elevates serum cytokine concentrations, and reduces the vasodilatory response to acetylcholine due, at least in part, to a decrease in vascular NO bioavailability (38). Additionally, in vitro treatment of endothelial cells and systemic in vivo infusion of IL-17 in mice attenuate eNOS activity and NO-mediated vasodilation (49). In vitro and in vivo treatment with TNF-α similarly impair NOS expression and endothelium-dependent vasodilation (72, 75, 77). While these animal models provide mechanistic evidence for detrimental effects of psoriasis-associated inflammatory mediators on vascular function, they are limited in their ability to replicate the pathogenesis of psoriasis.

In humans, macrovascular endothelial function, assessed via flow-mediated dilation of the brachial artery, is impaired in psoriasis, indicative of reductions in NO bioavailability (8, 17, 66). The present data also demonstrate impaired endothelial function in the cutaneous microvasculature in patients with psoriasis. Importantly, SNP-induced maximal vasodilation, an endothelium-independent stimulus, was not different between groups, consistent with the majority of previous reports (17, 66), although this may not be a universal finding (8). Moreover, through direct quantification of NO-dependent vasodilation by pharmacological targeting of the NO signaling pathway in the vasculature, these data clearly demonstrate reduced peripheral NO bioavailability in patients with at least moderate psoriasis, providing a potential mechanistic link between psoriasis and CVD. Reduced NO bioavailability increases vasoconstrictor tone due to a loss of NO-mediated relaxation and enhanced release of vasoconstrictor stimuli (68, 69). In
addition to its role in regulating vascular tone, NO inhibits platelet activation and the production of cytokines, growth factors, and cell adhesion molecules that attract inflammatory molecules to the vessel wall (67, 70). As such, diminished NO bioavailability promotes platelet attachment and vascular inflammation, which lead to lipoprotein oxidation, formation of atherosclerotic plaque, and clinical expression of CVD. Psoriasis severity is significantly correlated with a greater incidence of cardiovascular events and subclinical atherosclerosis (20, 21). Importantly, our data demonstrate a significant relation between percent lesional BSA and direct functional NO bioavailability, providing a mechanistic link to support the association between psoriasis severity and CVD risk.

Additionally, our data extend previous findings by examining potential mechanisms contributing to the impaired NO-mediated vasodilation in psoriasis. In animal models, overproduction of cytokines (e.g., IL-17) increases ROS production and reduces vascular NO bioavailability (38, 49). Perhaps surprisingly, NO-dependent vasodilation was not improved after local antioxidant administration, suggesting that ascorbate-sensitive oxidants do not appear to contribute to endothelial dysfunction in adults with psoriasis. Rather, upregulation of other vascular targets downstream of inflammatory cytokines (e.g., inducible NOS, arginase, and RhoA) likely mediates endothelial dysfunction in psoriasis; future studies directly investigating the contribution of these alternative mechanisms in patients with psoriasis are necessary.

To assess the potential contribution of heightened vasoconstrictor reactivity to cutaneous microvascular dysfunction, we measured peripheral adrenergic sensitivity in response to exogenous treatment with the sympathetic neurotransmitter NE. Contrary to our hypothesis, there were no differences in either vascular adrenergic sensitivity or maximal NE-induced vasoconstriction between groups. Our findings suggest that increased responsiveness to sympathetic neurotransmitters likely does not contribute to vascular dysfunction in psoriasis. While no other studies have examined vascular adrenergic sensitivity in psoriasis, elevated
endogenous NE concentrations at rest (36) and in response to stress (15) may contribute to psoriasis-associated vessel dysfunction. Future studies are necessary to more precisely elucidate potential alterations in sympathetically mediated vascular responsiveness.

While our study intentionally excluded systemically treated patients with psoriasis, the degree to which vascular function remains impaired in medicated patients with psoriasis is unclear. Studies investigating the effects of systemic treatment with a TNFα inhibitor and/or IL-17 inhibitors on vascular function and cardiovascular outcomes are equivocal (2, 4, 13, 27, 51, 53). Future studies examining the potential effects of immunomodulatory systemic psoriasis therapies on vascular function are warranted.

Limitations

While we did not definitively show that ascorbate reached the targeted ROS, local delivery of ascorbate through a microdialysis fiber has been shown to mitigate endothelial dysfunction (23, 29, 30, 33, 58, 60). Additionally, we used a concentration of ascorbate significantly higher (18%) than the concentration used for intravenous ascorbate infusions (0.4–10%) that have demonstrated reductions in markers of oxidative stress (37, 48, 52). Thus, we expect that delivery of 10 mM ascorbate solution directly to the cutaneous microvasculature for 1 h before data collection would be sufficient to scavenge ROS. It is possible that acute scavenging of ROS with local delivery of ascorbic acid may not be sufficient to mitigate chronic effects of ROS on vascular function, although ascorbate has been shown to improve endothelial function acutely in other disease states characterized by chronic oxidative stress (29, 30, 33, 60). The lack of an acute effect of ascorbate on endothelial function in psoriasis likely reflects the multifactorial mechanisms underlying endothelial dysfunction in psoriasis. That is, in psoriasis, the effects of ROS-mediated mechanisms on vascular function may be relatively small, at least in this moderate form of the disease, and/or entirely separate from the effects of other inflammatory signaling pathways.
Psoriasis is associated with a number of comorbidities, including hypertension, diabetes, and metabolic syndrome (54). In the present study, we excluded subjects with identified cardiometabolic comorbidities. Patients had normal lipid profiles (LDL < 150 mg/dl), blood pressure (systolic blood pressure < 140 mmHg and diastolic blood pressure < 90 mmHg), and blood glucose (HbA1c < 6.0%) and did not have a history of tobacco use. We did not conduct neuropsychiatric interviews for screening of depression, a condition that is similarly associated with increased CVD risk (1). However, subjects completed an extensive medical history questionnaire to identify self-reported history of depression, reducing the likelihood that depression contributed to the vascular impairments.

**Conclusions**

In summary, reductions in NO bioavailability mediate impaired endothelium-dependent vasodilation, thus contributing to cutaneous microvascular dysfunction in adults with psoriasis. Furthermore, it appears that the outward manifestation of the severity of psoriasis (e.g., percent lesional BSA) is directly related to greater reductions in NO-mediated vasodilation. Finally, augmented cutaneous adrenoreceptor-mediated vasoconstrictor reactivity was not evident in adults with psoriasis, suggesting that this mechanism does not contribute to impaired vascular function in the cutaneous microcirculation. Given the complexity of the disease and the multiplicity of effects mediated by inflammatory cytokines that are central to the pathogenesis of psoriasis (e.g., TNF-α and IL-17) (49, 61), additional research is needed to determine the contribution of specific inflammatory mediators to the impairment in microvascular function in this clinical cohort.
Grants

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions


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References


### Appendix B

**NUTRIENT COMPOSITION OF CONTROLLED FEEDING DIETARY INTERVENTIONS**

<table>
<thead>
<tr>
<th>Diet Intervention</th>
<th>Energy (kcal)</th>
<th>Total Fat (g)</th>
<th>Total Carbohydrate (g)</th>
<th>Total Protein (g)</th>
<th>Animal Protein (g)</th>
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<th>Total Sugars (g)</th>
<th>Omega-3 Fatty Acids (g)</th>
<th>Manganese (mg)</th>
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<th>Omega-3 Fatty Acids (g)</th>
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Title of Project: Milk and Cheese Consumption and Human Microvascular Function

Principal Investigator: Lacy Alexander, Ph.D.
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University Park, PA 16802
Phone: 814-867-1781

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Phone: 814-863-8556

Susan Slimak, RN
Phone: 814-863-8556

Jane Pierzga, M.S., Research Assistant
Phone: 814-865-1236

This is to certify that I, __________________________ have been given the following information with respect to my participation as a volunteer in a program of investigation.

1. Purpose of the study: High blood pressure (hypertension, HT) can lead to disease in the body’s blood vessels (cardiovascular disease or CVD). CVD is the leading cause of death in developed countries. High blood pressure and CVD cause harmful changes to the blood vessels in the kidney, heart, and other organs. These changes also occur in the blood vessels in human skin.

People can make changes to their diet as one way to help lower blood pressure without using drugs. Increased dairy intake improves the results of tests that measure the health of blood vessels. Having dairy in the diet for a long time reduces the rise in blood pressure often seen in people as they age.
People who had mild HT saw a modest drop in blood pressure when they increased their dairy intake. No one knows how dairy improves the function of blood vessels and lowers blood pressure in humans. The researchers plan to explore the effects of fluid milk and cheese intake on the function of blood vessels. Salt in the diet can cause blood pressure to rise. Cheese contains salt. The researchers will explore how that salt affects the way that cheese intake improves the health of blood vessels. The researchers recruit people who have normal to mild high blood pressure for this study.

It is much easier to see and study the effects of high blood pressure in the blood vessels of the skin. The researchers use “microdialysis” (MD) in this study. This technique involves placing very thin plastic tubing between the layers of the skin. The largest part of the tubing is about 6x the diameter of a human hair. They pump fluid like that found in the body’s tissues through the tubing. The tubing acts like very small blood vessels in the skin. Like those vessels, it allows some substances to pass between the fluid in the tubing and the fluid in the skin. During the experiment, they will add substances to the fluid in the tubing. The substances can only reach a 2.5 cm² (0.4 inch²), nickel-sized area of skin at each tube. Some of these substances are like natural chemicals found in the body. Some of these substances block the actions of natural chemicals found in the body.

The substances used for these experiments are:

1. L-NAME (N\textsuperscript{G}-nitro-L-arginine methyl ester) – blocks a substance made by your body that helps to make blood vessels get bigger
2. Vitamin C—blocks substances made by your body that make your blood vessels get smaller
3. BEC ((s)-2-boronoethyl)-L-cysteine-HCl – blocks a substance made by your body that can prevent blood vessels from getting bigger
4. 1400W - blocks a substance made by your body that can prevent blood vessels from getting bigger
5. SNP (sodium nitroprusside) causes your blood vessels to get as large as they can.
6. Lactated Ringers – a fluid like that which bathes the tissues in your body

This research study has two parts: (1) Fluid Milk and (2) Cheese. You can be in one or both parts. The Milk-part has 4 experiments. The Cheese-part has 6 experiments. There is at least 1-week between experiments. Each experiment includes blood draws and the MD technique.

2. **Procedures:** Please read the descriptions of the days. Then write your initials by the days.

You could be asked to repeat a trial, procedure, or test. This could include blood draws with your OK. This could happen for many reasons such as equipment failure, power outage, inconclusive test results, etc. You do not have to repeat a trial, procedure, and/or test if you do not wish to do so.
**initial Blood Pressure Readings**

If the researchers think that you have high blood pressure, they make a series of blood pressure readings. They make these readings during 2 visits to the Noll Lab including your screening visit. These readings occur within a 2-week period. This makes sure that they put you in the correct group. Also, you wear a monitor to record your blood pressure for 24 hours. The monitor has a cuff that goes around your arm. A control unit hangs on a strap around your waist or on your shoulder.

**initial Screening Day:** Drink only water and do not eat for 12 hours before your visit to the Noll Lab. The research and/or Clinical Research Center staff performs the screening. During the screening, the staff measures blood pressure, heart rate, weight, height, and waist circumference. They also take a medical history and 12-lead resting ECG. The nurse draws about 30 ml (2 Tbsp) of blood from a vein in your arm. They send the blood to labs that test it for blood cells, fats in the blood, and blood chemistry. They test for other proteins in which they are interested. If you take thyroid hormone, they may draw an extra 3.5 ml (0.2 Tbsp). They do not perform genetic tests on the blood. They do not test the blood for the presence of disease (e.g. HIV). Women who have not gone through menopause have a urine pregnancy test.

**initial Visit 3 Experiment:**

**Preparation for Experiment:** You eat a low-dairy diet (≤ 1 serving daily) on the 2 days before the experiment.

The researchers conduct all of the experiments in the same way. The pre-treatments are the only difference.

**initial Milk Pre-Treatments**

(random order).

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<th>No.</th>
<th>Description</th>
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<tr>
<td>1</td>
<td>2 servings of lowfat milk (1%)*</td>
</tr>
<tr>
<td>2</td>
<td>2 servings of isocaloric rice milk</td>
</tr>
<tr>
<td>3</td>
<td>4 servings of lowfat milk (1%)**</td>
</tr>
<tr>
<td>4</td>
<td>4 servings of isocaloric rice milk</td>
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</tbody>
</table>

*1/2 L or 2 cups (8 oz each)  **1 L or 4 cups

**initial Cheese Pre-Treatments**

(random order).
When you arrive at the lab, the researchers measure your heart rate and blood pressure. Women who have not gone through menopause have a urine pregnancy test if they have not had one within 2 weeks.

The nurse inserts a tube in your arm and draws a baseline blood sample (15 ml, 1Tbsp). The tube stays in your arm until the experiment nears an end.

**Microdialysis (MD):** The researchers place a tight band around your upper arm so they can easily see your veins. For each MD site, the researchers make pairs of pen-marks on your forearm. The marks are 2.5 cm (1 inch) apart and away from veins. Then the researchers remove the tight band. The MD tubing enters and exits your skin at the marks. The researchers clean your arm with an orange fluid (povidone iodine) and alcohol. They place an ice bag on your arm for 5 minutes to numb your skin. Then the researchers insert a thin needle into your skin at each entry mark. The needle’s tip travels between the layers of skin for 2.5 cm (1 inch). The needle leaves your skin at the matching exit mark. The researchers thread the MD tubing through the needle. Next, they withdraw the needle leaving the tubing in your skin. Any redness of your skin subsides in about 60 – 120 minutes. The researchers insert MD tubing at 4 sites on your forearm.

You drink or eat one of the **Pre-treatments (see above).** The nurse draws a blood sample (15 ml, 1Tbsp) every 30 minutes after the pre-treatment until the experiment nears an end.

The researcher places a local heater and laser probe over each MD site. The weak laser measures skin blood flow. They tape 3 ECG wires to your chest to measure heart rate. They place a cuff on your upper arm for taking blood pressure readings. The researchers record heart rate and skin blood flow throughout the experiment. They record skin temperature at the same time. They measure blood pressure every 5-7 minutes.

During a 20-minute baseline, Lactated Ringer’s flows through the MD tubing. Then the researchers add treatments to 3 of the 4 MD sites:

1. Lactated Ringer’s (only)
2. Lactated Ringer’s + Vitamin C
3. Lactated Ringer’s + 1400W
4. Lactated Ringer’s + BEC

After 20 minutes, the researchers increase the temperature at the MD sites to 42°C (104°F). When
the skin blood flow becomes stable (about 40 minutes), they add LNAME to the tubing at each MD site. When the skin blood flow becomes stable again (about 40 minutes), they stop the treatments. They add only SNP to the Lactated Ringer’s and increase the local heating to 43°C (108°F) at all sites. This makes your blood vessels as big as possible. The nurse stops drawing blood samples for this experiment when the SNP starts. After 30 minutes, the researchers remove the local heaters and the MD probes. They apply a sterile dressing over the sites. They measure blood pressure and heart rate.

You consume your normal amount of dairy (≤ 2 servings daily) until 2 days before the next experiment.

3. Discomforts and risks:

**Microdialysis (MD):** The risks are less than that for a blood draw because MD uses only a small, local area of skin. In contrast, a blood draw involves not only skin, but also large blood vessels and blood. MD is likely to cause some pain and bruising like that of a blood draw. However, the researchers use ice to numb the arm before they insert the tubing. Also, the small needle helps to reduce pain. Most people do not feel pain after the tubing is in place. You may feel a little pain when they remove the tubing at the end of the experiment. If you are nervous about needles, blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, or may faint. Sometimes the tubing can break during removal from the skin. Then the researchers remove the tubing by pulling on the other end of it. This produces no added risk for you. The tubing could break so that a small piece is left under the skin. This has not occurred in any of the studies in this lab. If this happened, they would treat any tubing remaining in the skin like a splinter. In this case, they would cut the thin layer of skin over the tubing to remove the tubing. Mild pressure with sterile gauze stops any slight bleeding that may occur. Aseptic technique and sterile supplies like those used in hospitals keep the risk of infection minimal. Infection has not occurred with MD in this lab or others that the researchers know of. They apply a sterile bandage after the experiment. They tell you how to take care of the site.

**Fluid flowing through the tubing:** The substances flowing through the tubing only go to a 2.5 cm² (0.4 inch²) area of skin at each tubing site. The amount that enters the skin is very small. However, there is a chance of a bad reaction to the substances. This reaction could produce redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, blood pressure change and/or fainting. If a bad reaction should occur, medical help is summoned right away.

**Lactated Ringer’s Solution:** This fluid is similar to the natural fluids in the skin. This fluid contains salt, potassium, lactate, and chloride. The acid content is like that of the body’s fluids. A bad reaction to this fluid is highly unlikely.

**L-NAME, Vitamin C, BEC, 1400W, and SNP:** Only minute amounts of these substances enter the nickel-sized area of skin around the MD tubing. These and other researchers have used the substances in humans before. There have been no reports of bad reactions.
**Blood Draw:** Blood draws often cause mild pain, bruising, swelling, or bleeding. There is also a slight chance of infection or a small clot. If you are nervous about needles, blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, or may faint. Using the same techniques used in hospitals keeps the chance of infection minimal. Do not exercise hard for 24 hours before a blood draw.

**Special note about blood draws for the experiments:** A trained nurse inserts a thin tube into a vein in your arm or hand through which she can draw blood. To do this, the nurse inserts a needle with the tube around it into your vein. Then the nurse removes the needle. The tube stays in the vein during the whole experiment. The tube allows the nurse to take more than one blood sample without sticking you with a needle each time. If the first attempt to insert the tube does not work, the nurse may need to try again. She will try again only if you allow. The nurse uses sterile saline to flush the blood out of the tube between blood draws. Sometimes the tube can stop letting the nurse draw blood through it. If this occurs, the nurse removes the tube. You may stop the experiment if you wish or you may proceed. If you allow, the nurse may insert a new tube in your vein. Or you may have the nurse stick you with a needle for each of the rest of the blood draws. The tube does not stay in your vein longer than 4 hours. At the end of the experiment, the nurse removes the tube from your vein. The maximum, total volume of blood drawn over the 10 experiments is 1.2 L. This is just a little over 2.5 pints. You will take at least 11-12 weeks to complete the 10 experiments. A typical Red Cross blood donation is 500 ml (1 pint) drawn in less than 15 minutes.

**ECG:** This machine measures the electrical activity of the heart. The researchers tape 3-12 wires from the machine to spots on your body. There have been no adverse effects. The tape may irritate the skin.

**Blood Pressure (manual, CardioCap):** The researchers measure blood pressure with the method used in a doctor’s office and they can use a machine. A cuff inflates on the upper arm. As the cuff slowly deflates, the researchers listen with a stethoscope at the bend in the elbow or the machine takes a reading. During the short time the researchers inflate the cuff, your arm may feel numb or tingly. The cuff could cause mild bruising.

**Medical Screening:** You may feel shy about giving health information. The staff collects the information in a private and professional manner. You may feel shy about being measured. You may request someone of the same sex to conduct the screening. The researchers will make their best effort to provide one.

**Phone screening / Food Frequency Questionnaire:** Only the researcher uses these forms. The forms help them to decide if you are a good candidate for the study. You may feel shy about answering questions. They ask the questions in a private and professional manner. The completed forms are kept confidential and secure.
**Laser Doppler Flowmetry:** Weak lasers can hurt your eye if you stared into the light for a long time. The researchers do not turn on the laser until the probes are taped to a surface. The tape may irritate the skin.

**Local Heating:** The researchers measure the temperature of the skin under the holders. The skin feels very warm but does not hurt. The heating makes the skin of the arm under the holders red like when you take a hot bath. The redness will not last more than several hours. Some people may be more sensitive to the heating than others. If your arm feels too hot, you tell the researchers, and they reduce or stop the heating.

**Povidine Iodine:** Hospitals and researchers use this orange-colored fluid to clean the skin. You could have a bad reaction to povidone iodine if you are allergic to iodine. You inform the researchers if you have this allergy so that they use only alcohol instead. A bad reaction could cause redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or blood pressure change and/or fainting.

**Latex:** Some gloves and medical materials are made of latex rubber. Inform the researchers if you are allergic to latex and decline to participate in the study.

**4. a. Benefits to me:** You will receive a medical screening that could inform you about your health. You will learn your blood pressure and blood cholesterol levels. This is important knowledge. High blood pressure and blood cholesterol contribute to many serious health problems. If you have high blood pressure or blood cholesterol, we will advise you to work with a health care provider to keep your blood pressure controlled.

**b. Potential benefits to society:** One in three adults under the age of 65 and one out of two adults over the age of 60 has high blood pressure. 40% of all deaths in the United States are due to CVD. This health care burden is greater than $350 billion/year and growing. As the population ages, these problems will increase. This project will add to the knowledge of how high blood pressure can progress to CVD. This knowledge could lead to methods for early intervention. Also, the study will explore dairy as a candidate for early intervention through a change in diet. This could reduce the need for drug treatment and its attending side effects and cost. The projects provide valuable experience, education and partial fulfillment of degree-work for graduate and undergraduate students of The Pennsylvania State University.

**5. Time duration of the procedures and study:** The circled statements apply to you. Please read the circled statements. Then write your initials by the circled statements.

_____ initial Blood Pressure visit: about ½ hour
6. **Statement of confidentiality:** Volunteers are coded by an identification number for statistical analyses. All records are kept in a secure location. All records associated with your participation in the study will be subject to the usual confidentiality standards applicable to medical records (e.g., such as records maintained by physicians, hospitals, etc.), and in the event of any publication resulting from the research no personally identifiable information is disclosed. The Office of Human Research Protections in the U.S. Department of Health and Human Services, the U.S. Food and Drug Administration (FDA), The Penn State University Office for Research Protections (ORP) and The Penn State University Institutional Review Board may review records related to this project. Your confidentiality is kept to the degree permitted by the technology used. No guarantees can be made regarding the interception of data sent via the Internet by any third parties.

7. **Right to ask questions:** Please contact Anna Stanhewicz (W: 814-863-8556, M: 845-551-3869), Susan Slimak (W: 814-863-8556, H: 814-237-4618), or Jane Pierzga (W: 814-865-1236, H: 814-692-4720) with questions, complaints or concerns about this research. You can also call these numbers if you feel this study has harmed you. If there are findings during the research that could relate to you wanting to help with the study, you will be told of the findings. If you have any questions, concerns, or problems about your rights as a research participant or would like to offer input, please contact Penn State University’s Office for Research Protections (ORP) at (814) 865-1775. The ORP cannot answer questions about research procedures. All questions about research procedures can only be answered by the research team.

8. **Compensation:**

Each Experiment: $15.00 for completing the experiment.

$60.00 for MD tubing ($15.00 for each MD tube inserted x 4 MD sites)

$75.00 total for each experiment

Total for all 10 experiments: $750.00

You receive a bonus if you complete all 10 experiments: $100
Total (maximum): $850.00

In addition, you may choose one of the following: lab T-shirt, bag, or other item we may offer.

For each trial, you are paid an amount of money equal to the part of the trial that you complete. For instance, if you complete only half of an experiment you will be paid for each probe that was inserted plus $7.50 for that trial. This is because $7.50 is one-half of $15.00. You may be asked to repeat a trial. If you agree to repeat a trial, you will be paid for the repeated trial as stated above.

Total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS. This may require you to claim the compensation that you receive for participation in this study as taxable income.

9. Injury Clause: In the unlikely event you become injured as a result of your participation in this study, medical care is available. Please call Jessica Kutz (W: 814-865-2432, M: 570-490-1426), Susan Slimak (W: 814-863-8556, H: 814-237-4618), and Jane Pierzga (W: 814-865-1236, H: 814-692-4720). It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

10. Voluntary participation: Your being in this study is voluntary. You may withdraw from this study at any time by telling the researcher. If you decide to withdraw, you will not have a penalty or loss of benefits you would receive otherwise. You may decline to answer certain questions. You may decide not to comply with certain procedures. However, your being in the study may be contingent upon answering these questions or complying with the procedures. The researcher may end your role in the study without your consent if the researcher deems that your health or behavior adversely affects the study or increases risks to you beyond those approved by the Institutional Review Board and agreed upon by you in this document. You have been given an opportunity to ask any questions you may have, and all such questions or inquiries have been answered to your satisfaction.

11. In the event that abnormal test results are obtained, you will be apprised of the results immediately and advised to contact a health care provider for follow-up.

This is to certify that I am 18 years of age or older and I consent to and give permission for
my participation as a volunteer in this program of investigation. I understand that I will receive a signed copy of this consent form. I have read this form, and understand the content of this consent form.

I have been given an opportunity to ask any questions I may have, and all such questions or inquiries have been answered to my satisfaction.

________________________________________________________________________
Volunteer Date

I, the undersigned, have defined and explained the studies involved to the above volunteer.

________________________________________________________________________
Investigator Date
CONSENT FOR RESEARCH
The Pennsylvania State University

Title of Project: Cheese Consumption and Human Microvascular Function

Principal Investigator: Lacy M. Alexander, Ph.D.
Address: 113 Noll Laboratory
Telephone Number: 814-867-1781

Subject’s Printed Name: _____________________________

We are asking you to be in a research study. This form gives you information about the research.

Whether or not you take part is up to you. You can choose not to take part. You can agree to take part and later change your mind. Your decision will not be held against you.

Please ask questions about anything that is unclear to you and take your time to make your choice.

1. Why is this research study being done? High blood pressure (hypertension, HT) can lead to disease in the body’s blood vessels (cardiovascular disease or CVD). CVD is the leading cause of death in developed countries. High blood pressure and CVD cause harmful changes to the blood vessels in the kidney, heart, and other organs. These changes also occur in the blood vessels in human skin.

Diet changes can help to lower blood pressure without using drugs. Increased dairy intake improves the results of tests that measure the health of blood vessels. People who had mild HT saw a modest drop in blood pressure when they increased their dairy intake. No one knows how dairy improves the function of blood vessels and lowers blood pressure in humans. We plan to explore the effects of cheese intake on the function of blood vessels. Salt in the diet can cause blood pressure to rise. Cheese contains salt. We explore how cheese intake improves the health of blood vessels despite the salt present in the cheese.

It is much easier to see and study the effects of high blood pressure in the blood vessels of the skin. We use “microdialysis” (MD) in this study. With MD, we perfuse some research drugs into nickel-sized areas of skin on your arm. The drugs remain in the small areas and do not go into the rest of your body. The research drugs are not approved by the FDA to treat disease. However, the FDA has approved our using the drugs in this study. We and others have used these drugs in people in research studies for many years without problem.
We recruit people who have normal to mild high blood pressure for this study. We are asking you to be in this research because you fit our criteria for being a subject.

2. What will happen in this research study?

You participate on the circled days or procedures. Please read the descriptions of the circled days. Then write your initials by the circled days or procedures.

We may ask you to repeat a trial, procedure, or test. This could happen for many reasons such as equipment failure, power outage, inconclusive test results, etc. You do not have to repeat a trial, procedure, and/or test if you do not wish to do so.

Note: This study involves the use of drugs that are not approved by the FDA to treat disease. All of the drugs have been used in humans by us or others. The FDA approved the use of the drugs for this study. We dilute the drugs in Lactated Ringer’s, a type of saline fluid like that found throughout your body. The drugs are:

- Acetylcholine (ACh) – like a substance made by your body; causes blood vessels to dilate
- Apocynin - antioxidant
- Ascorbate (Vitamin C) - found in many foods such as citrus fruit; antioxidant
- L-NAME – blocks the production of nitric oxide
- Sodium nitroprusside (SNP) – supplies nitric oxide; causes blood vessels to dilate
- Tempol – antioxidant

_______ initial A. Screening Visit

1. You drink only water and do not eat for 12 hours before the screening.
2. The research nurse and/or Clinical Research Center (CRC) staff perform the screening. The staff measures your height and weight, blood pressure (BP), and heart rate (HR). They measure waist circumference. The staff reviews your medical history. Women of childbearing age have a urine pregnancy test.
3. The staff draws 30 ml (2 Tbsp) of blood from a vein in your arm. We send some of the blood to a lab to see if the proteins, blood cells, electrolytes, etc. are within normal levels. We may test the blood for other substances of interest. The researchers do not perform genetic analyses on the blood nor look for presence of disease (e.g. HIV).
4. If you take a thyroid drug, please tell the nurse your thyroid stimulating hormone (TSH) level. If you do not know your TSH level and/or you have not had it measured within 6 months, the nurse draws a blood sample (3.5 ml; 0.2 Tbsp) to measure TSH.

5. You wear a monitor that measures blood pressure for 24 hours. The monitor has a cuff that goes around your arm. A control unit hangs on a strap around your waist, shoulder, or upper arm.

You meet with our registered dietitian. This meeting helps us to plan the food you eat during the controlled feeding-part of this study. The meeting includes recalling your physical activity on the 7 days before the meeting. Also, you discuss food preferences and issues. (The meeting can occur on a separate day.)

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**initial B. Baseline Experiments** (Separate days, the order of the Local Heating and ACh Dose Response MD experiments is random)

1. You complete a 3-day food record.
2. **Preparation for all experiments**
   a. We give you printed and verbal instructions listing what you need to do before you arrive at the lab. Please follow the instructions with care and arrive prepared. If you have questions, please contact us right away.
   b. Do not consume alcohol, niacin supplements, or fish oils for 48 hours before the experiment.
   c. Do not consume caffeine (ex. coffee, tea, Coca Cola, chocolate) for 12 hours before the experiment.
   d. Do not eat anything for 8 hours before the baseline experiments.
   e. Drink only water for 8 hours before the baseline experiments.
   f. **On the day of the experiment**
      i. Do not eat anything the morning of experiment.
      ii. It is important for you to be well hydrated for the experiment so you should drink at least 1 glass (8 ounces) of water before you arrive. (Drink only water the morning of experiment.)
      iii. Refrain from hard exercise, physical labor, and any other task in which you to exert yourself more than a leisurely walk.
      iv. We measure your blood pressure, heart rate, and oral temperature.
      v. Women of childbearing age have a urine pregnancy test if they have not had a test within 2 weeks of the experiment.
      vi. We insert the MD probes: You wash the skin on your forearm. We place a tight band around your arm so we can easily see your veins. For each MD site, we make pairs of pen-marks 2.5 cm (1 inch) apart and away from veins. The MD tubing enters and exits your skin at the marks. We remove the tight band. We clean your arm with an orange-colored fluid and alcohol. We place an ice bag on your arm for 5 minutes to numb your skin. Then we insert a thin needle into your skin at each entry mark. The needle’s tip travels between the layers of skin for 2.5 cm (1 inch). It leaves your skin at the matching exit mark. We thread the MD tubing through the needle. We withdraw the needle leaving the tubing in your skin. Any redness of your skin subsides in about 60 – 120 minutes.
vii. We tape a thin probe and its holder over each site where there is MD tubing in your skin. The thin probe measures skin blood flow (SkBF) with a weak laser light. We control the temperature of the holders. The holders start at 34°C (93°F). During the experiment, we measure blood pressure and heart rate.

3. **Baseline Experiments – “Day 1”**
   a. **FMD:** FMD measures the health of blood vessels.
      i. We place a blood pressure cuff around your forearm.
      ii. We place gel on your upper arm just above the elbow.
      iii. We place a Doppler ultrasound probe on the gel. The ultrasound makes sound waves to measure the size of blood vessels and the speed of the blood.
      iv. We make a “resting” measurement before we inflate the cuff.
      v. The cuff inflates for 5 minutes to stop blood flow to and from the forearm.
      vi. We deflate the cuff and perform a second reading for 3 minutes.

   b. **Sublingual nitroglycerin:** This test also measures the health of blood vessels. Nitroglycerin causes blood vessels to dilate.
      i. The nurse is present throughout the procedure.
      ii. You lie on a bed or recliner.
      iii. We apply a blood pressure cuff on your upper arm.
      iv. As with FMD, we use an ultrasound probe during the test. We place the probe on an artery near your elbow.
      v. A nurse places a 0.4 mg nitroglycerin tablet under your tongue. Then you close your mouth right away. The tablet dissolves in 15-90 seconds. Do not swallow until the tablet dissolves. The effect lasts for 5-10 minutes.
      vi. You lie still for 20 minutes after you received the nitroglycerin. You remain in the lab at least 20 minutes after you receive the nitroglycerin.
      vii. You stay in the lab for up to 60 minutes after you received the nitroglycerin if you have a bad or very strong reaction (e.g. drop in blood pressure that lasts longer than usual). We monitor you during this time.

   c. **MD Experiment - Local Heating (or ACh Dose Response)**
      i. This experiment has 5 MD sites.
      ii. Fluid starts flowing through the tubing in your arm while we wait for the redness to end.
      iii. We collect baseline data for 20 minutes.
      iv. We add the drugs to plain fluid at the MD sites.
         - Probe 1. Lactated Ringer’s only (control)
         - Probe 2. Lactated Ringer’s + Ascorbate
         - Probe 3. Lactated Ringer’s + Tempol
         - Probe 4. Lactated Ringer’s + Apocyanin
      v. We collect second baseline data for 20 minutes.
      vi. Then, we slowly increase the temperature at the MD sites to 42°C (107.6°F).
      vii. When the skin blood flow becomes stable (about 40 minutes), we add LNAME to the tubing at all MD sites.
      viii. When the skin blood flow becomes stable again (about 40 minutes), we stop the drugs.
ix. We keep skin’s temperature at 42°C (107.6°F) at all MD sites. At the same time, only plain fluid + SNP flows through the tubing at all sites. Heating and adding SNP to the fluid help the blood vessels in your skin to dilate.

x. After about 30 minutes, the experiment ends. We remove the MD tubing from your skin and place sterile bandages over the sites. If you want, we can place a bag of ice on the sites for 10 minutes to reduce any bruising that may occur.

xi. We measure your blood pressure and heart rate before you leave the lab.

   a. MD Experiment - ACh Dose Response (or Local Heating)
      i. This experiment has 5 MD sites.
      ii. Fluid starts flowing through the tubing in your arm while we wait for the redness to end.
      iii. We collect baseline data for about 20 minutes.
      iv. We add the test substances to plain fluid in Probe 3, 4, and 5. After about 30 minutes we add LNAME to Probe 2.
         Probe 1. Lactated Ringer’s only (control)
         Probe 2. Lactated Ringer’s + LNAME
         Probe 3. Lactated Ringer’s + Ascorbate
         Probe 4. Lactated Ringer’s + Tempol
         Probe 5. Lactated Ringer’s + Apocyanin
      v. When the SkBF is stable, we collect a second baseline data for about 20 minutes.
      vi. We add the first amount of ACh to the plain fluid flowing through each probe.
      vii. As the SkBF becomes stable at each probe (about 5 minutes), we proceed to the next amount of ACh.
      viii. Each probe receives 11 increasing amounts of ACh.
      ix. After the last amount of ACh ends, we increase the skin’s temperature to 42°C (107.6°F) at all MD sites. At the same time, we add SNP to all MD probes. Heating and adding SNP to the fluid help the blood vessels in your skin to dilate.
      x. After about 30 minutes, the experiment ends. We remove the MD tubing from your skin and place sterile bandages over the sites. If you want, we can place a bag of ice on the sites for 10 minutes to reduce any bruising that may occur.
      xi. We measure your blood pressure and heart rate before you leave the lab.

b. You wait at least 3 days before beginning the Controlled Feeding protocols described below.

---------initial C. Controlled Feeding Protocols and Experiments

(the order of the Local Heating and ACh Dose Response is randomized)

1. Controlled Feeding Protocol
   a. Following baseline experiments outlined above, you participate 4 Controlled Feeding Periods. Each period lasts 8 days. After each period, you repeat the experiments described above on Days “8” and “9”. You maintain the assigned feeding protocol through the first day of experiments. The feeding protocols occur in random order:
low sodium diet devoid of dairy products (L-Na)
low sodium diet containing cheese (L-Na+C)
high sodium diet devoid of dairy products (H-Na)
high sodium diet containing cheese (H-Na+C)

b. The special kitchen in the CRC prepares all food and drink for the feeding protocol.
c. During the Controlled Feeding Period, you eat only the food and drink provided by us.
d. It is important that you eat all of the food we give to you. You must eat all of the cheese. If you have a consistent problem with eating all of the food, please talk to us about this issue.
e. During the Controlled Feeding Period, refrain from antioxidants (e.g. vitamin C) and drugs containing salts (e.g. antacids).
f. You may consume the food / drink at home.
g. Fill out a daily survey about the food and drink that you consumed during the previous day.
h. Visit the CRC kitchen every 1-2 days to pick up food / drink and drop off containers from the previous day or 2.
i. Return any unconsumed food / drink to the kitchen in the containers provided. We measure the unconsumed portions to find out your nutrient intake.

2. Preparation for Experiments

a. We give you printed and verbal instructions telling you to do during the 8-day Controlled Feeding periods and before the experiments.
b. You consume the assigned food / drink for 8 days.
c. Day 7 of each Controlled Feeding period
   i. Collect all urine you produce for 24-hour into a container that we supply.
   ii. You undergo 24-hour blood pressure monitoring.
   iii. You wear a monitor that measures blood pressure for 24 hours. If your blood pressure goes up too much after eating the high sodium food, your participation in the study ends. Refrain from caffeine (ex. coffee, tea, Coca Cola, chocolate) for 12 hours before experiments.
   iv. Do not eat anything for 8 hours before the experiments.
   v. Drink only water for 8 hours before the experiments.
d. On the day of the experiment
   i. Do not eat anything the morning of experiment.
   ii. It is important for you to be well hydrated for the experiment so you should drink at least 1 glass (8 ounces) of water before you arrive. (Drink only water the morning of experiment.)
   iii. We measure your blood pressure, heart rate, and oral temperature.
   iv. Women of childbearing age have a urine pregnancy test if they have not had a test within 2 weeks of the experiment.
   v. We insert MD probes in the same manner as that described for the Baseline Experiments.
   vi. We tape a thin probe and its holder over each site where there is MD tubing in your skin. The thin probe measures skin blood flow (SkBF) with a weak laser light.
We control the temperature of the holders. The holders start at 34°C (93°F). During the experiment, we measure blood pressure and heart rate.

3. **Experiments “Day 8”**
   a. The nurse draws a blood sample 30 ml (2 Tbsp) blood draw.
   b. FMD (described above)
   c. MD Experiment - Local Heating (or Acetylcholine Dose Response) as described above.
   d. After Day 8’s experiment ends, eat the meals we supply for Day 8. Eat as much of Day 8’s meals as you can.

4. **Experiments “Day 9”**
   a. MD Experiment - Acetylcholine Dose Response (or Local Heating) as described above.
   b. After Day 9’s experiment ends, eat your normal diet.

5. You wait at least 3 days before beginning the next Controlled Feeding period.

6. You repeat the 3-day food record during one of the washout periods between feeding protocols.

3. **What are the risks and possible discomforts from being in this research study?**

   Microdialysis: The risks are less than that for a blood draw because microdialysis uses only a small, local area of skin. In contrast, a blood draw involves not only skin, but also large blood vessels and blood. You are likely to have some pain and bruising like that from a blood draw. However, we use ice to numb your arm when we insert the tubing. Also, the small needle reduces pain when we insert the tubing. You are not likely to have pain after the tubing is in place. You may feel a little pain when we remove the tubing from your skin. Needles make some people feel sick to their stomach, lightheaded, or may cause them to faint. Although rare, the tubing could break as we remove it from the skin. Then we remove the tubing still in your skin by pulling on the other end of it. This presents no added risk for you. Even rarer, the tubing could break so that a piece of the tubing is left under your skin. In this case, we treat any tubing still in your skin like a splinter. We stop any mild bleeding with mild pressure and sterile gauze. Infection is possible. We keep the risk of infection very small by using sterile techniques and supplies like those used with blood draws. We apply a sterile bandage to the site after the experiment. We tell you how to take care of the site.

   Fluid flowing through the tubing: The substances flowing through the tubing only go to a 2.5 cm² (0.4 inch²) area of skin at each tubing site. The amount that enters the skin is very small. However, there is a chance of having a bad reaction to the substances. This reaction could produce redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or fainting. We and other
researchers have used these substances with microdialysis in skin. There have been no reports that these substances caused bad reactions. If a bad reaction should occur, we summon medical help.

Lactated Ringer’s Solution and normal saline: These fluids are similar to the natural fluids in your skin. The fluids contain salt, potassium, lactate (Ringer’s only), and chloride. The acid content is like that of your body’s natural fluids. A bad reaction to these fluids is highly unlikely.

ACh, Apocynin, ascorbate, LNAME, SNP, and Tempol: These substances stop or mimic the action of your body’s natural chemicals upon the blood vessels in the skin. A small amount of these substances enter the skin around the tubing. This only affects the blood flow in the vessels in that nickel-sized area of skin. The effect of these substances is gone within an hour after the experiment.

Laser Doppler Flowmetry: Weak lasers can hurt your eye if you stare into the light for a long time. We do not turn on the laser until the probes are taped to a surface. The tape may irritate your skin.

Blood Pressure (manual, Cardiocap): We measure blood pressure with the method used in a doctor’s office. A cuff inflates on the upper arm. As the cuff slowly deflates, we listen with a stethoscope at the bend in the elbow. Or the Cardiocap monitors blood pressure through the cuff on your upper arm. During the short time cuff inflates, your arm may feel numb or tingly. The cuff could cause mild bruising.

Povidone Iodine: Researchers and hospitals use this orange-colored fluid to clean the skin. You could have a bad reaction to the fluid if you are allergic to iodine. You inform us if you have this allergy. In this case, we use only alcohol instead. A bad reaction could cause redness, itching, rash, and/or swelling. A worse reaction could also cause fever, breathing problems, changes in pulse, convulsions, and/or fainting.

Blood Draw: Blood draws often cause mild pain, bruising, swelling, or bleeding. There is also a slight chance of infection or a small clot. If you are nervous about needles, blood pressure and heart rate may increase for a little while. You may also feel lightheaded, sick to your stomach, or may faint. We keep the chance of infection minimal with the same techniques used in hospitals. Do not exercise hard for 24 hours before a blood draw.

Tape and sticky disks: The tape or sticky disks could cause a rash. During screening, you tell us if you are sensitive to tape. If a disk sticks very strongly, removing the disk could cause an abrasion like a rug-burn on your skin. An abrasion can feel tender or slightly painful, and can increase risk of infection. If you are sensitive to tape, you may have an increased chance for abrasion. An abrasion has occurred only twice during the years that the disks have been used in similar studies in our lab. We may use an adhesive remover like that used in a doctor’s office to remove the disks. If you get an abrasion a nurse checks the site. Antibiotic ointment and a sterile bandage are applied. We tell you how to take care of the site. You could have an allergic reaction to the adhesive remover. The reaction could include rash, itching, fever, or breathing problems. Also, it could include changes in pulse, and/or blood pressure, convulsions, shock, and/or fainting. If a bad reaction should occur, we summon medical help right away.
Medical Screening: You may feel shy about giving health information. The staff collects the information in a private and professional manner. You may feel shy about being measured. You may request someone of the same sex to conduct parts of the screening.

Initial screening form: Only members of our lab group use this form. We use the form to help decide whether you are a good candidate for the study. You may feel shy about answering questions. You may request someone of the same sex to ask you the questions. We collect the information in a private and professional manner. We keep the completed form confidential and secure.

ECG: This machine measures the electrical activity of your heart to record heart rate. You have 3 wires from the machine taped to spots on your chest. There are no adverse effects. The tape may irritate.

Local heating: We measure the temperature of your skin under the holders. During heating, the skin feels very warm but does not hurt. The heating makes the skin under the holder red like when you take a hot bath. The redness goes away within several hours. Some people may be more sensitive to heating. If your arm feels too hot, tell us, and we reduce or stop the heating.

Controlled Feeding Protocol: Food allergies can produce redness, itching, rash, and/or swelling. A severe reaction (anaphylactic shock) could cause fever, problems breathing, and changes in pulse. A severe reaction could include convulsions, and/or loss of consciousness. Please inform the nurse during screening of your known food allergies. Also, inform the dietician when you meet with her. We may be able to replace the problem-food with a food to which you are not allergic without affecting the study. If this is not possible, you cannot be in the study.

FMD Test / Doppler Ultrasound: There is a small chance the probe could irritate the skin. Minor redness may occur where the researchers place the probe against the arm. This is temporary. While the researchers inflate the cuffs, the arms and feet may feel numb or tingly, and the color of the skin may change slightly. The cuffs could cause mild bruising. The gel is the same as that used with medical ultrasound tests. The gel may feel cool or cold on the skin. A bad reaction to the gel is highly unlikely.

Sublingual Nitroglycerine: The research-use of nitroglycerin for artery measurements is not an FDA-approved use of this drug. However, nitroglycerin has been used in this way in many research studies without problem. Nitroglycerin is FDA approved for the treatment of angina (heart pain). The drug is often prescribed for heart patients who have, or are at risk for, angina.

You may have some of the following reactions to the nitroglycerine:
- headache
- lightheadedness
- dry mouth
- flushing
- irregular heart beat
- weakness
- nausea
- vomiting
- 5-10 minute drop in blood pressure
- sweating
- fainting
- dizziness

You may also notice a sweet taste and/or tingling in your mouth while the tablet dissolves. All these effects are usually short-lived. We can reduce some of them by having you lie down for 20 minutes after you receive the tablet. If your blood pressure drops, it is likely to return to within 10 mmHg of your starting level by the time the test ends. We monitor you
for up to an hour after you receive the nitroglycerin if you have a strong or bad reaction. If your blood pressure does not return to baseline, and you have related symptoms (e.g. dizziness) we advise you to see your doctor. You could have a mild or severe allergic response to the drug. This response could include rash, itching, difficulty breathing, and swelling of your face, lips, tongue, or throat. If you have a severe reaction (e.g. severe allergic response) we call 911.

The effects of nitroglycerin on pregnant or nursing women are unknown. You are not to be in the study if you are pregnant or nursing.

Fasting: You may feel hungry after fasting for the experiments and during the experiments. There is a risk of loss of confidentiality if your information or your identity is obtained by someone other than the investigators, but precautions will be taken to prevent this from happening. The confidentiality of your electronic data created by you or by the researchers will be maintained to the degree permitted by the technology used. Absolute confidentiality cannot be guaranteed.

4. What are the possible benefits from being in this research study?

4a. What are the possible benefits to you?

You receive a medical screening that could inform you about your health. You learn your blood pressure and blood cholesterol levels. This is important knowledge. High blood pressure and blood cholesterol contribute to many serious health problems. If you have high blood pressure or blood cholesterol, we advise you to work with a health care provider to keep your levels controlled.

4b. What are the possible benefits to others?

One in three adults under the age of 65 and one out of two adults over the age of 60 has high blood pressure. 40% of all deaths in the United States are due to CVD. This health care burden is greater than $350 billion/year and growing. As the population ages, these problems will increase. This study explores dairy as a candidate for early intervention through a change in diet. This could reduce the need for drug treatment and its attending side effects and cost. The projects provide valuable experience, education and partial fulfillment of degree-work for graduate and undergraduate students of The Pennsylvania State University.

5. What other options are available instead of being in this research study?

You may decide not to participate in this research.

6. How long will you take part in this research study?

Screening (1 Visit) less than 1.5 hour
FMD/MD Experiments (5 Visits) less than 7 hours each
MD Experiments (5 Visits) 5 hours
Obtain Food, 24-hour BP monitor & urine container, etc. (~14 Visits) about 15 minutes each

Total: about 75 Hours (about 25 visits; It could take about 8 weeks to complete the study.)

7. How will your privacy and confidentiality be protected if you decide to take part in this research study?

   We make efforts to limit the use and sharing of your personal research information to people who have a need to review this information.
   
   • We keep the list that matches your name with your code number in a locked file or password protected file on a computer in a room that is locked when unoccupied. Only authorized members of the lab have access to the list.
   
   • We label your research records with your code number and keep them in a locked file or password protected computer in a room that is locked when unoccupied.

   We label your research samples with your code number. We keep the samples in a dedicated ultralow freezer in Noll Lab until analysis.

   In the event of any publication or presentation resulting from the research, we do not share your personally identifiable information.

   We will do our best to keep your participation in this research study confidential to the extent permitted by law. However, it is possible that other people may find out about your participation in this research study. For example, the following people/groups may check and copy records about this research.

   • The Office for Human Research Protections in the U.S. Department of Health and Human Services
   
   • The research study sponsor, Dairy Management, Inc.
   
   • The Institutional Review Board (a committee that reviews and approves research studies) and

   • The Office for Research Protections.

   Some of these records could contain information that personally identifies you. Reasonable efforts will be made to keep the personal information in your research record private. However, absolute confidentiality cannot be guaranteed.

8. What are the costs of taking part in this research study?

8a. What will you have to pay for if you take part in this research study?

Nothing.
8b. What happens if you are injured as a result of taking part in this research study?

In the unlikely event you become injured as a result of your participation in this study, medical care is available. It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

9. Will you be paid or receive credit to take part in this research study?

Experiments (1 baseline + 4 controlled feeding periods = 5 of each type of experiment)

<table>
<thead>
<tr>
<th>Experiment Type</th>
<th>Payment</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMD/Sublingual Nitroglycerin</td>
<td>$250.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$50.00 each</td>
<td></td>
</tr>
<tr>
<td>MD local heating</td>
<td>$425.00</td>
<td>$85 each ($15.00 for each MD probe + $25 for completing MD experiment)</td>
</tr>
<tr>
<td>MD ACh dose response</td>
<td>$575.00</td>
<td>$115.00 each ($15.00 for each MD probe + $40 for completing MD experiment)</td>
</tr>
<tr>
<td>Bonus for completing all experiments</td>
<td>$200.00</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>$1,450.00</td>
<td></td>
</tr>
</tbody>
</table>

For incomplete experiments, we pay an amount of money equal to the part completed. For instance, if you complete half of an ACh dose response MD experiment, you receive $15.00 for each MD probe inserted + $20 ($20.00 is one-half of $40.00). We may ask you to repeat a trial. If you agree to repeat a trial, you receive payment for the repeated trial as stated above. We reimburse for gasoline if you live more than 20 miles from Noll Lab.

10. Who is paying for this research study?

Dairy Management, Inc. is paying for this study.

11. What are your rights if you take part in this research study?

- You do not have to be in this research.
- If you choose to be in this research, you have the right to stop at any time.
- If you decide not to be in this research or if you decide to stop at a later date, there will be no penalty or loss of benefits to which you are entitled.
- If you choose to withdraw from the study, all data collected up to the point of withdrawal will remain part of the study and may not be removed.
The person in charge of the research study or the sponsor can remove you from the research study without your approval. We remove you from the study if your blood pressure increases too much during a Controlled Feeding period. Other possible reasons for removal from the study include if we deem that your health or behavior adversely affects the study or increases risks to you beyond those approved by the Institutional Review Board and agreed upon by you in this document. You may decline to answer certain questions. You may decide not to comply with certain procedures. However, your being in the study may be contingent upon answering these questions or complying with the procedures.

During the course of the research you will be provided with any new information that may affect your health, welfare or your decision to continue participating in this research.

12. If you have questions or concerns about this research study, whom should you call?

Please call:

- Study head, Lacy M. Alexander, Ph.D.  (W: 814-867-1781)
- The research nurse, Susan Slimak RN     (W: 814-863-8556, H: 814-237-4618)
- Dr. Alexander’s assistant, Jane Pierzga   (W: 814-865-1236, H: 814-692-4720)

if you:

- Have questions, complaints or concerns about the research.
- Believe you may have been harmed by being in the research study.

You may also contact the Office for Research Protections at (814) 865-1775, ORProtections@psu.edu if you:

- Have questions regarding your rights as a person in a research study.
- Have concerns or general questions about the research.
- You may also call this number if you cannot reach the research team or wish to talk to someone else about any concerns related to the research.

INFORMED CONSENT TO TAKE PART IN RESEARCH

Signature of Person Obtaining Informed Consent

Your signature below means that you have explained the research to the subject or subject representative and have answered any questions he/she has about the research.

Signature of person who explained this research Date Printed Name

(Only approved investigators for this research may explain the research and obtain informed consent.)

Signature of Person Giving Informed Consent

Before making the decision about being in this research you should have:

- Discussed this research study with an investigator,
- Read the information in this form, and
• Had the opportunity to ask any questions you may have.
Your signature below means that you have received this information, have asked the questions you
currently have about the research and those questions have been answered. You will receive a copy of
the signed and dated form to keep for future reference.

Signature of Subject

By signing this consent form, you indicate that you voluntarily choose to be in this research and
agree to allow your information to be used and shared as described above.

___________________________  __________  ________________
Signature of Subject     Date   Printed Name
VITA
Billie K. Alba

Education

Ph.D. The Pennsylvania State University, Department of Kinesiology 2018
M.B.A. University of Florida, Warrington College of Business 2013
B.S. University of Florida, Department of Chemistry 2011
B.A. University of Florida, Department of Economics 2011

Fellowships

Predoctoral Fellowship, American Heart Association, Great Rivers Affiliate 2016
University Graduate Fellowship, The Pennsylvania State University 2013

Honors & Awards

Alumni Association Dissertation Award, The Pennsylvania State University 2018
Caroline tum Suden/Frances Hellebrandt Professional Opportunity Award, American Physiological Society 2017
College of Health and Human Development Professional Development Endowment, The Pennsylvania State University 2015
MARC ACSM Doctoral Student Investigators Award 2015
Research Training in Physiological Adaptations to Stress Award, T32GM108563, The Pennsylvania State University 2014
Rock Research Ethics Fellow, The Pennsylvania State University 2014

Peer Reviewed Publications

