The Dissertation Committee for Michelle Lorraine Harrison Certifies that this is the approved version of the following dissertation:

Cutaneous and Cerebral Microvascular Response to the Ingestion of Flavanols in Young and Older Humans: Role of Nitric Oxide

Committee:

R. Matthew Brothers, Supervisor

Hirofumi Tanaka

Roger P. Farrar

Dee U. Silverthorn

David M. Keller

Cutaneous and Cerebral Microvascular Response to the Ingestion of Flavanols in Young and Older Humans: Role of Nitric Oxide

by

Michelle Lorraine Harrison, B.S., M.A.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

August 2014

Cutaneous and Cerebral Microvascular Response to the Ingestion of Flavanols in Young and Older Humans: Role of Nitric Oxide

Michelle Lorraine Harrison, Ph.D.

The University of Texas at Austin, 2014

Supervisor: R. Matthew Brothers

These studies explored interactions between flavanols and nitric oxide in order to investigate implications for vascular health. Study 1 investigated acute effects of flavanol consumption on cutaneous microvascular endothelial function in young and older individuals along with chronic exposure in older individuals. This was accomplished by assessing skin blood flow response to local heating (thermal reactivity, TR); skin was clamped at 34°C and 40°C and values were normalized to those attained at 43°C. Older individuals demonstrated an attenuated TR at baseline during the entire local heating phase (58.4 ± 2.5% versus 49.3 ± 2.6%, $p<0.05$). Acutely following flavanol ingestion there was a significant increase in TR (52.4 ± 2.1% versus 56.1 ± 2.0%, $p=0.05$) that was not different with age. There was no effect of chronic flavanol exposure on TR in older individuals; however, there was a significant decrease in mean arterial pressure (95 ± 3 mmHg versus 91 ± 3 mmHg, $p<0.001$). These results contribute to research regarding flavanols increasing NO bioavailability; acutely via an improvement in cutaneous microvascular endothelial function and chronically via a reduction in blood pressure. Study 2 investigated the acute effects of flavanol consumption on cerebrovascular endothelial function in young and older individuals along with chronic flavanol exposure

in older individuals. This was accomplished by assessing basal cerebral blood flow indices (cerebral vascular conductance index, CVCi) and CBF response to hypercapnia (cerebral vasomotor reactivity; CVMR). At baseline older individuals demonstrated a reduced CVCi (0.85 ± 0.04 cm/s*mmHg versus 0.55 ± 0.04 cm/s*mmHg p=0.001) and CVMR (8.6 ± 0.6 versus 6.9 ± 0.4, p=0.05). An unexpected finding was that flavanol ingestion led to an acute decrease in CVCi (0.71 ± 0.04 cm/s*mmHg versus 0.62 ± 0.04 cm/s*mmHg $p<0.05$) and CVMR (8.6 ± 0.6 versus 6.1 ± 0.5, p=0.001) that was not different with age. In older individuals, chronic exposure led to a significant increase in CVCi (0.60 ± 0.05 cm/s*mmHg versus 0.72 ± 0.06 cm/s*mmHg, $p<0.05$) but had no effect on CVMR. These data provide evidence for an improvement in cerebral hemodynamics following chronic exposure in older individuals.

Table of Contents

List of Tables

Study 1

Study 2

List of Figures

Study 1

Study 2

Chapter I: Introduction

The number of individuals over 65 years of age increased from 34.8 million in 2000 to 40.2 million in 2010 and is projected to be 88.5 million by 2050. Accordingly, this population will go from accounting for 13% of the overall population in 2010 to 20% of a much greater population in 2050 (212). Cardiovascular disease (CVD) is currently the number one cause of mortality in the US and its incidence increases significantly with age in both males and females (167). The term CVD includes diseases of the circulatory system with the main public health concerns being hypertension, heart disease, stroke, and peripheral artery disease. Taking into account only coronary heart disease, hypertension and stroke, which together account for 75% of CVD deaths, the estimated direct and indirect costs associated with the treatment of these diseases in 2008 was already about 300 billion dollars annually (167). Currently, an estimated 83 million adults in the US alone suffer from CVD and, though the deaths from CVD are falling, the number of individuals with symptoms is not following the same trend. This suggests that lives are being saved by the availability of new drugs and development of new procedures while the prevalence of the disease remains unaltered (167).

One of the primary underlying pathologies of CVD is atherosclerosis, a disease process that develops sub-clinically in childhood and then presents with clinical symptoms such as angina, myocardial infarction, stroke or death during middle age or beyond. One of the initiating events in the development of atherosclerosis is endothelial dysfunction. Extensive research in this area has revealed that one of the most relevant physiological changes that occurs with aging and is the underlying cause of endothelial dysfunction, is a reduction in production and/or bioavailability of NO, a ubiquitous and potent vascular vasodilator (155, 184). However, it is important to note that this

progression from sub-clinical to clinical manifestations is related, not only to aging, but also to other modifiable, lifestyle-related risk factors.

There is compelling evidence that environmental factors, including nutrition, are key to the transition from an asymptomatic process to clinical sequelae (156). A number of meta-analyses have supported a negative correlation between the consumption of plant-based foods, including fruits and vegetables, and the risk of CVD (53) and have even supported a dose-response (85). These positive vascular benefits are believed to be due to the presence of 'bioactive' molecules, termed polyphenols that are present in plant-based foods.

Polyphenols are nonessential molecules that, by definition, are not considered vitamins or minerals; however, they do demonstrate disease-preventative properties. Flavanols, a family of polyphenols found predominantly in many fruits, green tea, red wine and cocoa products have been shown to confer vascular benefit. They have been shown to demonstrate cardio protective effects in epidemiological, observational and dietary intervention studies (8, 31, 56, 105). Furthermore, a number of randomized, placebo-controlled trials involving the ingestion of a flavanol-containing cocoa beverage have shown improvements in endothelial function via an increase in NO bioavailability (12, 67, 87, 90, 147, 180).

As a result of this interplay between diet and vascular health, this dissertation investigated the acute and chronic effects of flavanol ingestion on cutaneous microvascular and cerebral vascular function in aging. Investigations such as these have the potential to guide more precise age-related, evidence-based public health dietary recommendations as well as to provide preliminary insight for future mechanistic studies exploring novel pharmacological agents.

Chapter II: Statement of the Problem

- The projected increase in the number of individuals over 65 years of age, their correlated increase in CVD risk, and the associated financial burden attest to the importance of developing ways to delay or prevent the onset of CVD.
- The long subclinical progression of atherosclerosis, evidence that dietary choices favorably alter this progression along with data that polyphenol-rich foods provide vascular benefit attest to the importance of continuing with this line of research.
- Therefore, the overall purpose of these studies was to examine the effects of flavanol consumption on endothelial function and NO bioavailability in aging in order to explore implications for human CV health.

Study 1 focused on investigating the acute effects of flavanol consumption on cutaneous microvascular function in young and older individuals and explored the same effects following chronic flavanol exposure in older individuals.

Study 1 Specific Aims. To determine the acute and chronic effects of flavanol ingestion on cutaneous microvascular function in both young and older humans.

Study 2 focused on investigating the acute effects of flavanol consumption on cerebral vascular function in young and older individuals along with the effects of chronic flavanol consumption on cerebral vascular function in older individuals.

Study 2 Specific Aims. To determine the acute and chronic effects of flavanol ingestion on cerebral vascular function in both young and older humans.

Chapter III: Experimental Design

In order to accomplish the aims of this dissertation, two studies utilizing a randomized, double-blind, placebo-controlled cross-over study design were conducted. These studies allowed the assessment of whether a dietary flavanol intervention could acutely improve endothelial function in microvascular and cerebrovascular vessels, presumably via an increase in NO bioavailability, in two groups: individuals over 60 years of age and a group of younger individuals. In addition, these studies explored whether chronic exposure to flavanols in older individuals would have an effect on endothelial function in microvascular and cerebrovascular vessels, again presumably through enhanced basal NO bioavailability.

The acute portion of both these investigations included young and older individuals and required 2 days, separated by a minimum of 3 days, as subjects received either a flavanol-containing drink or a placebo. All parameters were assessed pre-ingestion and then 2 hours post-ingestion of the test drink. The chronic portions included a smaller cohort of older individuals. This phase was a double-blind, cross-over design that required that these individuals consume a test drink at home for 28 days with a washout period of at least two weeks between test periods. Subjects came in for testing prior to the first intervention and then again within 3 days of consuming their 28^{th} beverage. Following the chronic intervention only fasting, baseline measures were assessed.

Study 1. The purpose of this study was to investigate the acute and chronic effects of flavanol ingestion on cutaneous microvascular function in young and older individuals. This was accomplished by assessing skin blood flow response to a standard local heating

protocol (thermal reactivity, TR) as an assessment of endothelial function. We hypothesized the following:

1) At baseline, older individuals would demonstrate an impaired TR compared with the young,
2) Both groups would demonstrate an increase in TR two hours following flavanol consumption; however, the magnitude of improvement would be greater in the older individuals,
3) There would be an increase in baseline TR in the older individuals following chronic exposure to the flavanols, and
4) None of these effects would be seen following ingestion of the placebo.

Study 2. The purpose of this study was to investigate the acute and chronic effects of flavanol ingestion on cerebral vascular function in young and older individuals. This was done by assessing cerebral blood flow response to hypercapnia (cerebral vasomotor reactivity, CVMR). We hypothesized the following:

1) At baseline, older individuals would demonstrate a reduced CVMR compared with the young,
2) Both groups would demonstrate an increase in CVMR two hours following flavanol consumption; however, the magnitude of increase would be greater in the older individuals,
3) There would be an increase in baseline CVMR following chronic exposure to the flavanols, and
4) None of these effects would be seen following ingestion of the placebo.

Chapter IV: Study #1

Cutaneous Microvascular Response to the Acute and Chronic Ingestion of Flavanols in Young and Older Humans: Role of Nitric Oxide

ABSTRACT

Aging is associated with a progressive decline in endothelial function that contributes to cardiovascular disease (CVD) risk. Lifestyle choices, including nutrition, are known to modulate the progression of CVD. The cutaneous circulation represents an easily accessible microvascular bed and evidence suggests that impairments in the microcirculation precede overt clinical signs of disease. Therefore, this study investigated the effect of flavanols (~ 528 mg, provided by The Hershey Company) on cutaneous microvascular endothelial function with aging following both acute and chronic exposure. It was hypothesized that older individuals would exhibit a reduced cutaneous vasodilatory response to a local heating stimulus (thermal reactivity, TR) compared with the young at baseline, that this reduction would be acutely restored following flavanol ingestion and it would also be restored following chronic flavanol exposure in older individuals. For TR, skin blood flow (SkBF) was assessed while the skin was clamped at 34°C and 40°C and values were normalized to a maximal value obtained during 43°C heating. Cutaneous vascular conductance (CVC) was calculated as the ratio of SkBF to mean arterial pressure (MAP). For the acute portion of the study, 15 young (19-28 yrs) and 15 older (62-78 yrs) individuals were tested on two separate days; they were provided either a nutrient-matched placebo or a flavanol-containing test drink (randomized order, double-blinded) and TR was assessed pre- and 2 hours post-ingestion. For the chronic portion of the study, 11 older individuals (62-72 yrs) were recruited and TR was assessed at baseline and then following 28 days of flavanol consumption. In accordance with prior studies, at baseline older individuals demonstrated a reduced CVC

during the last 5 minutes of the stable plateau phase at 40°C (72.1 ± 2.9% versus 62.0 ± 3.0%, $p<0.05$) but not 34°C (10.8 ± 0.6% versus 11.9 ±0.7%, $p>0.05$) compared with the young. Acutely following flavanol ingestion there was a significant increase in SkBF response when considering the entire 30 minutes of 40°C heating (52.4 ± 2.1% versus 56.1 ± 2.0%, $p=0.05$) but, contrary to the hypothesis, this response did not differ between the young and older individuals. There was no effect of chronic flavanol ingestion on TR in the older individuals, either the last 5 minutes of the plateau phase or over the 30 minutes of local heating; however, there was a significant decrease in mean arterial pressure (MAP, Baseline: 95 ± 3 mmHg, Flavanols: 91 ± 3 mmHg, $p<0.001$). This suggests that, though chronic exposure to flavanols did not affect TR, flavanols consumption was long enough to exert positive vascular effects. These results contribute to existing research regarding flavanols having vascular health benefit; acutely via an improvement in cutaneous microvascular endothelial function and chronically via a reduction in blood pressure. Further studies are required to elucidate underlying mechanisms.

INTRODUCTION

Cardiovascular disease (CVD) is the number one cause of mortality in the US and its incidence increases significantly with age in both males and females (167). Taking into account only coronary heart disease, hypertension and stroke, which together account for 75% of CVD deaths, the estimated direct and indirect costs associated with the treatment of these diseases in 2008 was about 300 billion dollars annually (167). Currently, an estimated 83 million adults suffer from CVD in the US alone and, though the deaths from CVD are falling, the number of individuals with symptoms is not

following the same trend. This suggests that lives are being saved by the availability of new drugs and development of new procedures while the prevalence of the disease remains unaltered (167). Aging is one of the primary risk factors for CVD. Hence, of considerable concern is that the number of individuals over 65 years of age is projected to increase from the 40.2 million in 2010 to 88.5 million by 2050. Accordingly, this population will go from accounting for 13% of the overall population in 2010 to 20% of a much greater population in 2050 (212).

One of the principal underlying pathologies of CVD is atherosclerosis. Atherosclerosis is a process that develops over a lifetime; it can remain subclinical for many years prior to presentation of clinical symptoms including angina, myocardial infarction, stroke or death. The likelihood of clinical manifestations is especially prevalent in older individuals who have life-style associated risk factors such as hypertension, hypercholesterolemia, smoking, diabetes, and obesity.

Endothelial dysfunction is believed to be the primary event in the development of atherosclerosis as the endothelium plays an integral role in maintaining vascular homeostasis. Endothelial dysfunction is associated with atherosclerotic risk factors (38, 213) and is also a predictor of adverse CV events (80, 197). This dysfunction is due, in large part, to a decreased bioavailability of nitric oxide (NO) as NO mediates many of the protective functions exerted by a healthy endothelium. Aging is associated with reduced bioavailability of NO, a progressive decline in endothelial function, and increased risk of CVD (39, 155).

There is compelling evidence that environmental factors, including nutrition, are key to the transition from an asymptomatic process to clinical manifestations of atherosclerosis (156). The endothelium constitutes the predominant tissue to which any molecule is exposed following digestion and absorption into the blood stream and thus

represents a potential site of action for ingested compounds. A number of meta-analyses, assessing numerous large scale epidemiological studies have shown that increased consumption of plant-based foods, including fruits and vegetables, decreases the risk of CVD and have even supported a dose-response (52, 53, 85).

Current thinking is that the health benefit of plant-based foods is not due solely to vitamins and minerals contained in these foods, but also to the presence of bioactive compounds called polyphenols: small, nonessential molecules that possess disease-preventative properties. Over the past decade flavanols, one of the more prevalent family of polyphenols, have become the focus of intense research. Flavanols are found in a high concentration in certain fruits and in foods such as cocoa products, green tea, red wine and soy products (154).

Numerous studies have shown that flavanols increase NO bioavailability. This has been demonstrated in cultured endothelial cells and in rodents (161, 178, 193, 194) as well as humans (12, 67, 87, 90, 147, 180). There is evidence to suggest that the underlying mechanism accounting for the increase in NO bioavailability may differ between acute and chronic consumption of flavanols. This belief is supported by studies that demonstrate an increase in basal NO following chronic flavanol consumption then an even further increase following an acute dose of flavanols (12, 88, 180).

Endothelium-dependent vasomotor function reflects the integrity of the endothelial layer and is used as a surrogate of NO bioavailability. The skin is emerging as an ideal site for evaluation of endothelial function as it has been suggested that the cutaneous microcirculation can serve as a model for generalized microvascular dysfunction (94, 108). Skin blood flow (SkBF) response to a standard local heating protocol (thermal reactivity; TR) is a common, non-invasive research technique used to assess cutaneous microvascular function. This response is predominantly NO-mediated

(106, 144) and is reduced in a variety of at-risk and diseased populations. TR is also reduced with aging (95, 127, 145).

This study was designed to investigate both the acute and chronic effects of flavanol ingestion on TR. The acute portion of the study involved a cohort of young and older individuals while the chronic portion only included an older population. The majority of studies have demonstrated an improvement in larger arteries such as the brachial artery (12, 90, 147). However, whether or not endothelial function can be restored in the cutaneous microcirculation following flavanol ingestion remains unknown. We hypothesized that the older individuals would have an impaired TR compared with the young prior to flavanol consumption (i.e. at baseline) but TR would be acutely restored to the level of the young following flavanol ingestion. In addition, we hypothesized that this impairment would be improved following chronic flavanol consumption in the older individuals.

METHODS

Subjects. For the acute portion of the study, 15 young (range: 19-28 yr old; 8 men) and 15 older individuals (range: 62-78 yr old; 7 men) were recruited to participate (see Table 2 for subject descriptive information) and each visited the laboratory on 2 separate days (days 1 & 2). In order to control dietary intake of flavanols prior to testing subjects were asked to do the following: 1) avoid flavanol-rich foods including chocolate/cocoa products, green tea, red wine and most fruits in the 3 days prior to the study and 2) document all food intake on the day prior to their testing day so they could consume a similar diet prior to day 2 of testing (see Appendix A for Acute Study Instructions) (157, 158). Subjects came into the laboratory at approximately the same time for their 2 testing

days following a 12 hour fast, having refrained from caffeine and exercise for 24 hours. None of the young women were on birth control and all were tested during the early follicular phase of their menstrual cycle (41). All of the older women were postmenopausal. All subjects were healthy, nonsmokers with no diagnosed cardiovascular disease. None of the younger subjects were taking medications; however, 11 of the older subjects were taking medications, mainly for allergies, thyroid and cholesterol and one older female was taking hormone replacement therapy. Those subjects on medications refrained from taking them on the morning of each of their testing days.

For the chronic portion of the study, 11 older individuals (range: 62-72 yr old; 3 men) were recruited, 9 of whom were also part of the acute portion of the study. Subjects were provided with enough drink powder to consume a test drink at home for 28 days. They were not asked to follow any specific dietary restrictions during that time; however, they were asked to consume the test drink at approximately the same time each day (morning, afternoon or evening). They were also asked to return to the laboratory with all empty canisters and unused product. Subjects came in for testing within 3 days of consuming their 28th drink, following a 12 hour fast, having refrained from caffeine and exercise for 24 hours (see Appendix A for Chronic Study Instructions).

Prior to its commencement, the consent form and study procedures were approved by the Institutional Review Board at the University of Texas at Austin.

Instrumentation. Upon arrival in the laboratory all study procedures and risks were explained, questions were answered and then all participants provided informed consent. On each study day, height and weight were assessed using a medical–grade seca769 scale (seca corp., CA, USA). Subjects were then asked to lie semi-recumbent on a patient bed

and a fasting blood sample was drawn from the antecubital fossa for the measurement of glucose and lipids. Following the blood draw, subjects were instrumented with 5 electrodes. Both cardiac rhythm and heart rate were continuously monitored throughout the experimental protocol from an electrocardiogram on a patient monitor (GE DASH 4000, General Health Care). A blood pressure cuff was placed on the left arm and intermittent blood pressure measurements were obtained by auscultation of the brachial artery via electrosphygmomanometry (Tango+; SunTech, Raleigh, NC). Following a minimum of 20 minutes of rest, baseline heart rate and blood pressure were assessed in all subjects. Mean arterial blood pressure (MAP) was calculated as one-third pulse pressure plus diastolic blood pressure.

Skin blood flow (SkBF) was indexed using cutaneous red blood cell flux via laser-doppler flowmetry. Subjects were instrumented with 2 integrating probes (MoorLab Laser Doppler Perfusion Monitor, Moor Instruments) inset within 2 heating elements (3-cm diameter, Peritemp4005; Perimed) placed at 2 sites on the dorsal side of the right forearm. The product of average speed and concentration of moving red blood cells, represented by 'flux,' was used to represent SkBF. Skin temperature was assessed at the interface between the skin and the heating element using a thermocouple probe taped to the skin underneath one of the heating elements, randomly chosen (IT-18, Physitemp Instruments, Inc. New Jersey, USA).

Local Heating Protocol. The manipulation of local skin temperature at these sites of cutaneous blood flow measurement allowed for the assessment of TR. Temperature of the heating elements was initially set to 34°C for 15 minutes. Local heating element temperature was then increased to 40°C for 30 minutes and then 43°C for a final 30 minutes (rate of 1.0°C every 10 seconds). This rate of temperature increase and the length

of time for measurements have been shown to induce a cutaneous vasodilatory response that is primarily NO-mediated (106, 144). None of the subjects reported feeling pain in response to this heating protocol. This is important because feeling pain leads to a cutaneous vasodilatory response that is not primarily NO-mediated (106). The final heating stage of 43°C was chosen as it elicits a maximal vasodilatory response (106). A blood pressure was taken via auscultation of the brachial artery during the last 3 minutes of each heating stage so that cutaneous vascular conductance (CVC) could be calculated. CVC was calculated as the ratio of SKBF red cell flux to MAP. All testing was conducted in a temperature controlled room (21-24°C).

Acute Effects of Flavanol Ingestion. At the end of the heating protocol, the heating elements were turned off and removed from the skin. They were removed to avoid 'desensitization' that may occur in the skin with prolonged heating (43, 72). As this was a placebo-controlled, double-blinded study subjects were then provided a test drink (randomized order), either flavanol-containing (~528mg) or a nutrient-matched placebo, (see Table 1.1 for nutritional composition). Both drinks were provided by The Hershey Co., in powdered form so they were reconstituted by a third party with 8oz of warm, purified water just prior to consumption (see Appendix A for Test Drink Mixing Instructions). All subjects consumed the beverage within 10 minutes and a timer was started immediately upon completion. Subjects were asked to remain resting in the semi-recumbent position after consuming the test drink.

Seventy-five minutes following ingestion of the test drink, subjects were re-instrumented with the laser-doppler probes and heating units. This timing was chosen so that the end of the 30 minutes at 40°C would coincide with 2 hours post ingestion of the test drink. Plasma flavanols and associated metabolites have been shown to peak 2 hours

following consumption (12, 87, 180). The local heating protocol was repeated as outlined above.

Young male and older subjects were scheduled for their second day of testing after allowing for a minimum of 3 days of 'washout' as this length of time has been used in other similar studies (12, 147, 180) Young females had to wait for their menstrual cycle to begin the following month. For day 2 the experimental protocol was identical to the first study day with the only difference being that the test drink administered was the one not given on day 1. The drinks were given in a double-blinded and randomized fashion.

Chronic Effects of Flavanol Ingestion. Subjects came in for testing within 3 days of consuming their 28th drink with a minimum of 2 weeks between the interventions to avoid potential carry over effects (88, 157). All instrumentation along with the local heating protocol was identical to the assessment of the acute effects of flavanol ingestion. However, during these study days the testing measures were done only once while the subject was fasted.

Blood Analysis. Glucose and lipids were assayed simultaneously using the Cholestech LDX analyzer (Inverness Medical, Biosite Inc, CA, USA) within 30 minutes of collection into a heparinized vacutainer tube. This point of care analyzer has been validated against a hospital reference laboratory (34).

Data Analysis. Analogue output signals from cardiac rhythm, heart rate, both lasers and the thermocouple were converted, via a data acquisition system, to a digital signal allowing for the continual monitoring of these signals (Biopac System, Santa Barbara, CA). Data was then saved on a computer for subsequent analysis.

Absolute SkBF response at baseline and during the plateau phase was initially assessed by averaging over a stable 2 and 5 minute period of red cell flux at the end of 34 and 40°C respectively. The initial peak response was calculated as the average of the maximal one minute response during the peak period for both 40 and 43°C. CVC was then calculated as the ratio of SKBF red cell flux to MAP. Subsequent data analysis involved averaging red cell flux over the entire 30 minutes of the 40°C heating stage. In both cases, data was then normalized by comparing it to either absolute SkBF or CVC during the last 5 minutes at 43°C (as a percentage of maximal response) and this represents TR.

Statistical Analysis. All data are presented as means ± SEM unless otherwise stated. Baseline subject characteristics were those obtained under fasting conditions during rest on day 1 and were compared using unpaired t-tests. The acute effect of flavanols on hemodynamic variables, absolute SkBF response and CVC, along with relative SkBF response and CVC were assessed using a multi-factorial, repeated measures ANOVA with 1 between subject factor (age) and up to 3 within subject factors (treatment, time and stage). The chronic effect of flavanols on all variables was assessed using a mixed model ANOVA with 1 random effect (subject) and 2 fixed effects (treatment and stage). A mixed model was used because it allowed us to keep one of the subjects in the model who was only able to complete one of the chronic interventions. In all cases, when a significant interaction was observed, a Bonferroni adjustment for multiple comparisons was used to identify significant mean differences in the applicable pair wise comparisons. In cases where the assumption of sphericity was violated a Huynh-Feldt corrected degrees of freedom was used to determine significance. The level of significance was set a priori at $p<0.05$.

RESULTS

Baseline Differences in Young and Older Individuals. Subject characteristics are presented in Table 1.2. Baseline systolic, diastolic and mean arterial blood pressure along with total cholesterol and low-density lipoprotein were all significantly higher in the older individuals ($p<0.05$). There was a significant difference in mean skin temperature at the interface between the heating unit and skin surface for the different heating stages as expected (34.1 ± 0.04°C, 38.9 ± 0.07°C and 41.0 ± 0.10°C, $p<0.001$ for all pair wise comparisons). However, there was no significant difference in skin temperature for a given stage under any other condition: flavanol versus placebo, pre- versus post-ingestion or young versus older individuals ($p>0.05$ for all).

There was no significant difference in absolute SkBF response to local heating between young and older individuals during any stage of heating ($p>0.05$ for all). However, there was a significant difference in absolute CVC response to local heating between young and older individuals during the plateau phase of 40°C heating ($p<0.05$) but not during 34°C or the final 5 minutes of 43°C heating ($p>0.05$ for both). Figure 1.1 demonstrates the effect of age on relative TR; it shows no difference between young and older individuals in relative CVC in response to local heating at 34°C (10.8 ± 0.6% versus 11.9 ±0.7%, $p>0.05$); however, older individuals demonstrated a reduced response during the plateau phase at 40°C (72.1 ± 2.9% versus 62.0 ± 3.0%, $p<0.05$). This same response was seen in relative SkBF.

Acute Effects of Flavanol Ingestion. There was no treatment effect on any hemodynamic variables (HR, SBP, DBP or MAP) or on absolute or relative SkBF or CVC responses. Figure 1.2 shows the unexpected finding that there was a significant difference pre- versus post-ingestion of flavanols on relative initial peak SkBF response to 40°C (62.5 ±

1.7% versus 67.6 ± 2.4%, $p<0.05$) that was not present following ingestion of the placebo (64.0 ± 2.0% versus 60.7 ± 2.1%, $p>0.05$). Further investigation of this interaction revealed that there was also a significantly different initial peak response at 40°C post-ingestion when comparing flavanols and placebo (67.6 ± 2.4% versus 60.7 ± 2.1%, $p<0.01$) that was not present pre-ingestion of the test drink (62.5 ± 1.7% versus 64.0 ± 2.0% versus $p>0.05$). There was no difference in response between young and older individuals.

Figure 1.3 is a SkBF tracing from a representative subject pre- and post-flavanol ingestion. In order to investigate the unexpected finding that there was a significant effect of treatment during the relative initial peak SkBF response to 40°C heating but not during relative SkBF or CVC, both of which focus on the final 5 minutes of the plateau phase of the response, we re-analyzed the data. We averaged the entire SkBF response during the 30 minutes of 40°C heating and normalized it to the SkBF response achieved during the last 5 minutes at 43°C. Figure 1.4 demonstrates that this significant effect of flavanols on TR persists when the entire 30 minutes of heating at 40°C is considered. There remains a significant increase in SkBF response following flavanol ingestion (52.4 ± 2.1% versus 56.1 ± 2.0%, $p=0.05$) but not ingestion of the placebo (54.2 ± 2.4% versus 52.7 ± 2.1%, $p>0.05$). It is important to note that older individuals continue to show an attenuated response compared with the young when the entire response during 40°C is considered (58.4 ± 2.5% versus 49.3 ± 2.6%, $p<0.05$).

Chronic Effect of Flavanol Ingestion. Baseline subject characteristics are presented in Table 1.3. There was no effect of chronic flavanol ingestion on any of the blood analytes. There was a significant difference in mean skin temperature at the interface between the heating unit and skin surface for 34, 40 and 43°C as expected (34.1 ± 0.12°C, 38.9 ±

0.12°C and 41.2 ± 0.12°C, $p<0.001$ for all pair wise comparisons). However, there was no significant difference in skin temperature for a given stage following chronic ingestion of either flavanols or the placebo ($p>0.05$ for all). There was also no significant difference in TR following chronic ingestion of flavanols.

Figure 1.5 shows there was a main effect of treatment on systolic and diastolic blood pressure; both were significantly lower following chronic flavanol consumption compared with initial baseline values (Systolic Baseline: 126 ± 4mmHg versus Flavanols: 119 ± 4mmHg, $p<0.001$; Diastolic Baseline: 80 ± 3mmHg versus Flavanols: 77 ± 3mmHg, $p<0.05$). There was no difference in systolic or diastolic blood pressure following ingestion of the placebo. MAP also decreased following chronic ingestion of flavanols compared with baseline and this effect was not seen following ingestion of the placebo (Baseline: 95 ± 3mmHg, Placebo: 94 ± 3mmHg, Flavanols: 91 ± 3mmHg, $p<0.001$).

DISCUSSION

The significant findings of the present study were as follows: 1) at baseline older individuals demonstrated a reduced SkBF response to local heating compared with the young supporting the initial premise of impaired endothelial function in the older group, 2) consumption of flavanols led to an acute augmentation of the SkBF response to local heating; however, this effect was not different between the young and older individuals, and 3) there was no effect on SkBF response to local heating following chronic exposure to flavanols; however, there was a significant reduction in both systolic and diastolic blood pressures in older individuals.

Baseline Differences in Young and Older Individuals. Absolute CVC was reduced in response to 40°C local heating in older individuals but was not different from the young at either 34 or 43°C. This suggests that the young and older individuals did not differ at baseline and that both groups achieved the same maximal vasodilatory response to heating at 43°C. As expected, older individuals did demonstrate a reduced cutaneous vasodilatory response to local heating compared with the young (30, 145).

Acute Effects of Flavanol Ingestion. The goal of this study was to examine the acute effects of flavanol ingestion on the cutaneous vasodilator response to local heating and to determine if the response to heating differed in young versus older individuals. Contrary to our hypothesis there was no effect of flavanols on TR in either young or older individuals when only the final, stable 5 minutes of the plateau phase during 40°C were considered.

An unexpected finding was that flavanols did lead to an acute, significant increase in the relative initial peak SkBF response to local heating at 40°C. This effect was not seen following ingestion of the placebo nor was it present during the initial peak response to 43°C. Studies have shown that the initial peak response is primarily neurally-mediated with less of a contribution from NO, whereas the plateau phase has a much greater contribution from NO (106, 144). Therefore, we had initially hypothesized that we would see the effect of flavanols in the latter phase.

In order to further investigate this unexpected finding we averaged the entire SkBF response during the 30 minutes of 40°C local heating and normalized it to the SkBF response achieved during the last 5 minutes at 43°C. We hypothesized that we were detecting an effect of flavanols only during the initial peak because the overall response to local heating was less then than during the plateau phase so it represented a

greater fractional change. However, NO bioavailability was still being enhanced over the entire 30 minutes.

Figure 1.3 is a SkBF tracing from a representative subject pre- and post-flavanol ingestion that displays this hypothesized effect. In support of our hypothesis, the significant effect of flavanols was not lost when the entire SkBF response to 30 minutes of 40°C local heating was considered. We believe the effect of flavanols was occurring not only during the initial peak, though it may have appeared more robust, but rather there was some effect on cutaneous vasodilatory response throughout the 30 minutes at 40°C.

It is important to note that we observed the previously reported significantly attenuated SkBF response to 40°C in the older individuals compared with the young at baseline even when the SkBF response throughout the 30 minutes of 40°C local heating was considered. This method of data analysis differs from that reported in the literature, which involve the analysis of only the final 5 minutes of the plateau phase of the response and not the response over the entire 30 minutes. However, NO is shown to have some contribution to the cutaneous vasodilatory response throughout the entire heating phase as is evidenced by studies where the entire response is significantly blunted by the administration of L-NAME prior to local heating (106, 144).

We believe our results are strengthened by our study design: TR was assessed pre- and post-ingestion of the test drink on the same day under the same conditions; the study was placebo-controlled; and this augmentation in TR was not seen following ingestion of the placebo. In addition, the expected impairment in TR persisted prior to flavanol ingestion between our young and older groups.

The exact mechanism by which flavanols augment NO bioavailability has not yet been fully elucidated but a number of indirect pathways based on the synthesis and/or

degradation of NO have been hypothesized. These include: 1) interaction with endothelial nitric oxide synthase (eNOS) thereby maintaining the enzyme in an activated form so it is available to continually produce NO, 2) by decreasing arginase activity as it shares the substrate L-arginine with eNOS so this effectively leads to the preservation of intracellular concentration of L-arginine, and/or 3) by inhibiting NADPH oxidase (NOX) as it is the primary enzyme responsible for the generation of superoxide anion thereby reducing oxidative stress (71, 89, 177).

This study was not mechanistic by design so we can only speculate on which of these mechanisms may have been underlying this augmentation in TR following flavanol ingestion. eNOS has been shown to be the primary isoform responsible for the generation of NO during local heating; however its involvement in the initial peak SkBF response has been questioned (107, 183). In our study it appears as though flavanols were exerting some effect over the entire phase of 40°C local heating, including the initial peak, suggesting it is less likely that eNOS activation was the primary mechanism.

Dupont *et al.* demonstrated that the SKBF plateau phase of 40°C heating increased following the administration of both ascorbic acid and L-arginine whereas the initial peak response increased following the administration of ascorbic acid only (60). Free radicals, including the superoxide anion, lead to a reduction in NO bioavailability as they react with NO to form peroxynitrite (16, 17). Flavanols have been shown to improve NO bioavailability via the inhibition of NOX (20, 191, 193), a major producer of superoxide anions in the endothelium (33). In the study by Dupont *et al.*, the effects of ascorbic acid were seen during the initial peak along with the plateau, suggesting the possibility that the effect of flavanols in our study may be due to NOX inhibition. Mechanistic studies are required to confirm this.

There was no difference between the young and older individuals in their acute response to flavanol ingestion. It is known that older individuals have higher levels of oxidative stress so we might expect a greater effect of flavanols in older individuals compared with young if flavanols were working via inhibition of NOX. However, in order to limit confounders we specifically recruited older individuals who were taking little or no medication and who had not been diagnosed with CVD or any other metabolic illness. Not by coincidence, such individuals generally lead health-conscious lifestyles and their diet and physical activity levels reflect this. Though none of the older individuals in the study were highly trained, we believe they represented a healthier segment of the aging population.

Chronic Effects of Flavanol Ingestion. Contrary to our hypothesis there was no significant difference in cutaneous vasodilatory response to local heating following chronic ingestion of flavanols in older individuals. Compliance needs to be considered with long term, at home interventions such as this. In this study, subjects were asked on each of their testing days whether they had consumed all of the test drinks in the prior 28 days and whether the time of day of consumption was kept consistent. Only a single subject admitted to missing a drink and all subjects complied with the request to return empty canisters and unused product. This suggests that compliance was exceptional so we do not believe that a lack of compliance is playing a role in our results.

There is the possibility that the length of flavanol exposure was not long enough to lead to cutaneous microvascular effects; however, the 28-day study period did result in a decrease in blood pressure. This provides evidence that, though there was no effect on TR, the quantity of flavanols and the length of time they were taken was sufficient to lead to a beneficial vascular effect. A number of meta-analyses have concluded that flavanols

have a blood pressure lowering effect (164, 200). The theory behind this effect is that decreases in NO steady-state concentration can lead to a failure in smooth muscle relaxation and subsequent hypertension.

Limitations. One of the limitations in the current study is the use of 43°C local heating to elicit maximal vasodilatory response for data normalization. This study did not involve the insertion of microdialysis membranes so the administration of sodium nitroprusside was not possible. We believe this was not a true limitation because absolute CVC was not different between young and older individuals suggesting that they had the same vasodilatory capacity. There are a number of published studies that demonstrate that 43°C local heating elicits a maximal SkBF response (29, 48, 106).

A second limitation of our study was the inability to calculate relative CVC for the analysis of the response over the entire 30 minutes of 40°C heating. We took intermittent blood pressure in the final 3 minutes of each stage but we did not take beat-by-beat blood pressure throughout the entire 30 minutes so calculation of CVC was not possible. Calculation of conductance is important as changes in blood pressure can lead to changes in blood flow without vasodilation. However, analysis of the intermittent blood pressures taken at the end of each stage demonstrated no significant changes throughout the course of the heating protocol. This suggests that, in this case, flow would be representative of conductance. Furthermore, at baseline both relative SkBF response and relative CVC in the final 5 minutes of the plateau phase were significantly attenuated in older individuals compared to younger suggesting minimal effects of blood pressure. Our lab has also done some pilot work in which we have demonstrated that blood pressure does not change during the initial peak response to heating at 40°C (unpublished data).

Conclusions / Perspectives. Our main finding was that the ingestion of flavanols acutely improved the vasodilatory capacity of the cutaneous microvasculature in response to local heating and that this effect was not significantly different in young and older individuals. There was no effect on the cutaneous vasodilatory response to local heating following chronic exposure to flavanols, though beneficial vascular effects were seen by a reduction in blood pressure. The results from this study contribute to current research by providing further support for vascular health benefits associated with flavanols. Further mechanistic studies are warranted to elucidate underlying mechanisms as they could potentially guide future pharmacological research.

TABLES AND FIGURES

Table 1.1

Nutritional Composition of Test Drinks

Per serving	Flavanol	Placebo
Calories	149	153
Fat, g (calculated)	2	0
Sat fat, g	1	0
Trans fat, g	0	0
Cholesterol, mg	11	12
Sodium, mg	364	370
Carbohydrates, g	25	26
Dietary fiber, g	5	5
Sugar, g	17	20
Protein, g	14	14
Vitamin A, IU	10	8
Vitamin C, mg	2	3
Calcium, mg	429	486
Iron, mg	1	0
Magnesium, mg	113	41
Potassium, mg	780	676
Total Flavanols, mg	**528.0**	**0.0**
Proanthocyanidins 1-10, mg	247.2	0.0
PACs 1 mers	64.8	0.0
Catechin, mg	17.8	0.0
Epicatechin, mg	47.0	0.0
PACs 2 mers	39.8	0.0
PACs 3 mers	25.4	0.0
PACs 4 mers	26.4	0.0
PACs 5 mers	22.1	0.0
PACs 6 mers	20.6	0.0
PACs 7 mers	18.7	0.0
PACs 8 mers	12.0	0.0
PACs 9 mers	9.6	0.0
PACs 10 mers	7.7	0.0

Table 1.2

Subject Characteristics; Acute

	Young	Old
Sex, M/F	8/7	7/8
Age (yrs)	23 ± 3	69 ± 5
Height (m)	1.71 ± 0.09	1.68 ± 0.10
Weight (kg)	68.1 ± 13.0	65.9 ± 12.2
BMI (kg/m^2)	23.1 ± 2.8	23.1 ± 3.2
Glucose (mg/dL)	86 ± 4	90 ± 9
Total Cholesterol (mg/dL)	166 ± 30	200 ± 24#
Triglycerides (mg/dL)	76 ± 28	76 ± 29
LDL (mg/dL)	91 ± 25	117 ± 27*
HDL (mg/dL)	60 ± 15	69 ± 19
Heart Rate (bpm)	65 ± 10	67 ± 14
SBP (mmHg)	115 ± 12	125 ± 10*
DBP (mmHg)	71 ± 9	78 ± 9*
MAP (mmHg)	85 ± 9	94 ± 8#

Values are means ± SD, n=15 per group. M, male; F, female; BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure; MABP, mean arterial pressure. *$p<0.05$ and #$p<0.01$ compared with young.

Table 1.3

Effect of Chronic Ingestion of Flavanols

	Baseline	Placebo	Flavanol
Sex, M/F	3/8		
Age (yrs)	68 ± 3		
BMI (kg/m^2)	22.3 ± 2.8	22.0 ± 2.8	22.1 ± 2.6
Glucose (mg/dL)	87 ± 8	84 ± 8	82 ± 10
Total Cholesterol (mg/dL)	197 ± 20	189 ± 37	195 ± 30
Triglycerides (mg/dL)	64 ± 25	68 ± 30	68 ± 27
LDL (mg/dL)	110 ± 20	109 ± 31	111 ± 27
HDL (mg/dL)	74 ± 18	67 ± 19	71 ± 16

Values are means ± SD, n=11. M, male; F, female; BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein.

Figure 1.1

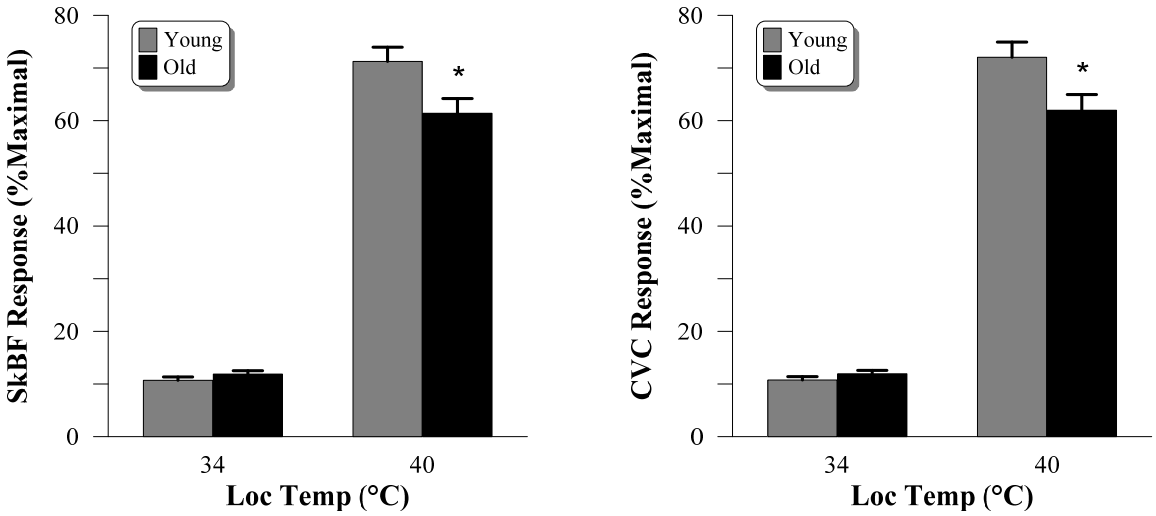

Figure 1.1: A summary of relative skBF and CVC responses to local heating in young and old individuals. The stage*age interaction was significant for both skBF and CVC, $p<0.01$. A pair wise comparison showed the mean difference in relative skBF and CVC responses at 40°C to be significantly different between young and old, after a bonferroni adjustment for multiple comparisons. *$p<0.05$ compared with young at same LocTemp. Values (means ± SEM) are expressed as a percentage of maximal skBF achieved during the last 5 minutes of 43°C heating.

Figure 1.2

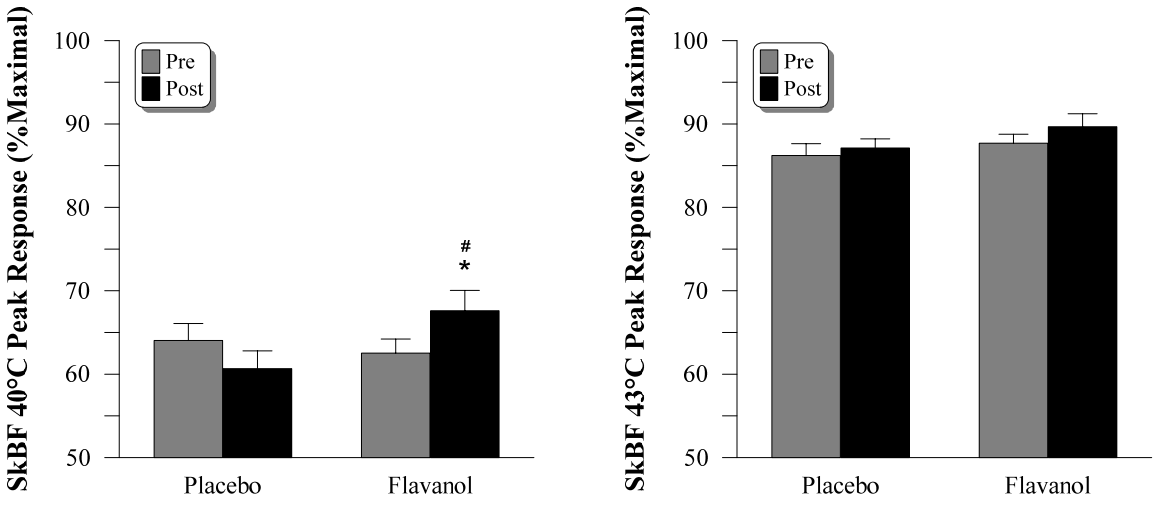

Figure 1.2: A summary of the relative initial peak SkBF response to 40°C pre- versus post-ingestion of the test drinks. The treatment*time*stage interaction was significant, $p<0.05$, and a pair wise comparison showed the mean difference in relative initial peak response to 40°C to be significantly higher following ingestion of flavanols, after a bonferroni adjustment for multiple comparisons. There was no treatment effect on the initial peak response to 43°C heating. *$p<0.05$ compared with pre in same treatment, $^{\#}p<0.01$ compared with placebo post-ingestion. Values (means ± SEM) are expressed as a percentage of maximal skBF achieved during the last 5 minutes of 43°C heating.

Figure 1.3

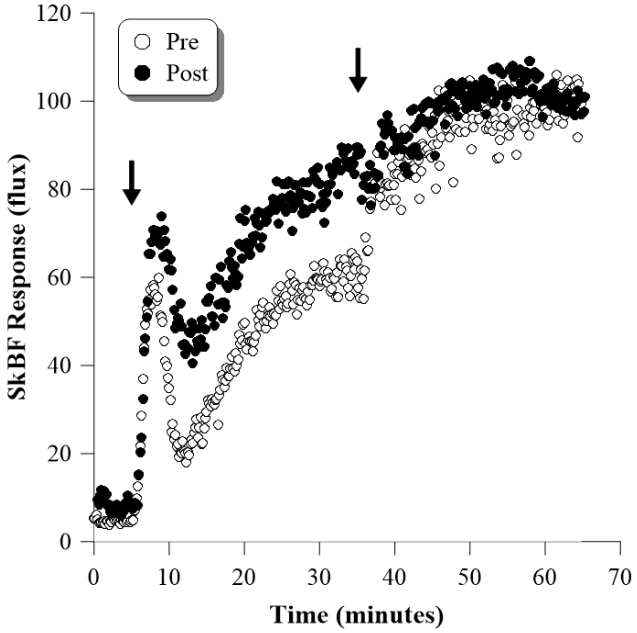

Figure 1.3: A representative tracing of the skin blood flow (skBF) response during the local heating protocol pre- and post-flavanol ingestion of a single subject. The arrows indicate the beginning and the end of 40°C heating. It appears as though there is an increase in skBF response following flavanol ingestion during the 30 minutes of 40°C heating that is not present at 34°C or 43°C .

Figure 1.4

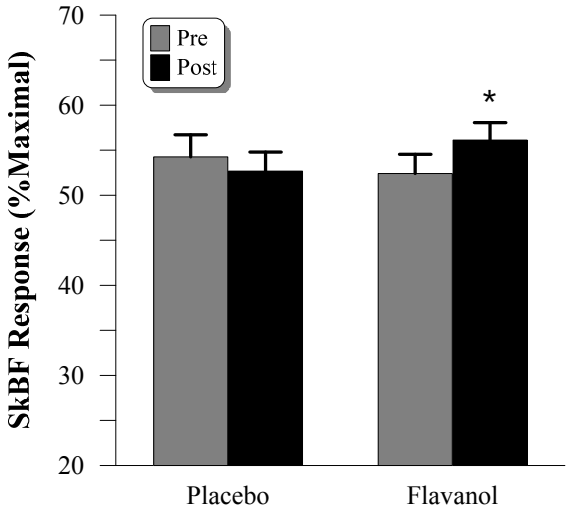

Figure 1.4: A summary of the relative SkBF response to 30 minutes of local heating at 40°C. There remained a significant interaction effect of treatment*time*stage, $p<0.05$ and a pair wise comparison showed the mean difference in relative skBF response to be significantly higher following flavanol consumption, after a bonferroni adjustment for multiple comparisons. There was no significant difference in response following ingestion of the placebo. *$p<0.05$ compared with pre of same treatment. Values (means ± SEM) are expressed as a percentage of the average response during 30 minutes of local heating at 40°C to the maximal skBF achieved during the last 5 minutes of 43°C heating.

Figure 1.5

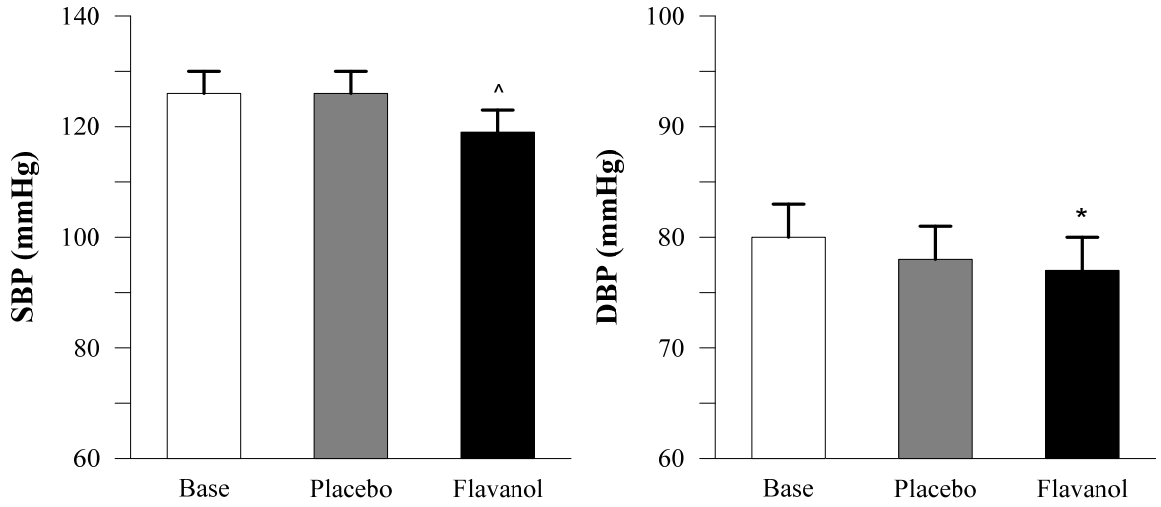

Figure 1.5: A summary of the effect of chronic flavanol ingestion on SBP and DBP. Both SBP and DBP decreased significantly following chronic exposure to flavanols but not the placebo. ^$p<0.001$ and *$p<0.05$ compared with baseline. Values are means ± SEM.

Chapter V: Study #2

Cerebrovascular Response to the Acute and Chronic Ingestion of Flavanols in Young and Older Humans: Role of Nitric Oxide

ABSTRACT

Aging is associated with a progressive decline in endothelial function that contributes to cardiovascular disease (CVD) risk. Lifestyle choices, including nutrition, are known to modulate the progression of CVD. Cerebral endothelial function has been shown to be impaired in individuals with CVD, it is correlated with the severity of the disease, and studies have shown that endothelial function is reversible with certain interventional strategies. NO has been shown to be a contributor to basal cerebral blood flow (CBF) tone and endothelium-dependent vasomotor function reflects the integrity of the endothelial layer and hence can be used as a surrogate of NO bioavailability. Therefore, in this study basal CBF and CBF response to hypercapnia (cerebral vasomotor reactivity; CVMR) were used to assess cerebral vascular endothelial function. This study investigated the effect of flavanols (~ 528mg, The Hershey Company) on cerebral vascular function with aging following both acute and chronic exposure. It was hypothesized that older individuals would exhibit both a reduced CBF and CVMR compared with the young at baseline, both of these measures would be acutely restored following flavanol ingestion in the old, and they would also be increased following chronic flavanol exposure in older individuals. Transcranial Doppler was used to assess baseline CBF and CBF response to hypercapnia (CVMR), achieved via a rebreathing technique. Cerebral vascular conductance index (CVCi) was calculated as the ratio of CBF velocity to MAP. For the acute portion of the study, 10 young (ages 19-28) and 10 older individuals (ages 65-77) were tested on two separate days. They were provided with either a nutrient-matched placebo or a flavanol-containing test drink (randomized order,

double-blinded) and CBF and CVMR were assessed pre-ingestion and 2.5 hours post-ingestion. For the chronic portion of the study, 6 older individuals (ages 65-71) were recruited. CBF and CVMR were assessed at baseline and then following 28 days of flavanol consumption. As expected, prior to flavanol ingestion older individuals demonstrated a reduced CVCi compared with the young (0.85 ± 0.04 cm/s*mmHg versus 0.55 ± 0.04 cm/s*mmHg p=0.001) along with a reduced CVCi-$CVMR_{max}$ (8.6 ± 0.6 versus 6.9 ± 0.4, p=0.05). This supports the premise of impaired endothelial function in the older individuals. Acutely following flavanol ingestion there was a significant decrease in CVCi (0.71 ± 0.04 cm/s*mmHg versus 0.62 ± 0.04 cm/s*mmHg p<0.05) and CVCi-$CVMR_{max}$ (8.6 ± 0.6 versus 6.1 ± 0.5, p=0.001). This unexpected effect was seen in both young and older individuals and requires further investigation. There was a significant increase in CVCi following chronic exposure to flavanols in older individuals (baseline, 0.60 ± 0.05 cm/s*mmHg versus flavanols, 0.72 ± 0.06 cm/s*mmHg p<0.05) suggesting an increase in basal NO bioavailability. However, there was no effect of chronic flavanol ingestion on CVMR in older individuals. Though further mechanistic studies are required, these data provide preliminary evidence for cerebral vascular health benefits associated with chronic flavanol ingestion.

INTRODUCTION

Cardiovascular disease (CVD) is currently the number one cause of mortality in the US and its incidence increases significantly with age in both males and females (167). Taking into account only coronary heart disease, hypertension and stroke, the estimated direct and indirect costs associated with the treatment of these diseases were already about 300 billion dollars annually in 2008 (167). As aging is one of the primary risk

factors for CVD it is of considerable concern that that the number of individuals over 65 years of age is projected to go from accounting for 13% of the overall population in 2010 to 20% of a much greater population in 2050 (212).

One of the underlying pathologies of CVD is atherosclerosis. Arterial endothelial dysfunction is believed to be one of the primary events in the development of atherosclerosis as the endothelium plays an integral role in maintaining vascular homeostasis (80). Endothelial dysfunction has been shown to be associated with atherosclerotic risk factors (38, 213) and is believed to be a predictor of adverse CV events (80, 197). This dysfunction is believed to be due to a decreased bioavailability of nitric oxide (NO) as it is the release of NO that mediates many of the protective functions exerted by an intact endothelium (152). In parallel with the increased risk of CVD, aging is also associated with a progressive decline in endothelial function that is evident by a reduced bioavailability of NO (39, 155). This impairment extends to the cerebral circulation; NO has been shown to be involved in CBF tone (99, 102, 116) and has been shown to be reduced with aging (13, 163, 168). Specifically, there is evidence that endothelial dysfunction results in impaired vasodilation in the cerebral vasculature thereby leading to dysfunction in CBF regulation (117, 185, 211). Thus, endothelium-dependent vasomotor function in the cerebral circulation, CBF response to hypercapnia (CVMR), reflects the integrity of the endothelial layer and can be used as a surrogate of NO bioavailability.

There is compelling evidence that environmental factors such as nutrition are key modulators to the progression of CVD (156). The endothelium constitutes the predominant tissue to which nutrient molecules are exposed following digestion and absorption and thus it represents a potential site of action for ingested compounds. Meta-analyses have shown that increased consumption of plant-based foods decreases risk of

CVD, including stroke (52). Health benefit is not believed to be due solely to vitamins and minerals, but also to the presence of bioactive compounds called polyphenols; small, nonessential molecules that demonstrate disease-preventative properties. Over the past decade flavanols, one of the more prevalent family of polyphenols, have become the focus of intense research. Flavanols are found in a high concentration in certain fruits and in foods such as green tea, red wine, soy, and cocoa products (154). Numerous studies have shown that flavanols increase NO bioavailability; in conduit arteries as assessed by flow mediated dilation (12, 87, 90, 147) and, in a smaller number of studies, in the microcirculation as assessed by peripheral arterial tonometry (67, 180). There is evidence to suggest that the underlying mechanism accounting for the increase in NO bioavailability may differ between acute and chronic consumption of flavanols.

Consequently, we investigated both the acute and chronic effects of flavanol ingestion on basal CBF and CVMR. The acute portion of the study involved young and older individuals and the chronic portion included only an older cohort. A number of studies have demonstrated an improvement in endothelial function associated with flavanol ingestion; however, whether or not endothelial function can be restored in the cerebral circulation following flavanol ingestion remains unknown. We hypothesized that the older individuals would have a reduced basal CBF and an impaired CVMR at baseline compared with the young but that they would both be acutely restored to the level of the young following flavanol ingestion. In addition, we hypothesized that these impairments would be improved at baseline following chronic flavanol consumption in the older individuals.

METHODS

Subjects. For the acute portion of the study, 10 young (ages 19-28; 5 men) and 10 older (ages 65-77; 5 men) individuals were recruited to participate (see Table 2.1 for subject characteristics). Each visited the laboratory for testing on 2 separate days. In order to control dietary intake of flavanols prior to testing subjects were asked to do the following: 1) avoid flavanol-rich foods including chocolate/cocoa products, green tea, red wine and most fruits in the 3 days prior to the study and 2) document all food intake on the day prior to their testing day so they could consume a similar diet prior to day 2 of testing (157, 158). Subjects came into the laboratory at approximately the same time for their 2 testing days following a 12 hour fast, having refrained from caffeine and exercise for 24 hours. None of the young women were on birth control and all were tested during the early follicular phase of their menstrual cycle (41). All of the older women were postmenopausal. All subjects were healthy, nonsmokers with no diagnosed cardiovascular disease. None of the younger subjects were taking medications; however, 7 of the older subjects were on medications and they refrained from taking them on the morning of each of their testing days.

For the chronic portion of the study, 6 older individuals (ages 65-71; 1 man) were recruited, 4 of whom were also part of the acute portion of the study. Subjects were provided with enough drink powder to consume a test drink at home for 28 days. They were not asked to follow any specific dietary restrictions during that time; however, they were asked to consume the test drink at approximately the same time each day (morning, afternoon or evening). They were also asked to return to the laboratory with empty canisters and unused product. Subjects came in for testing within 3 days of consuming their 28^{th} drink, following a 12 hour fast, having refrained from caffeine and exercise for 24 hours.

Prior to its commencement, the consent form and study procedures were approved by the Institutional Review Board at the University of Texas at Austin.

Instrumentation. Upon arrival in the laboratory all study procedures and risks were explained, questions were answered and then all participants provided informed consent. On each study day, height and weight were assessed using a medical–grade seca769 scale (seca corp., CA, USA). Subjects were then asked to lie semi-recumbent on the bed and a fasting blood sample was drawn from the antecubital fossa so that glucose and lipids could be assessed. Following the blood draw, subjects were instrumented with 5 electrodes. Both cardiac rhythm and heart rate were continuously monitored throughout the experimental protocol from an electrocardiogram on a patient monitor (GE DASH 4000, General Health Care). A blood pressure cuff was placed on the left arm and intermittent blood pressure measurements were obtained by auscultation of the brachial artery via electrosphygmomanometry (Tango+; SunTech, Raleigh, NC). Mean arterial blood pressure (MAP) was calculated as one-third pulse pressure plus diastolic blood pressure. A finger blood pressure cuff was placed on the right hand, inflated and calibrated allowing for the assessment of continuous beat-by-beat blood pressure using the Penaz method (NIBP100D, BIOPAc Systems). A 2-MHz Doppler probe (Multi-flow, DWL Elektronische Systeme, Singen, Germany) was adjusted over the temporal window of the right middle cerebral artery (MCA). The placement of the Doppler probe allowed for the assessment of the MCA mean blood velocity, which will be denoted as cerebral blood flow velocity (CBFV) for the remainder of the document. Once an optimal signal was identified the probe was secured to a specially designed head set so it would remain stable throughout the rebreathing protocol. Following a minimum of 20 minutes of rest, baseline systemic and cerebral hemodynamic measures were assessed in all subjects.

Rebreathing Protocol. Following the collection of baseline measures, subjects were fitted with a nose clip and were asked to put in a mouthpiece attached to a specialized 3-way valve joined to a rubber bag (2100 series, Hans Rudolph, Kansas City, MO). Manipulation of the valve directed whether the subject breathed room air or their own expired air from the bag. A sampling line attached to this valve assembly allowed for the continuous assessment of PET_{CO2} using a capnograph (VitalCap Capnograph Monitor, Oridion, Needham, MA). Subjects were instructed to remain relaxed while breathing room air through the mouthpiece for about 2 minutes at which point they were asked to take in a larger breath and, prior to expiration, the valve was switched allowing them access to only their own expired air for subsequent breathing. The rebreathing procedure continued until there had been a minimum of 15 mmHg change in PET_{CO2}, about 2-3 minutes. During the rebreathing protocol, oxygen was continuously supplied (amount calculated based on height, weight and age) and saturation was continually monitored to ensure Pa_{O2} remained unaffected (83). All testing was conducted in a temperature controlled room (21-24°C).

Acute Effects of Flavanol Ingestion. At the end of the baseline rebreathing protocol, all of the instrumentation was removed from the subject except for the ECG leads and the brachial blood pressure cuff. As this was a placebo-controlled, double-blinded study subjects were then provided a test drink, either flavanol-containing (~528mg) or a placebo, by a third party (same nutritional composition as study 1). Drinks were provided in powdered form by The Hershey Co., PA, USA, so they were reconstituted with 8oz of warm, purified water just prior to consumption as per instructions. All subjects consumed the beverage within 10 minutes and a timer was started immediately upon completion.

Subjects were asked to remain resting in the semi-recumbent position after consuming the test drink.

Two hours and twenty minutes following ingestion of the test drink, subjects were re-instrumented with the finger blood pressure cuff. An optimal CBFV signal was once again identified with the Doppler probe and the probe was then secured to the headset. This timing was strictly adhered to because plasma flavanols and associated metabolites have been shown to peak 2 hours following consumption, and physiological responses to flavanol ingestion have been shown to remain maximal for 2-3 hours post-ingestion (12, 87, 180). The rebreathing protocol was repeated as outlined above.

Young male and older subjects were scheduled for their second day of testing after allowing for a minimum of 3 days 'washout.' This length of time has been used in other similar studies (12, 147, 180). Young females had to wait for their menstrual cycle to begin the following month. For day 2 the experimental protocol was identical to the first study day, with the only difference being that the test drink administered was the one not given on day 1. The order in which these drinks were provided was randomized.

Chronic Effects of Flavanol Ingestion. Subjects came in for testing within 3 days of consuming their 28th drink. There was a minimum of 2 weeks between the interventions to avoid potential carry-over effects (88, 157). All instrumentation along with the rebreathing protocol was identical to the acute portion of the study. However, during these study days the testing measures were done only once while the subject was fasted as there was no additional test drink provided.

Blood Analysis. Glucose and lipids were assayed simultaneously using the Cholestech LDX analyzer (Inverness Medical, Biosite Inc, CA, USA) within 30 minutes of collection

into a heparinized vacutainer tube. This point of care analyzer has been validated against a hospital reference laboratory (34).

Data Analysis. Analogue output signals from cardiac rhythm, heart rate, continuous blood pressure measures and the ultrasound probe were converted, via a data acquisition system, to a digital signal allowing for the continual monitoring of these signals throughout the testing protocol (Biopac System, Santa Barbara, CA). Data was then saved on a computer for subsequent analysis.

Blood pressures taken at the brachial artery are the reported baseline measures. The continuous blood pressure measurements taken at the finger allowed for the breath-by-breath determination of MAP and the ratio of CBFV to MAP were then used to calculate cerebrovascular conductance index (CVCi). Baseline CBFV and CVCi measures were assessed by averaging over the final minute of resting prior to the start of the rebreathing protocol. CVMR, the response of CBFV (and CVCi) to breath-by-breath changes in PET_{CO2}, was assessed by plotting CBFV (or CVCi) as a function of PET_{CO2}. The resulting curvilinear relationship was analyzed using a four parameter logistic function as outlined by Claassen *et al.* (44). This allowed for the quantification of model parameters that are suggested to have physiological implications: *a* the range in change of CBFV (or CVCi), *y0* the maximum of CBFV (or CVCi) achieved during hypercapnia, *x0* the level of PET_{CO2} when the slope of the curve is maximal and *b* the curvilinear properties of the line of best fit. Additionally, the first order derivative of the logistic function used for the curve fitting generated a bell-shaped curve and the peak of that curve represented CBFV-$CVMR_{max}$ (or CVCi-$CVMR_{max}$). Nonlinear curve fitting was performed using Simplex & Levenberg-Marquardt algorithms (Model & Analyze, VUV Analytics). Nonlinear curve

fitting was chosen over linear regression as it has been shown to be a better model of the physiological response of CBFV (or CVCi) to hypercapnia (44).

Statistical Analysis. All data are presented as means ± SEM unless otherwise stated. A paired t-test showed no significant differences in systemic and cerebral hemodynamic variables obtained under fasting conditions during rest on each of the study days so these data were averaged and represent baseline values. Differences between young and older individuals were then compared using unpaired t-tests and are presented in Tables 2.1 and 2.2. The acute effect of flavanols on systemic and hemodynamic variables were assessed using a multi-factorial, repeated measures ANOVA with 1 between subject factor (age) and 2 within subject factors (treatment and time). The chronic effect of flavanols on all variables was assessed using a repeated measures ANOVA with treatment as the main effect. In all cases, when a significant interaction was observed, a Bonferroni adjustment for multiple comparisons was used to identify significant mean differences in the applicable pair wise comparisons (SPSS, version 19.0; IBM, New York, NY). The level of significance was set a priori at $p<0.05$.

RESULTS

Baseline Characteristics. Subject characteristics at baseline are presented in Table 2.1 while Table 2.2 lists the baseline values for CBFV and CVCi as well as for model parameters obtained during the rebreathing protocol. Baseline systolic and MAP were significantly higher in the older group ($p<0.01$) as were total cholesterol and diastolic blood pressure ($p<0.05$). The older group had a significantly lower CBFV and CVCi at baseline compared with younger individuals ($p=0.001$ for both). In response to the

baseline rebreathing protocol, older subjects also demonstrated more of a linear response (both CBFV-b and CVCi-b were decreased, $p<0.05$) and a reduced CVMR (CVCi-$CVMR_{max}$, $p=0.05$).

Acute Effects of Flavanol Ingestion. There was no significant difference in BMI between young and older individuals at baseline or between study days. There was also no significant difference in any systemic hemodynamic variables (HR, SBP, DBP, and MAP) following the acute ingestion of flavanols. Figure 2.1 demonstrates there was a significant decrease in CBFV acutely following flavanol ingestion (62 ± 3 versus 54 ± 3 cm/s, $p<0.01$) that was not present following ingestion of the placebo (60 ± 3 versus 59 ± 3 cm/s, $p>0.05$). The same response was seen with CVCi; a significant decrease acutely following flavanol ingestion (0.71 ± 0.04 versus 0.630 ± 0.04 cm/s*mmHg, $p<0.05$) but not the placebo (0.69 ± 0.04 versus 0.66 ± 0.04 cm/s*mmHg, $p>0.05$). This effect was not different between young and older individuals. The treatment*time interaction was only trending towards significance with CVCi, $p=0.15$, compared with CBFV, $p=0.05$; however, we felt the investigation of this former pair wise comparison was warranted due to the significance of CBFV.

Figure 2.2 demonstrates there was also a significant decrease in CBFV-$CVMR_{max}$ acutely following flavanol ingestion (9.1 ± 0.6 versus 7.0 ± 0.6, $p<0.01$) but not ingestion of the placebo (7.5 ± 0.5 versus 7.7 ± 0.5, $p>0.05$). Once again, the same response was seen with CVCi-$CVMR_{max}$ (flavanol: 8.6 ± 0.6 versus 6.1 ± 0.5, $p=0.001$ compared with placebo: 6.9 ± 0.5 versus 7.1 ± 0.6, $p>0.05$). With both these measures there was a significant treatment*time interaction, $p<0.05$, prior to investigation of these pair wise comparisons. Further investigation of this interaction revealed there was also a significant difference in the pre-ingestion measures on the 2 study days ($p<0.05$). The R^2 value was

not significantly different between any of these comparisons nor was the absolute range of PET_{CO2}. Once again, this effect was not different between young and older individuals.

Chronic Effect of Flavanol Ingestion. Baseline subject characteristics, blood analytes and systemic hemodynamic variables are presented in Table 2.3. There was no effect of chronic flavanol ingestion on any of the blood analytes or hemodynamic variables. However, there was a significant increase in CBFV following chronic flavanol ingestion (58 ± 2 versus 68 ± 3 cm/s, $p<0.01$) that was not present following chronic ingestion of the placebo (58 ± 2 versus 54 ± 3 cm/s, $p>0.05$).

Figure 2.3 demonstrates the same relationship in CVCi (flavanol: 0.60 ± 0.05 versus 0.72 ± 0.06 cm/s/mmHg, $p<0.05$ compared with placebo: 0.60 ± 0.05 versus 0.57 ± 0.05 cm/s/mmHg, $p>0.05$). Figure 2.4 shows the individual and group averaged CVCi responses of each of the 6 subjects at baseline and then following chronic ingestion of flavanols. There were no significant differences in any of the modified rebreathing parameters including the calculated $CVMR_{max}$ following chronic ingestion of flavanols.

DISCUSSION

The significant findings of this study were as follows: 1) at baseline older individuals demonstrated a reduced CBF and CVMR compared with the young, supporting the initial premise of endothelial dysfunction in the older individuals, 2) consumption of flavanols led to an apparent acute decrease in both CBF and CVMR in both groups, and 3) chronic exposure of flavanols led to an increase in CBF in older individuals but had no effect on CVMR.

Baseline Differences in Young and Older Individuals. As has previously been demonstrated (13, 130, 163, 168, 202), older individuals had a significantly lower CBFV and CVCi at baseline compared with younger individuals, along with a decreased CVMR. It has been shown that NO contributes to baseline CBF tone and is also involved in CVMR (99, 102, 116) so this supports the initial premise of impaired endothelial function in older individuals.

The sigmoidal curve relationship between hypercapnia and CBF indicates that CBFV increases steadily along with PET_{CO2} until it begins to level off at high levels of PET_{CO2}. In this protocol, older individuals demonstrated a more linear response (*b* model parameter was significantly lower) indicating they were less likely to plateau within the range of PET_{CO2} attained in this study. Claassen *et al.* suggested 2 possible mechanisms for the plateau response to hypercapnia: there is a peak cerebral vasodilatory response to increasing Pa_{CO2} or the vasoconstrictive effects of increasing sympathetic output eventually overcome vasodilation (44). We would surmise that it is not peak response because we would expect the older individuals to have a reduced vasodilatory capacity as a result of a reduction in NO bioavailability. If that were true, they would plateau sooner in the protocol, and this was not the case. Increasing sympathetic output overcoming vasodilation would only be possible if the older individuals were experiencing less sympathetic outflow or having reduced tissue responsiveness during the rebreathing protocol than the young. However, sympathetic nerve activity was not assessed in this study. Further investigation of the physiology associated with the *b* model parameter is required to determine if one of these hypothesized mechanisms is playing a role.

Acute Effects of Flavanol Ingestion. One of the goals of this study was to examine the acute effects of flavanol ingestion on cerebral function and to determine if these cerebral

effects differed in young versus older individuals. An unexpected finding was that flavanol ingestion resulted in an acute, significant decrease in baseline CBFV and CVCi, and that this response was the same in the young and older individuals. Our hypothesis was that flavanols would cause vasodilation due to an increase in NO bioavailability. However, we hypothesized the vasodilatory response would be further downstream in the microvasculature.

In this study we used CBFV as a surrogate for CBF because we could not accurately measure MCA diameter. We made this assumption based on evidence from previous studies showing that MCA diameter does not change with changes in Pa_{CO2} within the range used in this study (77, 182). However, the effect of flavanols on cerebral hemodynamics has not yet been investigated so the possibility exists that flavanols were causing vasodilation of the MCA. An increase in MCA diameter would provide a plausible explanation for these results but requires further investigation. Flavanols have been shown to have a vasodilatory affect on the brachial artery, also a conduit artery, in a number of studies (12, 87, 147). The fact that the acute effect of flavanols on CBFV and CVCi was not different between young and older individuals suggests that the impairment between young and older individuals at baseline was not the same mechanism being acutely affected by flavanol ingestion. The explanation requires further investigation.

Also contrary to our hypothesis, there was a significant decrease in calculated $CVMR_{max}$ acutely following flavanol ingestion. Again, this effect was not different between the young and older individuals. The R^2 values ranged from 0.96 – 0.97 and were not different between treatments indicating that the fit to the nonlinear regression was not a factor in these results. The absolute range of PET_{CO2} that subjects experienced during their rebreathing protocol was also not different between treatments indicating that

their hypercapnic stimulus was equivalent. A potentially confounding variable was that the pre-ingestion values on each of the treatment days were different; significantly higher on the day the flavanol-containing test drink was provided when compared with the placebo day.

We believe that our observed decrease is $CVMR_{max}$ remains significant based on the strength of the study design. On each testing day post-ingestion measures were assessed in relation to pre-ingestion measures taken on the same day under the same conditions. This should account for any variability between the 2 intervention days. Furthermore, this study was placebo-controlled and the decrease from pre-ingestion values was not seen following the ingestion of the placebo.

A number of studies have shown that CVMR is impaired in individuals with endothelial dysfunction and/or vascular disease (117, 185, 211) so the present findings would suggest that flavanols were having an acute adverse effect. However, the preponderance of evidence regarding flavanols suggests an acute positive vascular benefit so we believe that this effect warrants further investigation.

Chronic Effects of Flavanol Ingestion. Older individuals demonstrated a significant increase in both CBFV and CVCi following 28 days of daily flavanol consumption. Though this is opposite the response seen acutely following flavanol ingestion it is not surprising as there is evidence to suggest that the underlying mechanism accounting for the increase in NO bioavailability differs between acute and chronic consumption of flavanols. This belief is supported by studies that demonstrate an increase in basal NO following long-term flavanol consumption that increases even further following an acute dose of flavanols (12, 67, 88). Acute vascular effects are postulated to be mediated through the inhibition of NADPH oxidase thereby limiting oxidative stress. Chronic

effects are believed to lead to increased levels of activated eNOS or decreased arginase activity within endothelial cells (177, 178). The chronic effect is believed to be an adaptive response to continuous high-flavanol exposure and therefore, mediated by changes in gene expression and protein synthesis or breakdown (194).

As previously stated, NO contributes to tonic cerebral vascular tone so it is possible that the observed increase at baseline is due to an increase in basal NO having an effect on the cerebral microvasculature as hypothesized. Determination of which of the possible pathways is involved is outside the scope of the current study and requires further mechanistic investigation.

There was no effect on any of the CVMR model parameters or calculated $CVMR_{max}$ in older individuals following chronic exposure to flavanols. However, if the effect on NO bioavailability was not as robust as that seen with baseline CBFV and CVCi then there is a possibility that we did not detect it due to the small sample size. Four subjects had to be eliminated from analysis due to poor signal quality.

Methodological Considerations. As previously stated, the current investigation used MCA mean blood velocity to assess cerebral blood flow. It is recognized that velocity is not flow; however, if the diameter of the insonated vessel does not change then a linear relationship exists between the two. Previous reports indicate that the diameter of large cerebral arteries such as the MCA do not change during an array of perturbations that include changes in Pa_{CO2} (77, 182). Furthermore, other methods have validated that changes in CBFV correspond to changes in CBF following vasodilatory stimuli (22, 51). However, the effect of flavanols on MCA diameter has not specifically been studied so it is possible that there was an effect on the diameter of the MCA following acute flavanol ingestion.

A second consideration is whether PET_{CO2} was an accurate surrogate for Pa_{CO2} such that changes in PET_{CO2} reflected changes in Pa_{CO2}. As it has previously been demonstrated that Pa_{CO2} and PET_{CO2} are similar over a range of hypercapnic stimuli and respiratory rates, we believe this to be a reasonable assumption (101).

Limitations. We recognize that the significant increase in CBFV and CVCi following the chronic ingestion of flavanols involved only a small number of subjects (n=6). However, we believe this result is strengthened by the fact that an increase was seen in all six individuals so it was not being skewed by only a single individual. Additionally, this response was not seen following 28 days of ingesting the placebo.

Conclusions / Perspectives. Our main finding was that chronic exposure to flavanols resulted in a significant increase in baseline cerebral hemodynamics in older individuals though there was no effect on cerebral reactivity. Further studies are required regarding the acute effects of flavanol ingestion on the cerebral vasculature. The results from this study contribute to current research by providing support for cerebral vascular health benefits associated with chronic exposure to flavanols in older individuals.

TABLES AND FIGURES

Table 2.1

Baseline Subject Characteristics

	Young	Old
Sex, M/F	5/5	5/5
Age (yrs)	23 ± 3	68 ± 5
Height (m)	1.7 ± 0.1	1.7 ± 0.1
Weight (kg)	66.3 ± 14.9	68.1 ± 15.0
BMI (kg/m^2)	23.0 ± 3.0	23.6 ± 3.8
Glucose (mg/dL)	87 ± 4	90 ± 9
Total Cholesterol (mg/dL)	167 ± 32	201 ± 26*
Triglycerides (mg/dL)	71 ± 25	78 ± 31
LDL (mg/dL)	94 ± 25	119 ± 29
HDL (mg/dL)	58 ± 11	67 ± 21
Heart Rate (bpm)	65 ± 9	69 ± 17
SBP (mmHg)	111 ± 10	124 ± 6#
DBP (mmHg)	70 ± 8	78 ± 9*
MAP (mmHg)	84 ± 8	93 ± 6#

Values are means ± SD, n=10 per group. M, male; F, female; BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure. *$p<0.05$ and #$p<0.01$ compared with young.

Table 2.2

Baseline Cerebral Hemodynamics and Rebreathing Model Parameters

	Young	Old
CBFV (cm/s)	72 ± 11	50 ± 14#
CVCi (cm/s/mmHg)	0.85 ± 0.13	0.55 ± 0.15#
Baseline CVMR Model Parameters		
CBFV-a	78 ± 27	83 ± 24
CBFV-b	0.55 ± 0.16	0.40 ± 0.12*
CBFV-x0	47 ± 3	48 ± 4
CBFV-y0	62 ± 24	73 ± 25
CBFV-$CVMR_{max}$	8.8± 1.7	7.8 ± 1.4
CVCi-a	58 ± 20	62 ± 20
CVCi-b	0.67 ±0.20	0.48 ± 0.14*
CVCi-x0	46 ± 2	47 ± 4
CVCi-y0	49 ± 21	55 ± 21
CVCi-$CVMR_{max}$	8.6 ± 2.2	6.9 ± 1.4*

Values are means ± SD, n=10 per group. CBFV, cerebral blood flow velocity; CVCi, cerebral vascular conductance index; CVMR, cerebral vasomotor reactivity; *a* , range of change in CBFV or CVCi; *b* , curvilinear properties of the fitted curve; *x0* , end-tidal CO_2 value that exhibits highest slope; *y0* , maximum value of CBFV or CVCi; CVMRmax, when CBFV or CVCi becomes maximal. *p<0.05 and #p<0.01 compared with young.

Table 2.3

Effect of Chronic Ingestion of Flavanols

	Baseline	Placebo	Flavanol
Sex, M/F	1/5		
Age (yrs)	68 ± 3		
BMI (kg/m^2)	23.7 ± 2.7	23.1 ± 2.8	23.0 ± 2.8
Glucose (mg/dL)	88 ± 7	84 ± 4	82 ± 6
Total Cholesterol (mg/dL)	190 ± 20	186 ± 45	198 ± 29
Triglycerides (mg/dL)	61 ± 27	64 ± 20	65 ± 23
LDL (mg/dL)	102 ± 16	103 ± 30	111 ± 21
HDL (mg/dL)	77 ± 17	70 ± 20	75 ± 18
Heart Rate (bpm)	65 ± 7	67 ± 13	67 ± 15
SBP (mmHg)	131 ± 9	134 ± 8	124 ± 12
DBP (mmHg)	84 ± 8	82 ± 11	82 ±13
MAP (mmHg)	100 ± 8	99 ± 10	96 ± 12

Values are means ± SD, n=6. M, male; F, female; BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure.

Figure 2.1

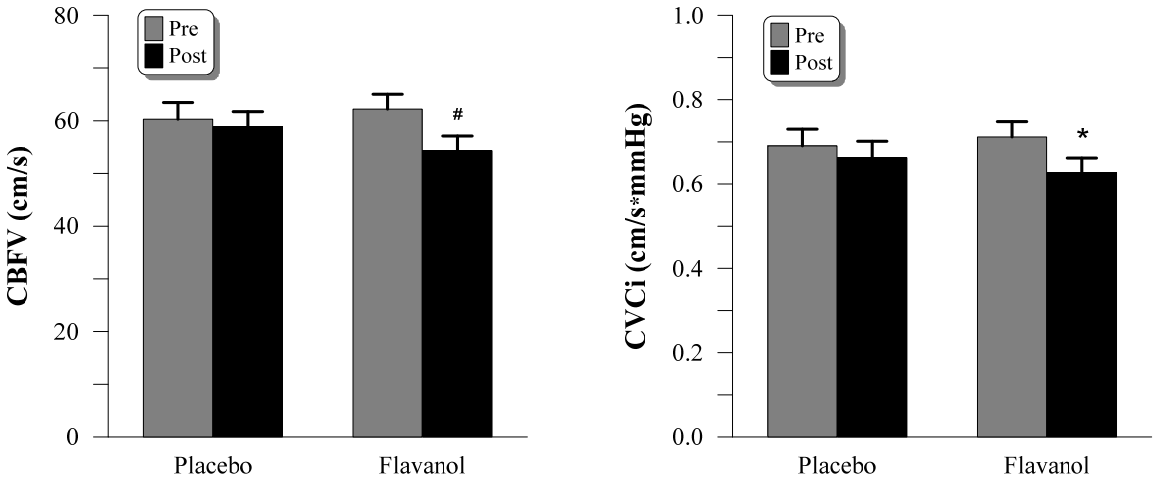

Figure 2.1: A summary of the acute effects of treatment on CBFV and CVCi. Measurements were taken 2.5 hours following ingestion of either placebo or flavanol-containing test drink while subjects were breathing room air. Young and old individuals showed the same response. *$p<0.05$, #$p<0.01$ compared with pre in same treatment. Values are means ± SEM.

Figure 2.2

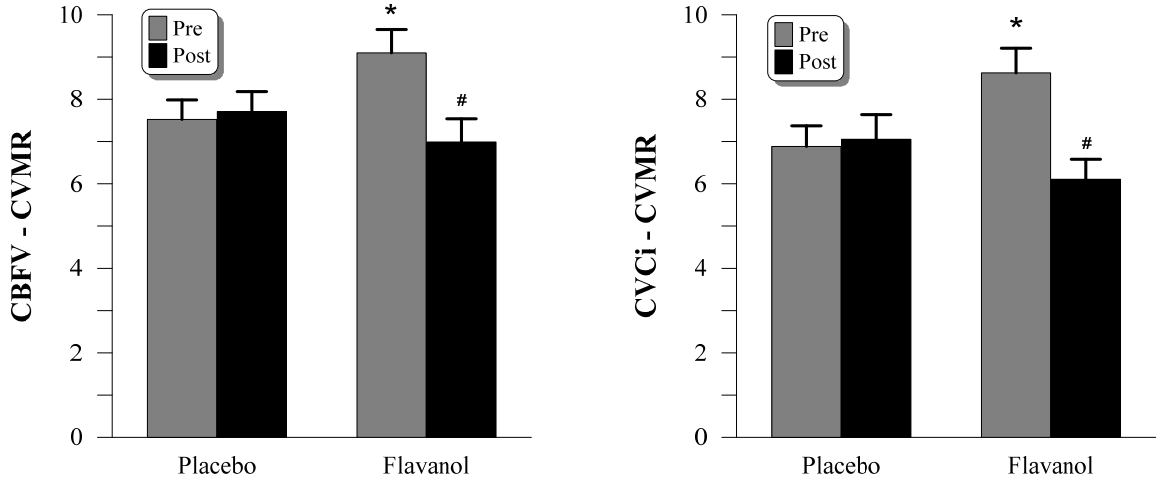

Figure 2.2: A summary of the acute effects of treatment on cerebral reactivity in response to the rebreathing protocol. Reactivity was assessed 2.5 hours following ingestion of either placebo or flavanol-containing test drink. Young and old individuals showed the same response. $^{*}p<0.05$ compared with placebo pre, $^{\#}p<0.01$ compared with pre in same treatment. Values are means ± SEM.

Figure 2.3

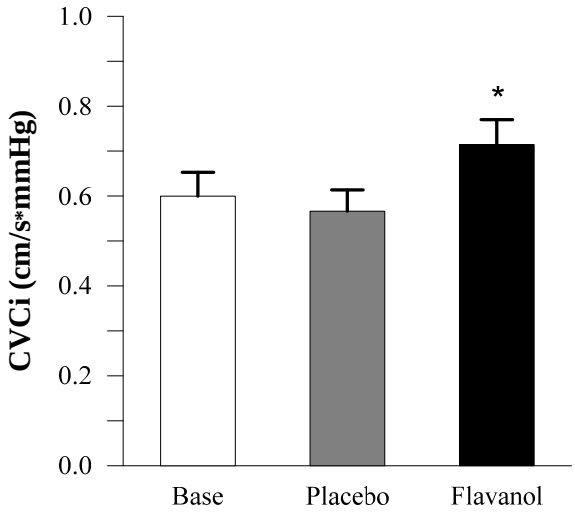

Figure 2.3: A summary of the chronic effects of treatment on cerebral blood flow in older individuals. Measurements were taken after daily consumption of either a placebo or a flavanol-containing test drink for 28 days. Subjects were breathing room air.*$p<0.05$ compared with baseline and the placebo. Values are means ± SEM.

Figure 2.4

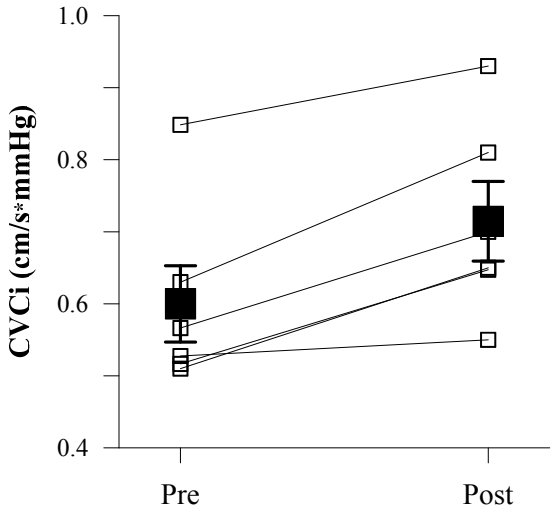

Figure 2.4: Individual cerebral blood flow responses following the chronic ingestion of flavanols. All 6 subjects showed an increase in CVCi from baseline. Hollow squares represent individual pre & post values and filled-in squares represent means ± SEM.

Chapter VI: Review of the Literature

PATHOPHYSIOLOGY OF CVD

The term CVD encompasses a number of different vascular diseases including hypertension, coronary heart disease, stroke, peripheral artery disease and diseases of the veins; all of which share a common underlying pathology, atherosclerosis. Atherosclerosis results from the complex interplay between structural elements of the arterial wall, circulating cells such as platelets & leukocytes, along with inflammatory cells such as monocytes/macrophages and can best be described, collectively, as an inflammatory disease (169). Central to this pathology is the vascular endothelium as it is the interface between blood and its elements and the underlying arterial wall. Histologically, there are 3 layers to the arterial wall and, beginning from the lumen, they are as follows: 1) an intimal layer that is composed of a single layer of squamous epithelial cells and is termed the endothelium, 2) a medial layer that consists mainly of circumferentially arranged layers of smooth muscle cells contained within internal and external elastic membranes, and 3) an adventitial layer that is mainly composed of longitudinally arranged collagenous tissue that eventually merges with the loose connective tissue that surrounds the vessel. With regards to CVD our focus will be on the innermost layer, the endothelium. This layer was initially believed to serve primarily as a physical barrier separating blood and its contents from underlying tissue; however, it is now well understood that endothelial cells are crucial to vascular health in that they maintain vascular homeostasis by secreting a number of vasoactive mediators in response to a very dynamic environment. With regards to atherosclerosis, it is a disruption in the functioning of the endothelium that is the initiating pathology.

It is important to note that under normal circumstances the atherosclerotic process is protective in that it follows an insult to the endothelium and/or smooth muscle cells located in the layer below so that repair is possible. However, in instances of repeated vascular insult there is a chronic, excessive, inflammatory-fibro-proliferative response that has the potential to lead to an occlusive, atherosclerotic lesion. Several clear phases have been identified in the development of an atherosclerotic plaque (152, 170).

1) Endothelial dysfunction: The primary event in the development of atherosclerosis is a disruption in endothelial function at an injury site. The most susceptible sites are areas near branch points in the arterial tree. Initial dysfunction results in the trapping of lipoproteins in the medial layer of the vessel wall and the expression of docking proteins on the endothelial cells apical membrane; changes that are under the influence of chemokines released by the altered endothelium and by other adherent leukocytes. Once these leukocytes have adhered to the docking proteins they then migrate between and across endothelial cells and gain access to the medial layer below.

2) Inflammatory Phase: The leukocytes that have gained entry migrate further beneath the arterial surface and the monocytes transform into macrophages as a result of more locally-released mediators. The macrophages then accumulate lipid, become foam cells, and together with accompanying lymphocytes, present as a 'fatty streak.' There is evidence that oxidized low-density lipoprotein (oxLDL) is fundamental to the atherosclerotic process: it is able to directly cause injury to the endothelium, it plays a role in the initial adherence and migration of both monocytes and T-lymphocytes, it plays a role in the transformation of monocytes to macrophages, and its uptake by macrophages leads to foam cell formation and the fatty streak (129, 195). The fatty streak is the earliest recognizable lesion of atherosclerosis and the ubiquity of this atherosclerotic process is supported by the finding that fatty streaks exist in the coronary arteries of 65% of autopsy

specimens from children 12-14 years old (135, 190). Continued leukocyte influx and proliferation lead to more advanced fibrous lesions that are distinguished by multiple layers of foam cells and smooth muscle cells.

3) Reparative Phase: The formation of fibrous lesions is a normal physiological response to injury; wound healing follows the same course. However, in atherosclerosis the cause of the injury, which is often lifestyle related, is most often chronic in nature. Chronic insult to this inflamed, focal site in the vessel by life style factors such as hypercholesterolemia, hypertension, smoking, obesity, and diabetes allows this process to propagate and become pathological. This persistent inflammatory condition has a number of deleterious vascular consequences. As smooth muscle cells lose their contractile properties and undergo change to a more fibroblastic phenotype, a fibrous cap forms over the injured area thereby providing some stability and this is collectively called plaque formation. However, though stable, potential consequences to this plaque formation include a thickening of the arterial wall, protrusion of the plaque into the arterial lumen where it can become restrictive to blood flow, and impaired diffusion of oxygen and nutrients to the tissue below.

4) Thrombotic Phase: Atherosclerotic plaques vary significantly in their final composition, particularly in the thickness and stability of the fibrous cap. Those with a thick cap often remain stable over time and may present with no clinical consequence providing they do not protrude into the lumen of the vessel and restrict blood flow. However, those with a thin cap are vulnerable to rupture (fully or partially). A ruptured fibrous cap has 2 possible sequelae: 1) leads to the exposure of collagen from within the vessel wall to blood elements and 2) leads to the leakage of pro-thrombotic proteins from within the atherosclerotic lesion out into the bloodstream. Either or both of these can then activate the coagulation cascade as well as platelets, resulting in the formation of a

thrombus. It is either a restriction in blood flow or formation of a clot that account for the clinical sequelae of atherosclerosis including angina, claudication, myocardial infarction, and/or stroke.

Endothelial dysfunction is the primary event in the pathology of atherosclerosis, a process that begins in childhood and continues throughout one's lifetime. However, the risk factors associated with progression to clinical manifestations are often related to lifestyle choices and thus investigations into ways to preserve or restore endothelial function could significantly and positively alter the pathophysiology of CVD.

ENDOTHELIAL FUNCTION AND THE ROLE OF NITRIC OXIDE

As previously stated, the endothelium is a major regulator of vascular homeostasis and does so by maintaining the balance between a number of physiological processes including: vasodilation vs. vasoconstriction, inhibition vs. stimulation of smooth muscle cell proliferation & leukocyte migration, and thrombogenesis vs. fibrinolysis. Accordingly, endothelial dysfunction results in an upset in one or all of these balances and is considered an early marker for atherosclerosis as it precedes angiographic or ultrasonic evidence of plaque formation (80).

The Endothelium and Vasomotor Regulation: The maintenance of vascular tone comes from the ability of the endothelium to release a number of vasodilators and vasoconstrictors in response to signals from its environment (57, 120). Some of the more prevalent vasodilators include prostacyclin, bradykinin and endothelium-derived hyperpolarizing factor; however, the most significant vasodilator produced by the endothelium is nitric oxide (NO). Interplay and synergism among these vasodilators also contributes to an anti-atherogenic environment: inhibiting smooth muscle cell

proliferation and leukocyte migration and promoting fibrinolysis (120). The most important endothelium-derived vasoconstrictors are endothelin and angiotensin II. These vasoconstrictors contribute to a pro-atherogenic environment; they promote smooth muscle cell proliferation, leukocyte migration and thrombogenesis (120). Any disruption in the balance between these vasodilators and vasoconstrictors leads to a number of physiological consequences including increased endothelial permeability, platelet aggregation, leukocyte adhesion and generation of cytokines, all of these factors favor atherosclerotic plaque formation (as previously outlined).

Functions of NO: NO was originally discovered and named endothelium-derived relaxing factor (73) before being specifically identified as NO (100, 148, 159). Since then, extensive research surrounding this molecule has demonstrated that NO is the primary paracrine involved in endothelium-dependent vasodilation. Along with this vasodilatory role, other functions of NO include inhibiting platelet adherence & aggregation, inhibiting leukocyte adhesions & infiltration, and inhibiting the proliferation of vascular smooth muscle cells (75, 111, 137). Furthermore, NO prevents the oxidative modification of LDL (171). As previously stated, oxLDL is believed to be one of the primary mechanisms underlying the atherosclerotic process and plasma concentrations of oxLDL have been shown to positively correlate with clinical manifestations of atherosclerosis (63). This suggests that NO bioavailability has the potential to directly modulate plasma levels of oxLDL. As a result of its inhibitory role, impaired production or reduced bioavailability of NO promotes events such as vasoconstriction, platelet aggregation, smooth muscle cell proliferation, and leukocyte adhesion with the deleterious consequence of atherosclerotic plaque formation and its associated clinical consequences.

Production of NO: NO is formed by the reaction of nitric oxide synthase (NOS) on its substrate L-arginine thereby liberating both NO and L-citrulline (159). A number of

cofactors contribute to its production including tetrahydrobiopterin (BH_4), nicotinamide adenine dinucleotide phosphate (NADPH), heme, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) (19). There are a number of constitutive isoforms of NOS including endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). Our focus is on eNOS as it is the primary enzyme responsible for the generation of endothelial-derived NO (70). When inactive, eNOS is located within invaginations in the endothelial cell membrane termed caveolae where the protein caveolin-1 binds calmodulin (CaM), an essential allosteric activator of eNOS. Increases in intracellular Ca^{2+} activate CaM, which in turn dissociates from caveolin-1 and binds to eNOS, leading to its release/solubilization. Therefore, any increase in intracellular Ca^{2+} concentration via either transmembrane influx and/or release from intracellular storage sites activates eNOS and leads to the production of NO (19, 32, 186). Accordingly, activators of eNOS also include molecules such as bradykinin, acetylcholine, adenosine triphosphate (ATP) along with shear stress on the arterial wall.

NO is a highly lipophilic and diffusible gas so it passes readily from the endothelial cell where it is produced to the smooth muscle cells in the medial layer below. Once in the smooth muscle cell, NO activates the enzyme guanylyl cyclase, which then converts GTP to cGMP, thereby inducing relaxation of the smooth muscle cell. Due to the short half-life of NO (3-5 seconds), tight temporal and spatial regulation of its production is essential. Several highly organized layers of regulation exist and the control of eNOS gene expression and post-translational modifications of the enzyme are crucial to this regulation. Phosphorylation of amino acid residues of eNOS appears to be one of the most important post-translational regulatory mechanisms and two of the most important sites appears to be the phosphorylation of Ser^{1177} and Thr^{495} (55, 149). The former of these enhances eNOS activity while the latter seems to be a major negative

regulatory site as it interferes with the bonding of CaM to eNOS (68, 132, 138). The critical role played by eNOS in vascular homeostasis is apparent in the fact that it is a highly regulated enzyme and its activity directly influences the production of NO; therefore, any bioactive molecule that has the potential to modulate the activity of eNOS also has the potential to effect vascular health. It is important to note that bioavailability of NO represents the balance between net production and usage.

NO Bioavailability: The tight regulation of eNOS controls production of NO and then other factors have the potential to affect its bioavailability following production. Some of these factors include: excessive production of the superoxide anion (O_2^-), an increase in the intracellular concentration of the enzyme arginase and/or, an increase in ADMA.

The presence of O_2^-, the one-electron reduction product of oxygen, provides an alternative pathway for NO as the two will react, in a nearly diffusion controlled reaction, to form peroxynitrite ($NO + O_2^{\cdot -} \rightarrow ONOO^-$) (16). This reaction has two potentially harmful physiological consequences: the oxidization of cell components by peroxynitrite and a reduction in NO bioavailability (17). In this manner, production of O_2^- has the potential to modulate cellular, steady-state levels of NO. There are a variety of O_2^- sources in the body, but with reference to endothelial cells, NADPH oxidase (NOX) and eNOS dependent O_2^- production are particularly relevant (33). Oxidases are enzymes that catalyze oxidation reactions and under normal conditions, NOX generates O_2^- by catalyzing the transfer of electrons from NADPH to molecular oxygen ($NADPH + 2O_2 \leftrightarrow NADP^+ + 2O_2^- + H^+$). There are a number of NOX isoforms that contribute to a variety of physiological functions including immunity, posttranslational processing of proteins, cellular signaling, regulation of gene expression and cell differentiation (18). However, the fact that NOX has a prominent role in vascular physiology is apparent in the observations that a moderate level of NOX is essential for optimal angiogenesis and

wound healing (205) but does become a risk factor for endothelial dysfunction when this level is exceeded (35). A second postulated source of excessive O_2^- production within the endothelial cell is eNOS. eNOS functions as a reductase enzyme as it catalyzes the flavin-mediated transfer of electrons from NADPH to a prosthetic heme group. This reaction requires BH_4 as a cofactor in order to complete the electron transfer from the heme group to L-arginine thereby forming NO. In the absence of substrate or cofactor, eNOS becomes 'uncoupled' and produces O_2^- along with hydrogen peroxide instead (209). This has been supported by the observation that intra-arterial infusion of BH_4 has been shown to improve endothelium-dependent vasodilation in chronic smokers, suggesting that depletion of BH_4 may turn eNOS into an $O_2^{-\cdot}$-generating enzyme in humans (91). The exact mechanisms whereby eNOS becomes uncoupled in vivo remains unclear; however, recent studies have suggested that peroxynitrite itself can oxidize BH_4 and lead to eNOS uncoupling (139). This brings forth the hypothesis that excessive production of O_2^- leads to an increased formation of peroxynitrite, which then oxidizes BH_4 leading to the uncoupling of eNOS and further generation of O_2^-.

An increase in the intracellular concentration of the enzyme arginase has also been implicated in reducing NO bioavailability. Arginase plays a role in NO bioavailability because it shares substrate with eNOS. In mammals, there are two isoforms of arginase, arginase I and II. Arginase I is a cytosolic enzyme, expressed primarily in the liver that is responsible for the conversion of L-arginine to urea and L-ornithine thereby playing a role in ammonia detoxification. Arginase II is a mitochondrial enzyme, expressed primarily in the kidney, that plays a role in protein biosynthesis by supplying L-ornithine (216). Human endothelial cells express predominantly arginase II, which has been hypothesized to play a role in the endothelial dysfunction associated with atherosclerosis (11, 142). It has been shown that the availability of L-arginine can

represent a rate-limiting factor in cellular NO production in both animals and humans (47, 49, 58) thereby suggesting that an increase in arginase, and the resulting decrease in L-arginine, has the potential to indirectly reduce NO bioavailability. However, it is important to note that since these initial studies performed in the early 1990's, further research has revealed inconsistent results suggesting that the link between arginase, eNOS and their common substrate, L-arginine, requires further investigation (119).

An increase in asymmetric dimethylarginine (ADMA) has also been postulated to affect NO bioavailability as it is an endogenous competitive antagonist for the substrate, L-arginine (46). The production of NO is selectively inhibited by guanidine-substituted analogs of L-arginine, including L-NG-nitro arginine methyl ester (L-NAME), L-NG-monomethyl-L-arginine (L-NMMA), and ADMA as they act as competitive inhibitors at the active site of the NOS enzyme (162). The first two of these analogues, L-NAME and L-NMMA, have been used extensively in vascular research and are often administered to ascertain the role of NO in a variety of research techniques that assess endothelial-dependent vasodilation in an array of vessels. L-NMMA and ADMA occur endogenously as a result of the degradation of methylated proteins and following their formation they are either excreted without significant catabolism in urine (134) or they can be metabolized by the endothelium (122). Vallance *et al.* initially demonstrated that ADMA is present in a high enough concentration in plasma and urine to inhibit NOS in chronic renal failure patients and he suggested that it is the major endogenous NO inhibitor compared with L-NMMA (207). Since then, data from other studies demonstrate that ADMA may play a role in vascular pathology as it can elicit an inflammatory response in rats (113), it can inhibit vasodilation in rat & rabbit cerebral vessels that is prevented following the administration of L-arginine (65), and it can inhibit endothelium-dependent vasodilation via the inhibition of eNOS in human cerebral arteries (181). There is also

some evidence to suggest that ADMA is synthesized in human endothelial cells and is capable of uncoupling eNOS (9, 25) much the same as L-NMMA or LDL cholesterol (160).

One of the earliest signs of atherosclerosis, and an indicator of endothelial dysfunction, is decreased production and/or reduced bioavailability of NO hence NO plays a pivotal role in optimal functioning of the endothelium and in preserving vascular health over the lifetime. Excessive production of superoxide anion, increased levels of arginase, and/or increased ADMA have the potential to reduce NO bioavailability; therefore, any bioactive molecule that interferes with one or all of these factors have the potential to effect vascular health.

FLAVANOLS AND VASCULAR HEALTH

It is well established that diet plays a major role in the primary and secondary prevention of many chronic diseases, including those with impaired endothelial function as their underlying pathology. The endothelium constitutes the predominant tissue to which any molecule is exposed following digestion and absorption into the blood stream and thus represents a potential site of action for ingested compounds. Current dietary recommendations include the suggestion to consume more fruits and vegetables (98, 118) as there is significant evidence attesting to the negative association between their consumption and risk of CVD. It is now believed that much of this benefit comes from the polyphenols contained within these foods, of which flavonoids are a predominant group (6, 110). Numerous epidemiological and correlational studies have demonstrated the same inverse relationship between flavonoid ingestion and decreased risk of CVD (64, 143, 167). Initial in vitro experiments suggested that this negative association

between fruit and vegetable consumption and risk of vascular disease was due to the antioxidant capacity of these polyphenols (110). However, recent in vivo evidence suggests that a direct antioxidant action of flavonoids is unlikely since data from animal experiments and human interventional studies offer conflicting results (93). Though the underlying mechanism for their vascular benefit remains unclear, current evidence suggests they do exert their effects through the modulation of CVD risk factors: by improving endothelial function, inhibiting low-density lipoprotein oxidation, and/or by decreasing blood pressure as a result of their interaction with molecular signaling pathways and related machinery that regulates cellular processes (151).

Flavonoids consist of a large family of phytochemicals that are present in a number of 'healthy' plant-derived foods. These 'bioactive' compounds are extra-nutritional constituents that typically occur in small quantities in foods and confer health benefit. All flavonoids contain the same 3-ring carbon skeleton and then the important subfamilies: flavanols (fruits, cocoa, tea and wine), flavonols (onion, broccoli, tomato, tea), flavones (herbs), isoflavones (soybean), flavanones (citrus fruits and juices), and anthocyanidins (berries and wine) differ based on which functional groups are attached to the 'C-ring' of this 3-ring carbon backbone. The arrangement and type of these attached chemical residues dictates how each of these different molecules will be absorbed and metabolized by the body following ingestion. During digestion, the process of conjugation occurs mainly in the small intestine and the liver and, in the case of flavonoids, leads to circulating metabolites that are mainly glucuronidated, sulphated and/or methylated so some of the resulting metabolites that reach the blood and tissues are chemically distinct from their parent compounds (64, 125). Accordingly, these different subfamilies give rise to different metabolic plasma profiles following digestion and; therefore, have differing physiological effects that determine their effects on health

(64, 126). It is important to note that there is no evidence for the long-term accumulation of these water-soluble metabolites, even when consumed in high doses. This rapid clearance implies that repeated and regular consumption of these foods is required to sustain plasma levels and derive vascular benefit.

One of these flavonoid subfamilies, the flavanols, are defined by the presence of a hydroxyl group at position 3 of ring C and they are highly concentrated in foods such as fruits, red wine, green tea and cocoa products (154). Flavanols have received individual attention because they have demonstrated, independent of the others, to have cardio protective effects in epidemiological, observational and dietary intervention studies (7, 31, 56, 105). In particular, research into the health benefits of cocoa were initiated by observations involving the Kuna Indians of Panama. These individuals reside on the island of San Blas, just off the coast of Panama, and they have a very low overall incidence of CVD and hypertension, positive health advantages that disappear when these island dwellers move to the mainland. A noteworthy difference between the Kuna Indians that remain on the island compared with those that move inland is their flavanol-rich cocoa consumption; cocoa is grown on the island and is one of the main beverages consumed by the island dwellers (15, 133, 180).

Much the same as with flavonoids, studies involving flavanols have suggested that they work via the modulation of CV risk factors, including blood pressure (BP). The conclusions of a number of meta-analyses examining the effects of chocolate or cocoa on blood pressure have been that flavanol ingestion leads to a decrease in both diastolic and systolic blood pressure (164, 200). The underlying concept is that, sufficient NO bioavailability is associated with normal vasodilation and therefore normal BP; whereas, decreases in NO steady-state concentration can lead to a failure in smooth muscle relaxation and subsequent hypertension. A number of randomized, placebo-controlled

trials involving the ingestion of a flavanol-containing cocoa beverage have shown improvements in endothelial function; in conduit arteries as assessed by flow mediated dilation (FMD) (12, 87, 90, 147) and, in a smaller number of studies, in the microcirculation as assessed by peripheral arterial tonometry (PAT)(67, 180). These improvements in endothelial function were hypothesized to be the result of increased NO bioavailability as they were abolished by the inhibition of eNOS and/or plasma concentrations of flavanols and its metabolites were paralleled by an increase in plasma nitrosylated species (67, 90, 180).

Though the specific profile of flavanols in the different food sources varies considerably and is dependent on food processing practices, they mostly exist as mixtures of the monomers, epicatechin (EP) and catechin (C), and the oligomers (procyanidins). Following digestion, the parent compounds EP and C can be found in plasma and urine along with a variety of their sulphated, methylated, and glucuronidated metabolites (10, 153, 157, 158). The preponderance of evidence surrounding flavanols suggests that EP and its associated methylated metabolites are primarily responsible for the vascular benefits (12, 90, 161, 180, 193). Studies involving murine aortic endothelial cells have demonstrated that endothelial cells are capable of accumulating EP, possibly through binding to cellular proteins (179). This suggests that the concentration of EP that reaches the vascular endothelium after oral intake may be considerably higher than what is being measured in plasma and, under physiological conditions, protection by EP may outlast its presence in the plasma.

The exact mechanism by which these molecules affect NO bioavailability has not yet been fully elucidated. Much the same as with their parent family, the flavonoids, their beneficial effects were also initially believed to be due to their role as antioxidants, that they were able to scavenge free radicals that would otherwise react with NO and

reduce its bioavailability via the formation of peroxynitrite (as previously outlined). However, recent reviews and position papers are suggesting that the role of flavanols as antioxidants is actually minimal and their underlying mechanism may be via more indirect pathways. Hypothesized pathways include: by maintaining a high concentration of activated eNOS, by inhibiting NOX, and/or by decreasing arginase activity thereby preserving intracellular concentrations of L-arginine (71, 89, 177).

Activation / Preservation of eNOS: The first of these postulated pathways is the role of flavanols in maintaining a high concentration of intracellular, activated eNOS thereby rendering it available for the production of NO. Ramirez-Sanchez et al. demonstrated in human coronary artery endothelial cells (HCAECs) that treatment with EP activates eNOS via phosphorylation of both Ser^{1177} and Ser^{633} along with the dephosphorylation of Thr^{495}. These authors suggested that this provided evidence for the presence of a cell surface receptor for EP and could be a primary mediator in its cardiovascular effects (161). Steffen *et al.* demonstrated that exposure of endothelial cells to oxLDL caused proteasomal breakdown of the eNOS protein and this loss was prevented by a 16-hour pretreatment of the cells with EP. The eNOS protein was found to be particularly prone to protein carbonylation upon exposure to oxidative stress and the authors suggested that this carbonylation may serve as a signal for proteasomal degradation (192, 194).

Inhibition of NOX: Another postulated pathway for the vascular benefits associated with flavanol ingestion is via inhibition of NOX, thereby leading to a reduction in O_2^- production. Aortas from rats treated with EP-containing, de-alcoholated, red wine demonstrated increased endothelial-dependent vasodilation and increased NO production along with a decreased superoxide production (20). Steffen *et al.* demonstrated that pure EP was associated with increased NO levels in human umbilical vein endothelial cells (HUVEC's). These authors provided evidence that endothelial cells convert EP to its O-

methylated metabolites and demonstrated these metabolites to be potent inhibitors of NOX. He concluded that this inhibition led to an elevation in the NO steady-state level in those endothelial cells. This conclusion was strengthened by the fact that the NO increase was prevented via inhibition of the enzyme catechol-O-methyltransferase, the enzyme responsible for the O-methylation of EP (193). These O-methylated metabolites have previously been reported in the plasma after flavanol ingestion by humans and rats and are structurally similar to apocynin, a well-known NOX inhibitor (191, 193).

Inhibition of arginase: The final postulated indirect pathway for the vascular benefits associated with flavanol consumption involves the enzyme arginase. Schnorr et al. did a series of studies looking at the effects of flavanols on arginase in vitro and in vivo by using HUVEC's, rat kidney and human erythrocytes. These authors demonstrated that there was a reduced arginase II mRNA expression in HUVECs following their incubation with EP and some of its common metabolites. This decrease was detectable in 2 hours but much more pronounced in 24 hours, and led to a detectable decrease in actual arginase II protein activity in those cells at 48 hours. Furthermore, these effects were dose-dependent and low levels of basal arginase 1 were unaffected. These same authors also demonstrated a decreased, renal arginase II activity in rats that had been fed a high flavanol diet for 28 days and a decreased arginase activity in human erythrocytes 24 hours after the consumption of a high flavanol cocoa drink (178). The authors concluded that flavanols have the potential to indirectly influence NOS activity by increasing the availability of its substrate L-arginine.

Acute vs. Chronic: There is evidence to suggest that the underlying mechanism accounting for the increase in NO bioavailability may differ between acute and chronic exposure to flavanols. This belief is supported by studies that demonstrate an increase in basal NO following long-term flavanol consumption that increases even further following

an acute dose of flavanols (12, 67, 88). Acute vascular effects are postulated to be mediated through the inhibition of NOX, whereas, chronic effects are believed to be mediated by maintaining a higher intracellular level of activated eNOS or by decreasing arginase activity within endothelial cell (177, 178). This longer term effect may be regarded as adaptation to continuous high-flavanol exposure and is possibly mediated by changes in gene expression and protein synthesis or breakdown (194).

There is significant evidence to suggest that flavanols influence endothelial NO steady state levels. Investigation of their cellular actions will not only increase our understanding of the regulation of endothelial NO production but could provide valuable clues for prevention and/or treatment of CVD.

AGING AND ENDOTHELIAL DYSFUNCTION

Aging is associated with a progressive decline in basic physiologic function thereby leading to an increased risk of chronic diseases, including CVD, and their associated complications. However, age itself is not synonymous with disease as there are many people that achieve old age without evidence of disease, coined in the literature as 'successful aging.' So the question becomes, what are possible explanations for this discrepancy and can interventions increase the number of individuals that fall into the latter category, that of successful aging? Some of the hypotheses put forth to explain the increased prevalence of CVD with age include: 1) risk factors associated with CVD increase in number and become more severe over time, 2) increasing age contributes to an increased exposure time to these same risk factors and/or, 3) CV structure and function change over time as a result of the aging process and this alters the substrate on which these same risk factors interact rendering individuals more susceptible to their

deleterious consequences. This latter hypothesis suggests that the increased incidence of CVD may be due to interactions between the vascular aging process and specific aspects of vascular disease pathophysiology; suggesting an age-disease interaction that increases in strength over time with both lifestyle related risk factors and genetics acting as contributors (114). Though there is considerable ongoing research into the contribution that genetics play in the pathology of CVD, our focus will be on the involvement of lifestyle risk factors, specifically diet.

Aging of the CV system leads to a number of pathological changes in the vasculature that are believed to contribute to the atherosclerotic process. A sustained effort has been made over the past 2 decades to characterize the effects of aging on multiple aspects of CV structure and function in a single study population, the Baltimore Longitudinal Study on Aging (BLSA)(184). Results from the BLSA, along with other smaller-scale studies, have demonstrated that age-related structural vascular changes include: increases in luminal diameter, increased intimal media thickness (IMT), and increasing arterial stiffness along with impaired endothelial function (155). Our focus will be on impaired endothelial function as that is where, as outlined above, flavanols are believed to exert their positive vascular effects.

It is well established that endothelium-dependent vasodilation, and therefore NO bioavailability, declines progressively with age. This has been supported by several studies involving rats that have demonstrated impaired endothelial function in both conduit and resistance vessels (76, 97, 112, 150). This same dysfunction has been demonstrated in numerous studies involving humans in conduit vessels such as the coronary arteries (62, 213, 217) and the brachial artery (121, 198) and also in resistance vessels (121, 187). However, many of these human studies examined only small numbers of subjects and/or subjects with multiple CVD risk factors. In a landmark study

investigating a much larger and apparently healthy cohort, Celermajer *et al.* demonstrated that aging is associated with progressive endothelial dysfunction in 238 volunteers from 15 – 72 years of age with no known CVD risk factors. In addition, they demonstrated that endothelial dysfunction occurs earlier in men than woman, though it was present in almost all subjects by 65 years of age (39). All of these observations taken together suggest that this impairment in endothelial function is systemic in that it occurs in conduit and resistance vessels along with the microcirculation (2) and this is supported by evidence suggesting a reduction in overall, total body NO production with age (121). It is noteworthy that though atherosclerosis does not affect vessels of the microcirculation those vessels do demonstrate impaired endothelial function thus suggesting that this dysfunction may occur in the absence of atherosclerosis and/or may precede its development. Many of the aforementioned studies also demonstrated that endothelium-independent relaxation to sodium nitroprusside remains unaffected by aging thereby suggesting that the impairment involves NO and does not involve the smooth muscle cells located in the medial layer below.

There are a number of postulated mechanisms underlying this age-related reduction in the production and/or bioavailability of NO and they include: increased oxidative stress, reduced expression or activation of eNOS, upregulation of the enzyme arginase, and an increase in ADMA.

Increased Oxidative Stress: It is well established that aging is associated with increased oxidative stress, the consequence of which is modification to cellular proteins, lipids and DNA. This results in an increased production of harmful metabolic byproducts such as reactive oxygen species (ROS), one of which is O_2^- (66, 82). Intracellular levels of ROS are tightly regulated because, under normal physiological conditions, they are important second messenger signaling molecules. However, it has been observed in human aortic

endothelial cells that excessive generation of ROS can result in unregulated inflammatory signaling, including the oxidation of circulating LDL and increased expression of inflammatory cytokines such as monocyte chemotactic protein-1 and vascular adhesion molecule-1 (74). In addition, as previously outlined, intracellular concentration of NO will decrease as a result of its reaction with O_2^- and the formation of peroxynitrite.

There are a variety of postulated sources of the increased production of ROS with aging including: dysfunctional mitochondria (204, 215), the transformation of eNOS into a radical generating enzyme (115), and excess production by the enzyme NOX (28, 103). There is also evidence that the endothelium itself appears to be an important source of O_2^- in the vascular wall and the enzyme responsible is NOX (28, 79, 81, 103). This is further supported by the observation that the stimulation of dermal microvessel endothelial cells with angiotensin II, a potent inducer and activator of NOX, leads to DNA fragmentation (131).

Reduced Expression and/or Activation of eNOS: At the molecular level, as endothelial cells age they exhibit a reduction in the expression of eNOS thereby reducing the production of NO and this has been demonstrated in both the aorta and in aortic endothelial cells from aged rats (14, 50, 199, 201) as well as in human endothelial cells (92, 128). Also, an age-induced loss of eNOS phosphorylation has been demonstrated in the aorta from aged rats (188, 189). eNOS expression is more dramatically reduced in areas such as the aorta, where there is significant hemodynamic stress compared with those areas that have a high endothelial cell concentration but experience less hemodynamic stress, such as the lungs (40). This pattern of reduced eNOS expression/activation and the resulting decrease in NO mimic the normal distribution of atherosclerosis. Numerous mechanisms can modulate eNOS activity but one of the most potent inducers for its transcription is hemodynamic shear stress, the frictional force

acting on the endothelial cell surface as a result of blood flow (45, 123, 124) as it increases eNOS mRNA expression and stability (54). As vessels age they are exposed to reduced shear stress as a result of the reduction in cardiac output that accompanies aging and this flow-induced reduction in NO has been demonstrated in rat coronary arterioles (104). Though response to shear stress is the primary stimuli, there are other factors that have been shown to affect the activity and expression of eNOS and they include: hormones such as estrogen (109), growth factors (27), and hydrogen peroxide(59). The secretion of many of these factors decreases with age so there is speculation that this decline may also affect eNOS expression and NO production. Studies have been done looking at the effects of the replacement of these factors on plaque formation and endothelial function in rats (5), rabbits (4, 78, 84), and humans (26) but results remain inconclusive.

Upregulation of Arginase: Evidence suggests that NO bioavailability in aged skin may be decreased by an age-related upregulation of the enzyme arginase (61, 173). As previously stated increased levels of arginase reciprocally regulate NO bioavailability by pilfering substrate (21, 86, 214). The expression of arginase is increased with age in rabbits and in rats (172, 174) and inhibition of arginase in the aorta rings from aged rats improves endothelium-dependent relaxation (21). Furthermore, Holowatz *et al.* demonstrated that the age-related deficit in reflex cutaneous vasodilation in older humans can be restored by either arginase inhibition or L-arginine supplementation (95, 96).

Increase in ADMA: ADMA is emerging as a novel CVD risk factor (23). Traditional risk factors account for the majority of risk for coronary events but there are certain individuals, lacking in these traditional risk factors, that still present with premature coronary artery disease suggesting that other, less defined, factors may also play a role. Increased ADMA levels are associated with reduced NO production in

hypercholesterolemic and in hypertensive patients, as judged by reduced urinary nitrate excretion and/or impaired endothelium-dependent, NO-mediated forearm vasodilation (24, 196). Studies involving both animal models and humans have suggested that an increase in ADMA occurs prior to vascular disease and it increases with aging. Miyazaki *et al.* assessed ADMA in the plasma of 116 apparently healthy human subjects, 26-77 years old, who had no signs of vascular disease, and they found that ADMA levels were positively correlated with age (146). In another study by Valkonen *et al.* they showed that middle-aged men with ADMA plasma levels in the highest quartile had a 3.9-fold increased risk for acute coronary events compared with the other quartiles (206) suggesting that ADMA is a predictor of acute coronary events. Studies are suggesting that ADMA may only become damaging to the vasculature once its concentration exceeds some threshold, though a normal range has not been established.

Flavanols exert their positive vascular benefits via some of the same postulated mechanisms that are accounting for the age-related reduction in NO bioavailability, thereby suggesting that the potential, positive health benefits that could be gained with habitual flavanol consumption may become more significant with age. Furthermore, ADMA has recently emerged as a novel CVD risk factor and it has been shown to be increased with aging so further investigation interaction with bioactive molecules may have the potential to guide future, more mechanistic studies.

PHYSIOLOGICAL CONSEQUENCE TO REDUCED NO

Impaired endothelial function is believed to be the result of decreased production and/or bioavailability of NO. As previously stated, in conjunction with its vasodilatory affects, NO also inhibits vascular inflammation, thrombotic events and aberrant cell

proliferation. Therefore, this age-related reduction in the physiological effects of NO greatly influences the vasculature as a whole; it affects vessels on a cellular level, mediating numerous changes in endothelial cell structure and function. As a result of its prominent role in normal vascular physiology, this decrease in NO results in detrimental physiological consequences, specific to the aging process, two of which include 1) increased oxidative stress and 2) induction of endothelial cell premature senescence. It is noteworthy to appreciate that there is some uncertainty as to whether or not increased oxidative stress and/or premature senescence are aging-associated or vascular disease-associated phenomenon but it is becoming apparent that, regardless of their association, a reduced NO bioavailability is common to both.

Increased Oxidative Stress: It was previously outlined that the increased oxidative stress associated with aging causes a reduction in NO bioavailability. However; a reduction in NO bioavailability by any mechanism also has the potential to increase oxidative stress as NO is no longer available to oxidatively inactivate (via the formation of peroxynitrite) newly formed radicals.

Induction of Endothelial Cell Senescence: Though different processes such as endothelial injury, wound healing and/or angiogenesis can initiate endothelial cell proliferation, under normal conditions endothelial cells rarely divide, maybe every 3 years or so. As a result of their limited capacity for division, endothelial cells eventually enter a state of irreversible growth arrest, termed senescence (69). Evidence is suggesting that a reduction in NO may cause endothelial cells to enter premature senescence. During each normal cell division segments at the end of the chromosome, called telomeres, are lost as a result of limitations in the cell replication machinery. At some pre-defined telomere length the cell quits replicating; a defense mechanism against the loss of genes that code for relevant proteins. Normally, telomere shortening is minimized by the enzyme

telomerase as it functions to extend the end of the telomeres during each replication cycle. There is believed to be interaction between NO and telomerase such that NO activates telomerase thereby preventing the shortening of telomeres and, therefore, preventing premature exit from the cell cycle (208). The speculation is that endothelial dysfunction leads to a reduction in the bioavailability of NO, which then results in less protection by telomerase, accelerated shortening of telomeres and premature endothelial cell senescence. Senescent cells are metabolically active but display an altered gene and protein expression compared with proliferating cells. Specifically, they exhibit increased expression of inflammatory adhesion molecules and decreased NO production thereby perpetuating endothelial dysfunction and atherosclerosis (141). Endothelial cells displaying the senescent phenotype have been found localized at sites of atherosclerotic plaque formation (140) and this is further supported by the fact that the elderly display impaired wound healing and angiogenesis (166).

ASSESSMENT OF ENDOTHELIAL FUNCTION

As previously stated, the endothelium plays an integral role in maintaining vascular homeostasis and it is NO that mediates many of the protective functions exerted by a functioning endothelium. Thus, endothelium-dependent vasomotor function reflects the integrity of the endothelial layer and is often used as a surrogate of NO bioavailability in a variety of research and clinical methodologies. In both the clinical and research setting, endothelial function has been shown to be associated with atherosclerotic risk factors (176, 213) and is believed to be a predictor of adverse CV events (80, 197). Furthermore, evidence suggests that endothelial dysfunction is reversible with certain interventional strategies making its assessment even more relevant (36). The most

pertinent site to assess endothelial function in relation to CVD is the coronary arteries but that requires invasive techniques and is not practical for routine assessment. However, studies have shown a positive correlation between vasodilator impairment in the coronary circulation to that of the peripheral circulation (3, 175) suggesting that endothelial dysfunction is a systemic disorder allowing for it to be measured non-invasively at more accessible sites (2, 37). Endothelial dysfunction in the microcirculation is also established as an early marker of vascular disease; it predisposes to the development of atherosclerosis and it is also a systemic process that occurs in multiple tissue beds throughout the body (175, 210). Consequently, identification of impaired microvascular blood flow and vasoreactivity by noninvasive means can provide a quantitative assessment of the effects of a given intervention (1).

The skin is emerging as an ideal site for evaluation of endothelial function and; furthermore, it has been suggested that the cutaneous microcirculation can serve as a model for generalized microvascular dysfunction (203). Additionally, changes in skin vascular reactivity are observed before clinical signs of microvascular dysfunction, during the early stages of many diseases, suggesting it precludes more globalized microvascular dysfunction (136). The cerebral circulation can also be used to assess endothelial function. Lavi *et al.* demonstrated that the chemoregulatory mechanisms of cerebral blood flow (CBF) are impaired in diseases affecting endothelial NO production and suggested that the CO_2-NO axis is a primary pathway in the chemoregulation of CBF in humans (116). Furthermore, it has been demonstrated that acute systemic administration of L-arginine can restore cerebral reactivity in patients at CV risk (218).

Thus, for these studies skin blood flow response to local heating (thermal reactivity; TR) and CBF response to hypercapnia (cerebral vasomotor reactivity; CVMR) were used as surrogates for NO bioavailability. These assessments allowed for the

investigation of the effects of flavanol ingestion on endothelial function in both the microvascular and cerebrovascular circulations. Skin blood flow response to a standard local heating protocol (TR) is a common, non-invasive research technique used to assess microvascular function and it has been shown to be predominantly NO-mediated (106, 144). CBF response to hypercapnia (CVMR) has also been shown to be primarily NO-mediated and, thus can be used as a surrogate of local cerebral endothelial function (117). Both TR and CVMR have been shown to be reduced with aging (13, 30, 145, 168); however whether or not endothelial function can be restored in these vascular beds following flavanol ingestion remains unknown.

Chapter VII: General Discussion and Future Directions

These studies explored interactions between flavanols and nitric oxide (NO) in order to investigate implications for vascular health and cardiovascular disease (CVD). We chose to investigate endothelial function, as an indicator of NO bioavailability, in 2 different vascular beds due to the systemic nature of CVD and to investigate the effect of flavanols in these different vascular beds. Study 1 investigated acute effects of flavanol consumption on cutaneous microvascular endothelial function in young and older individuals and explored the effects of chronic exposure in older individuals. This was accomplished by assessing skin blood flow response to local heating (thermal reactivity, TR); skin was clamped at 34°C and 40°C and values were normalized to those attained at 43°C. The skin was chosen as it is emerging as an ideal site for evaluation of endothelial function and it has been suggested that the cutaneous microcirculation can serve as a model for generalized microvascular dysfunction (94, 108). Study 2 investigated the acute effects of flavanol consumption on cerebrovascular endothelial function in young and older individuals along with chronic flavanol exposure in older individuals. This was accomplished by assessing basal cerebral blood flow indices (cerebral vascular conductance index, CVCi) and CBF response to hypercapnia (cerebral vasomotor reactivity; CVMR). CVMR can be used as a surrogate of local cerebral endothelial function (117, 165), it has been shown to be predominantly NO-mediated (116) and has been shown to be reduced with aging (130, 168).

There were a number of significant findings with study 1. Older individuals demonstrated a reduced TR at baseline compared with the young confirming an impairment in endothelial function as previous research has shown. There was an acute augmentation of SKBF response to local heating following flavanol ingestion; however,

it was not different between young and older individuals. Also, chronic exposure to flavanols had no effect on TR but did result in a significant decrease in blood pressure thereby still demonstrating vascular benefit.

One of the goals of this study was to examine the acute effects of flavanol ingestion on the cutaneous vasodilator response to local heating and to determine if it differed in young versus older individuals. There was no effect of flavanols on TR in either young or older individuals when only the final, stable 5 minutes of the plateau phase during 40°C was considered, which is the most common data analysis protocol used to assess TR. An unexpected finding was that flavanols did lead to an acute, significant increase in the relative initial peak SKBF response to local heating at 40°C. The validity of this finding is strengthened by study design; the same effect was not seen following ingestion of the placebo only flavanols nor was it present during the initial peak response to 43°C. This effect was initially surprising because studies have shown that the initial peak response is primarily axon-mediated with less of a contribution from NO, whereas the plateau phase has a much greater contribution from NO (106, 144). Therefore, we had initially hypothesized that we would see the effect of flavanols in the latter phase. However, there is some contribution from NO over the entire local heating protocol as is evidenced by mechanistic studies that have shown a contribution of NO from 70-75% during the initial peak and 87-92% during the plateau phase and these same studies have shown the entire cutaneous vasodilatory response to be significantly blunted by the administration of L-NAME prior to local heating (60, 106, 144). Though this study was not mechanistic in design, we used a similar local heating protocol so we would surmise that a comparable relative contribution of NO was occurring during these studies.

Consequently, we re-analyzed the data by averaging the entire SKBF response during the 30 minutes of 40°C local heating and normalizing it to the SKBF response

achieved during the last 5 minutes at 43°C. We hypothesized that we were detecting an effect of flavanols only during the initial peak because the overall response to local heating was less then than during the plateau phase so it represented a greater fractional change. This reasoning has been supported by a recent publication by Choi *et al.* that investigated different local heating protocols and concluded that a lower temperature resulted in less of a NO-mediated response and this may be more beneficial in interventional studies as it allowed for more room for interventions to 'improve' NO bioavailability by avoiding the potential ceiling effect (42). As we suspected, the significant effect of flavanols was not lost when the entire SKBF response to 30 minutes of 40°C local heating was considered. We believe the effect of flavanols was occurring not only during the initial peak, though it may have appeared more robust, but rather there was some effect on cutaneous vasodilatory response throughout the 30 minutes. In addition, the baseline impairment in TR between our young and older groups persisted with the re-analyzed data.

This study was not mechanistic by design so we can only hypothesize as to the underlying cause of the increased NO bioavailability. A number of indirect pathways have been hypothesized for the effect of flavanols on NO bioavailability including: 1) interaction with endothelial nitric oxide synthase (eNOS) thereby maintaining it in an activated form, 2) by decreasing arginase activity leading to the preservation of intracellular concentration of L-arginine, and/or 3) by inhibiting NADPH oxidase (NOX) thereby limiting the generation of superoxide anions (71, 89, 177). eNOS has been shown to be the primary isoform responsible for the generation of NO during local heating; however its involvement in the initial peak SkBF response has been questioned (107, 183). In this study it appears as though flavanols were exerting some effect over the entire phase of 40°C local heating, including the initial peak, suggesting it is less likely

that eNOS activation was the primary mechanism. Dupont et al. demonstrated that the plateau phase of heating at 40°C increased following the administration of both ascorbic acid and L-arginine whereas the initial peak response increased following the administration of ascorbic acid only (60). Free radicals, including the superoxide anion, lead to a reduction in NO bioavailability as they react with NO to form peroxynitrite (16, 17). Flavanols have been shown to improve NO bioavailability via the inhibition of NOX (20, 191, 193), a major producer of superoxide anions in the endothelium (33). Similar to the effect of flavanols in this study, in the study by Dupont *et al.* the effects of ascorbic acid were seen during the initial peak along with the plateau suggesting the possibility that the effect of flavanols in this study may be due to NOX inhibition. Future, more mechanistic studies are required to elucidate underlying mechanisms.

Contrary to our hypothesis, there was no significant age effect in this acute response to flavanol ingestion. However, as a result of the attenuation seen in the older individuals compared with the young at baseline, along with our initial hypothesis, and for discussion purposes we investigated the pair-wise comparisons to determine the magnitude of change in the young versus the older individuals. On both treatment days (placebo and flavanol), as expected, there was a significant decrease in TR in the older individuals compared with the young pre-ingestion of the test drink ($p<0.05$ for both). Following flavanol ingestion both the young and the older individuals increased, though the magnitude of the change was much greater in the older individuals (young: 58.1 ± 3.0% to 60.0 ± 2.7%; older: 46.7 ± 3.1% to 52.5 ± 2.8%, $p=0.07$). There was no increase in either group following ingestion of the placebo (young: 59.6 ± 3.4% to 56.3±3.0%; older: 49.0 ± 3.5% to 49.1 ± 3.1%, $p>0.05$). This increase was significant when age groups were combined but did not quite reach significance as individual groups. We believe this may be related to subject recruitment. In order to limit confounders we

specifically recruited older individuals that were taking either no medication at all or very little and had not been diagnosed with CVD or any other metabolic illness. Not by coincidence, such individuals generally lead health conscious lifestyles and their diet and physical activity levels reflect this. Though none of the older individuals in the study were highly trained we believe they represented a healthier segment of the aging population and this could explain our lack of significance between our groups. We believe that future studies into the acute effects of flavanols on aging are warranted and should include more representative segments of our aging population.

There was no significant difference in cutaneous vasodilatory response to local heating following chronic ingestion of flavanols in older individuals. There is the possibility that the quantity of flavanols provided or length of exposure time were not long enough to lead to cutaneous microvascular effects; however, this amount of time did result in a decrease in blood pressure. This provides evidence that, though there was no effect on TR, the quantity of flavanols and the length of time they were taken was sufficient to have a vascular effect. A number of meta-analyses have concluded that flavanols do have a blood pressure lowering effect and the results of this study support this previous research (164, 200). The underlying concept is that sufficient NO bioavailability is associated with normal vasodilation; whereas, decreases in NO steady-state concentration can lead to a failure in smooth muscle relaxation and subsequent hypertension. With long term, at home interventions such as this compliance needs to be a consideration. However, in this study subjects complied with requests to return empty canisters and unused product and they were asked about compliance prior to each testing session so we do not believe that this played a role in the negative results.

There were also a number of significant findings with study 2. Once again, at baseline older individuals demonstrated a reduced CBF and a reduced cerebral reactivity

compared with the young supporting the premise of cerebral endothelial dysfunction in the older individuals. The consumption of flavanols led to an apparent acute decrease in both CBF and cerebral reactivity and this response was not different in the young versus the older individuals and yet chronic exposure led to an increase in CBF in older individuals but had no effect on cerebral reactivity.

As has previously been demonstrated, older individuals had significantly lower CBFV and CVCi at baseline compared with younger individuals along with a decreased CVMR (13, 130, 163, 168, 202). It has been shown that NO contributes to baseline CBF tone and is also involved in CVMR (99, 102, 116) so this provides evidence for impaired cerebral endothelial function via a reduction in NO bioavailability in older individuals. Another significant difference at baseline was that the *b* model parameter was significantly lower in the older individuals compared with the young. The *b* model parameter is derived from the nonlinear logistic regression function that is used to assess CBFV response to hypercapnia and it represents the curvilinear properties of the resulting line of best fit. In response to hypercapnia, CBFV increases steadily along with PET_{CO2} until it begins to level off at high levels of PET_{CO2} thereby generating a sigmoidal curve response. In this protocol, older individuals demonstrated a more linear response indicating they were less likely to plateau within the range of PET_{CO2} attained in this study. Claassen et al suggested 2 possible mechanisms for the plateau response to hypercapnia; there is a peak cerebral vasodilatory response to increasing Pa_{CO2} or the vasoconstrictive effects of increasing sympathetic output are eventually overcoming vasodilation (44). Further investigation of the physiology associated with the *b* model parameter is required in order to determine if one of these hypothesized mechanisms is playing a role.

In this study, the curvilinear relationship resulting from the effects of hypercapnia on CBFV was analyzed using a four parameter logistic function as outlined by Claassen *et al.* as he suggests that this nonlinear curve fitting should be chosen over linear regression as it is a better model of the physiological response of CBFV (or CVCi) to hypercapnia (44). He suggests that there is added benefit to this method of analysis as it allows for the quantification of model parameters that may have physiological implications and should be considered when assessing CMVR: *a* the range in change of CBFV (or CVCi), *y0* the maximum of CBFV (or CVCi) achieved during hypercapnia, *x0* the level of PET_{CO2} when the slope of the curve is maximal and *b* the curvilinear properties of the line. Additionally, the first order derivative of the logistic function used for the curve fitting generates a bell-shaped curve and the peak of that curve represents CBFV-$CVMR_{max}$ (or CVCi-$CVMR_{max}$). In our experience further research is required regarding this type of analysis as there are some subjects, particularly those that do not plateau, that demonstrate model parameters (mainly *y0* and *a*) that are outside of normal physiological range and this reduces their usefulness and suggests that caution is required when interpreting such data. We suggest that further studies are required to investigate the physiological implications of these model parameters in different subject populations to assess their usefulness in contributing to the information gained via CVMR.

One of the goals of this study was to examine the acute effects of flavanol ingestion on cerebral vascular endothelial function and to determine if it differed in young versus older individuals. Contrary to our hypothesis was the finding that flavanol ingestion resulted in an acute, significant decrease in baseline CBFV and CVCi and that this response was the same in young and older individuals. Our hypothesis was that flavanols would cause vasodilation due to an increase in NO bioavailability; however, we hypothesized the vasodilatory response would be further downstream in the

microvasculature. In this study we used CBFV as a surrogate for CBF because we could not accurately measure MCA diameter. We made this assumption based on evidence from previous studies that have shown that MCA diameter does not change with perturbations such as changes in Pa_{CO2} within the range used in this study (77, 182). However, the effect of flavanols on cerebral hemodynamics has not yet been investigated so the possibility exists that flavanols were causing vasodilation of the MCA. An increase in MCA diameter would provide a plausible explanation for these results but requires further investigation. Flavanols have been shown to have a vasodilatory affect on the brachial artery, also a conduit artery, in a number of studies (12, 87, 147).

Also contrary to our hypothesis, there was a significant decrease in calculated $CVMR_{max}$ acutely following flavanol ingestion and, again, this effect was not different between the young and older individuals. The R^2 values ranged from 0.96 – 0.97 and were not different between treatments indicating that the fit to the nonlinear regression was not a factor in these results. In addition, the absolute range of PET_{CO2} that subjects experienced during their rebreathing protocol was not different between treatments indicating that their hypercapnic stimulus was equivalent. A more debatable finding was that the pre values on each of the treatment days were different; significantly higher on the day the flavanol-containing test drink was provided compared with the placebo. We believe that this decrease is $CVMR_{max}$ remains significant based on the strength of the study design: 1) on each testing day post measures were assessed in relation to pre measures taken on the same day under the same conditions so this should account for any variability between the 2 intervention days and 2) this study was placebo-controlled and this same decrease from pre-ingestion was not seen following the ingestion of the placebo. This would suggest that flavanols were having an acute adverse effect as the general consensus is that endothelial dysfunction results in an attenuated CVMR.

However, we believe the acute decrease in calculated $CVMR_{max}$ following flavanol ingestion may be due to differences in cerebral reactivity protocols and assessment techniques. We used a rebreathing protocol that assessed CBFV response to breath-by-breath changes in PET_{CO2} and then chose a relatively novel four parameter logistic curve to assess CVMR as it was a better choice for the sigmoidal curvilinear response compared with linear regression. As a result, we believe that the decrease in calculated $CVMR_{max}$ seen acutely following flavanol ingestion using this method of analysis requires further investigation to determine if it does indicate an impairment in cerebrovascular function. Other studies use stepped hypercapnia with or without a period of hypocapnia followed by linear regression to calculate CVMR (13, 116, 117, 211, 218). In this study, we chose not to include a hypocapnic stage as we were interested only in the vasodilatory effects of flavanols and our laboratory has previously shown that a prior hypocapnic stage has an effect on the subsequent vasodilatory response to hypercapnia (unpublished data). The preponderance of evidence regarding flavanols suggests a positive vascular benefit and further investigation is required to know if this benefit extends to the cerebral circulation.

Older individuals demonstrated a significant increase in both CBFV and CVCi following 28 days of daily flavanol consumption. Though this is opposite the response seen acutely following flavanol ingestion it is not surprising as there is evidence to suggest that the underlying mechanism accounting for the increase in NO bioavailability differs between acute and chronic consumption of flavanols. This belief is supported by studies that demonstrate an increase in basal NO following long-term flavanol consumption that increases even further following an acute dose of flavanols (12, 67, 88). Acute vascular effects are postulated to be mediated through the inhibition of NADPH oxidase thereby limiting oxidative stress, whereas, chronic effects are believed to lead to

increased levels of activated eNOS or decreased arginase activity within endothelial cells (177, 178). The chronic effect is believed to be an adaptive response to continuous high-flavanol exposure and therefore, mediated by changes in gene expression and protein synthesis or breakdown (194). As previously stated, NO contributes to tonic cerebral vascular tone so it is possible that this increase at baseline is due to an increase in basal NO having an effect on the cerebral microvasculature as hypothesized. Determination of which of the possible pathways is involved is outside the scope of the current study and requires further mechanistic investigation. There was no effect on any of the CVMR model parameters or calculated $CVMR_{max}$ in older individuals following chronic exposure to flavanols. However, if the effect on NO bioavailability was not as robust as that seen with baseline CBFV and CVCi then there is a possibility that we did not detect it due to the small sample size.

These studies explored interactions between aging, flavanols and NO in order to investigate implications for vascular health and cardiovascular disease (CVD) and they did so in two different vascular beds. Our main findings were that the ingestion of flavanols acutely improved cutaneous microvascular endothelial function in response to local heating but that this response was not different with aging and, though there was no effect on the cutaneous vasodilatory response to local heating following chronic exposure to flavanols, there was a beneficial vascular effect as was evidenced by a reduction in blood pressure. Our main finding with regards to cerebral endothelial function was that chronic exposure to flavanols resulted in a significant increase in baseline cerebral hemodynamics in older individuals but there was no effect on cerebral reactivity. The results from these studies contribute to current research by providing further support for vascular health benefits associated with flavanols. Further mechanistic studies are

warranted to elucidate underlying mechanisms as they could potentially guide future pharmacological research.

Appendix

ACUTE STUDY INSTRUCTIONS

Please follow these instructions before your next two visits (Study Days 1 & 2) to the laboratory where we will be assessing your vascular response to flavanol ingestion.

Your scheduled study day is _____ so please write down ALL of your food intake (see second page) from the day before, _____ as you will be asked to consume a similar diet on the day prior to your next study day. In addition, we ask that you consume a low-flavanol diet in the 3 days prior to coming into the laboratory for testing. You will begin this diet on _____ and end it on _____. Foods to avoid are listed below.

PLEASE FOLLOW A FLAVANOL-POOR DIET IN THE 3 DAYS PRIOR TO YOUR STUDY DAY SO AVOID THE FOLLOWING FOODS AS THEY ARE FLAVANOL-RICH.

- Chocolate and/or any cocoa products
- Green/Fruit tea
- Red wine
- Fruits (or juice made from any of the following fruits)
    - Apples
    - Apricots
    - Bananas
    - Grapes
    - Nectarines
    - Peaches
    - Pears
    - Plums
- Berries (blackberries, blueberries, raspberries, cherries, cranberries)
- Nuts (pecans, pistachios)
- Soybeans (anything made with soy)
- Pinto Beans
- Carob flour

During the ***24 hours*** before your testing session please do not engage in any strenuous physical activity and avoid alcoholic beverages.

During the ***12 hours*** before your testing session please do not eat or drink anything (water is ok).
Your testing time is _____ so please do not eat after _____ the night before.

Thank you; we appreciate our subjects and all that you do!!!

Subject ID#

Dietary Log

Please write down all of the food that you eat on the day prior to your scheduled visit to the laboratory.

Breakfast:

_____
_____
_____
_____
_____
_____

Midmorning Snack:

_____
_____
_____
_____

Lunch:

_____
_____
_____
_____
_____
_____

Afternoon Snack:

_____
_____
_____
_____

Supper:

_____
_____
_____
_____
_____
_____

CHRONIC INTERVENTION INSTRUCTIONS

Please follow these instructions over the next 28 days that you consume your daily test beverage.

To prepare daily test beverage:

- Add 1 level scoop of powder to a cup, making sure NOT to pack the scoop.
- Heat 8oz of water in microwave for about 1 minute (hot but not boiling).
- Add hot water to powder in the cup, stir until ALL powder is dissolved.
  Some people find it beneficial to mix the beverage into a container with a lid, for ease of mixing
- Drink entire beverage within 15 minutes.

Please try to consume the beverage at approximately the same time each day (either in the morning, afternoon or evening).

You will need to come in to the laboratory for your final testing day within 3 days of consuming your last test beverage. Your final scheduled study day is _____. Your testing time is _____ so please do not eat after _____ the night before.

During the ***24 hours*** before your final testing session please do not engage in any strenuous physical activity and avoid alcoholic beverages.

During the ***12 hours*** before your testing session please do not eat (including supplements/vitamins) or drink anything (water is ok).

Thank you; we appreciate our subjects and all that you do!!!

TEST DRINK MIXING INSTRUCTIONS

Below are the 'test drink' mixing instructions provided by The Hershey Company (product development branch). Instructions for mixing are the same for both the flavanol test drink and the placebo with the only difference being the size of the scoop (2 different size scoops were provided).

To prepare the test drink:

- Add 1 level scoop of powder to a cup, ensure that you do not pack the scoop.
- Heat 8oz of water in microwave for about 1 minute (hot but not boiling).
- Add hot water to powder in the cup, stir until ALL powder is dissolved.

You may choose to mix the beverage into a container with a lid so you can lightly shake it and ensure complete mixing

References

1. **Abularrage CJ, Sidawy AN, Aidinian G, Singh N, Weiswasser JM, and Arora S**. Evaluation of the microcirculation in vascular disease. *J Vasc Surg* 42: 574-581, 2005.
2. **Anderson TJ, Gerhard MD, Meredith IT, Charbonneau F, Delagrange D, Creager MA, Selwyn AP, and Ganz P**. Systemic nature of endothelial dysfunction in atherosclerosis. *Am J Cardiol* 75: 71B-74B, 1995.
3. **Anderson TJ, Uehata A, Gerhard MD, Meredith IT, Knab S, Delagrange D, Lieberman EH, Ganz P, Creager MA, Yeung AC, and et al.** Close relation of endothelial function in the human coronary and peripheral circulations. *J Am Coll Cardiol* 26: 1235-1241, 1995.
4. **Arad Y, Badimon JJ, Badimon L, Hembree WC, and Ginsberg HN**. Dehydroepiandrosterone feeding prevents aortic fatty streak formation and cholesterol accumulation in cholesterol-fed rabbit. *Arteriosclerosis* 9: 159-166, 1989.
5. **Ariznavarreta C, Castillo C, Segovia G, Mora F, Azcoitia I, and Tresguerres JA**. Growth hormone and aging. *Homo* 54: 132-141, 2003.
6. **Arts IC, and Hollman PC**. Polyphenols and disease risk in epidemiologic studies. *Am J Clin Nutr* 81: 317S-325S, 2005.
7. **Arts IC, Hollman PC, Feskens EJ, Bueno de Mesquita HB, and Kromhout D**. Catechin intake and associated dietary and lifestyle factors in a representative sample of Dutch men and women. *Eur J Clin Nutr* 55: 76-81, 2001.
8. **Arts IC, Jacobs DR, Jr., Harnack LJ, Gross M, and Folsom AR**. Dietary catechins in relation to coronary heart disease death among postmenopausal women. *Epidemiology* 12: 668-675, 2001.
9. **Azuma H, Sato J, Hamasaki H, Sugimoto A, Isotani E, and Obayashi S**. Accumulation of endogenous inhibitors for nitric oxide synthesis and decreased content of L-arginine in regenerated endothelial cells. *Br J Pharmacol* 115: 1001-1004, 1995.
10. **Baba S, Osakabe N, Yasuda A, Natsume M, Takizawa T, Nakamura T, and Terao J**. Bioavailability of (-)-epicatechin upon intake of chocolate and cocoa in human volunteers. *Free Radic Res* 33: 635-641, 2000.
11. **Bachetti T, Comini L, Francolini G, Bastianon D, Valetti B, Cadei M, Grigolato P, Suzuki H, Finazzi D, Albertini A, Curello S, and Ferrari R**. Arginase pathway in human endothelial cells in pathophysiological conditions. *J Mol Cell Cardiol* 37: 515-523, 2004.
12. **Balzer J, Rassaf T, Heiss C, Kleinbongard P, Lauer T, Merx M, Heussen N, Gross HB, Keen CL, Schroeter H, and Kelm M**. Sustained benefits in vascular function through flavanol-containing cocoa in medicated diabetic patients a double-masked, randomized, controlled trial. *J Am Coll Cardiol* 51: 2141-2149, 2008.

13. **Barnes JN, Schmidt JE, Nicholson WT, and Joyner MJ**. Cyclooxygenase inhibition abolishes age-related differences in cerebral vasodilator responses to hypercapnia. *J Appl Physiol (1985)* 112: 1884-1890.
14. **Barton M, Cosentino F, Brandes RP, Moreau P, Shaw S, and Luscher TF**. Anatomic heterogeneity of vascular aging: role of nitric oxide and endothelin. *Hypertension* 30: 817-824, 1997.
15. **Bayard V, Chamorro F, Motta J, and Hollenberg NK**. Does flavanol intake influence mortality from nitric oxide-dependent processes? Ischemic heart disease, stroke, diabetes mellitus, and cancer in Panama. *Int J Med Sci* 4: 53-58, 2007.
16. **Beckman JS, Beckman TW, Chen J, Marshall PA, and Freeman BA**. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* 87: 1620-1624, 1990.
17. **Beckman JS, and Koppenol WH**. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 271: C1424-1437, 1996.
18. **Bedard K, and Krause KH**. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 87: 245-313, 2007.
19. **Behrendt D, and Ganz P**. Endothelial function. From vascular biology to clinical applications. *Am J Cardiol* 90: 40L-48L, 2002.
20. **Benito S, Lopez D, Saiz MP, Buxaderas S, Sanchez J, Puig-Parellada P, and Mitjavila MT**. A flavonoid-rich diet increases nitric oxide production in rat aorta. *Br J Pharmacol* 135: 910-916, 2002.
21. **Berkowitz DE, White R, Li D, Minhas KM, Cernetich A, Kim S, Burke S, Shoukas AA, Nyhan D, Champion HC, and Hare JM**. Arginase reciprocally regulates nitric oxide synthase activity and contributes to endothelial dysfunction in aging blood vessels. *Circulation* 108: 2000-2006, 2003.
22. **Bishop CC, Powell S, Rutt D, and Browse NL**. Transcranial Doppler measurement of middle cerebral artery blood flow velocity: a validation study. *Stroke* 17: 913-915, 1986.
23. **Boger RH**. The emerging role of asymmetric dimethylarginine as a novel cardiovascular risk factor. *Cardiovasc Res* 59: 824-833, 2003.
24. **Boger RH, Bode-Boger SM, Szuba A, Tsao PS, Chan JR, Tangphao O, Blaschke TF, and Cooke JP**. Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: its role in hypercholesterolemia. *Circulation* 98: 1842-1847, 1998.
25. **Boger RH, Bode-Boger SM, Tsao PS, Lin PS, Chan JR, and Cooke JP**. An endogenous inhibitor of nitric oxide synthase regulates endothelial adhesiveness for monocytes. *J Am Coll Cardiol* 36: 2287-2295, 2000.
26. **Boger RH, Skamira C, Bode-Boger SM, Brabant G, von zur Muhlen A, and Frolich JC**. Nitric oxide may mediate the hemodynamic effects of recombinant growth hormone in patients with acquired growth hormone deficiency. A double-blind, placebo-controlled study. *J Clin Invest* 98: 2706-2713, 1996.

27. **Bouloumie A, Schini-Kerth VB, and Busse R**. Vascular endothelial growth factor up-regulates nitric oxide synthase expression in endothelial cells. *Cardiovasc Res* 41: 773-780, 1999.
28. **Brandes RP, Barton M, Philippens KM, Schweitzer G, and Mugge A**. Endothelial-derived superoxide anions in pig coronary arteries: evidence from lucigenin chemiluminescence and histochemical techniques. *J Physiol* 500 (Pt 2): 331-342, 1997.
29. **Brothers RM, Wingo JE, Hubing KA, and Crandall CG**. Methodological assessment of skin and limb blood flows in the human forearm during thermal and baroreceptor provocations. *J Appl Physiol* 109: 895-900, 2010.
30. **Bruning RS, Santhanam L, Stanhewicz AE, Smith CJ, Berkowitz DE, Kenney WL, and Holowatz LA**. Endothelial nitric oxide synthase mediates cutaneous vasodilation during local heating and is attenuated in middle-aged human skin. *J Appl Physiol (1985)* 112: 2019-2026.
31. **Buijsse B, Feskens EJ, Kok FJ, and Kromhout D**. Cocoa intake, blood pressure, and cardiovascular mortality: the Zutphen Elderly Study. *Arch Intern Med* 166: 411-417, 2006.
32. **Busse R, and Mulsch A**. Calcium-dependent nitric oxide synthesis in endothelial cytosol is mediated by calmodulin. *FEBS Lett* 265: 133-136, 1990.
33. **Cai H, and Harrison DG**. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 87: 840-844, 2000.
34. **Carey M, Markham C, Gaffney P, Boran C, and Maher V**. Validation of a point of care lipid analyser using a hospital based reference laboratory. *Ir J Med Sci* 175: 30-35, 2006.
35. **Cave AC, Brewer AC, Narayanapanicker A, Ray R, Grieve DJ, Walker S, and Shah AM**. NADPH oxidases in cardiovascular health and disease. *Antioxid Redox Signal* 8: 691-728, 2006.
36. **Celermajer DS**. Endothelial dysfunction: does it matter? Is it reversible? *J Am Coll Cardiol* 30: 325-333, 1997.
37. **Celermajer DS, Sorensen KE, Bull C, Robinson J, and Deanfield JE**. Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interaction. *J Am Coll Cardiol* 24: 1468-1474, 1994.
38. **Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, Lloyd JK, and Deanfield JE**. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet* 340: 1111-1115, 1992.
39. **Celermajer DS, Sorensen KE, Spiegelhalter DJ, Georgakopoulos D, Robinson J, and Deanfield JE**. Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. *J Am Coll Cardiol* 24: 471-476, 1994.
40. **Challah M, Nadaud S, Philippe M, Battle T, Soubrier F, Corman B, and Michel JB**. Circulating and cellular markers of endothelial dysfunction with aging in rats. *Am J Physiol* 273: H1941-1948, 1997.

41. **Charkoudian N, Stephens DP, Pirkle KC, Kosiba WA, and Johnson JM**. Influence of female reproductive hormones on local thermal control of skin blood flow. *J Appl Physiol* 87: 1719-1723, 1999.
42. **Choi PJ, Brunt VE, Fujii N, and Minson CT**. New approach to measure cutaneous microvascular function: an improved test of NO-mediated vasodilation by thermal hyperemia. *J Appl Physiol (1985)* 2014.
43. **Ciplak M, Pasche A, Heim A, Haeberli C, Waeber B, Liaudet L, Feihl F, and Engelberger R**. The vasodilatory response of skin microcirculation to local heating is subject to desensitization. *Microcirculation* 16: 265-275, 2009.
44. **Claassen JA, Zhang R, Fu Q, Witkowski S, and Levine BD**. Transcranial Doppler estimation of cerebral blood flow and cerebrovascular conductance during modified rebreathing. *J Appl Physiol* 102: 870-877, 2007.
45. **Collins C, and Tzima E**. Hemodynamic forces in endothelial dysfunction and vascular aging. *Exp Gerontol* 46: 185-188.
46. **Cooke JP**. Does ADMA cause endothelial dysfunction? *Arterioscler Thromb Vasc Biol* 20: 2032-2037, 2000.
47. **Cooke JP, Singer AH, Tsao P, Zera P, Rowan RA, and Billingham ME**. Antiatherogenic effects of L-arginine in the hypercholesterolemic rabbit. *J Clin Invest* 90: 1168-1172, 1992.
48. **Crandall CG, Levine BD, and Etzel RA**. Effect of increasing central venous pressure during passive heating on skin blood flow. *J Appl Physiol (1985)* 86: 605-610, 1999.
49. **Creager MA, Gallagher SJ, Girerd XJ, Coleman SM, Dzau VJ, and Cooke JP**. L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. *J Clin Invest* 90: 1248-1253, 1992.
50. **Csiszar A, Ungvari Z, Edwards JG, Kaminski P, Wolin MS, Koller A, and Kaley G**. Aging-induced phenotypic changes and oxidative stress impair coronary arteriolar function. *Circ Res* 90: 1159-1166, 2002.
51. **Dahl A, Lindegaard KF, Russell D, Nyberg-Hansen R, Rootwelt K, Sorteberg W, and Nornes H**. A comparison of transcranial Doppler and cerebral blood flow studies to assess cerebral vasoreactivity. *Stroke* 23: 15-19, 1992.
52. **Dauchet L, Amouyel P, and Dallongeville J**. Fruit and vegetable consumption and risk of stroke: a meta-analysis of cohort studies. *Neurology* 65: 1193-1197, 2005.
53. **Dauchet L, Amouyel P, Hercberg S, and Dallongeville J**. Fruit and vegetable consumption and risk of coronary heart disease: a meta-analysis of cohort studies. *J Nutr* 136: 2588-2593, 2006.
54. **Davis ME, Cai H, Drummond GR, and Harrison DG**. Shear stress regulates endothelial nitric oxide synthase expression through c-Src by divergent signaling pathways. *Circ Res* 89: 1073-1080, 2001.
55. **Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, and Zeiher AM**. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 399: 601-605, 1999.

56. **Ding EL, Hutfless SM, Ding X, and Girotra S**. Chocolate and prevention of cardiovascular disease: a systematic review. *Nutr Metab (Lond)* 3: 2, 2006.
57. **Drexler H**. Factors involved in the maintenance of endothelial function. *Am J Cardiol* 82: 3S-4S, 1998.
58. **Drexler H, Zeiher AM, Meinzer K, and Just H**. Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. *Lancet* 338: 1546-1550, 1991.
59. **Drummond GR, Cai H, Davis ME, Ramasamy S, and Harrison DG**. Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. *Circ Res* 86: 347-354, 2000.
60. **Dupont JJ, Farquhar WB, Townsend RR, and Edwards DG**. Ascorbic acid or L-arginine improves cutaneous microvascular function in chronic kidney disease. *J Appl Physiol (1985)* 111: 1561-1567.
61. **Durante W, Johnson FK, and Johnson RA**. Arginase: a critical regulator of nitric oxide synthesis and vascular function. *Clin Exp Pharmacol Physiol* 34: 906-911, 2007.
62. **Egashira K, Inou T, Hirooka Y, Kai H, Sugimachi M, Suzuki S, Kuga T, Urabe Y, and Takeshita A**. Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans. *Circulation* 88: 77-81, 1993.
63. **Ehara S, Ueda M, Naruko T, Haze K, Itoh A, Otsuka M, Komatsu R, Matsuo T, Itabe H, Takano T, Tsukamoto Y, Yoshiyama M, Takeuchi K, Yoshikawa J, and Becker AE**. Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. *Circulation* 103: 1955-1960, 2001.
64. **Erdman JW, Jr., Balentine D, Arab L, Beecher G, Dwyer JT, Folts J, Harnly J, Hollman P, Keen CL, Mazza G, Messina M, Scalbert A, Vita J, Williamson G, and Burrowes J**. Flavonoids and heart health: proceedings of the ILSI North America Flavonoids Workshop, May 31-June 1, 2005, Washington, DC. *J Nutr* 137: 718S-737S, 2007.
65. **Faraci FM, Brian JE, Jr., and Heistad DD**. Response of cerebral blood vessels to an endogenous inhibitor of nitric oxide synthase. *Am J Physiol* 269: H1522-1527, 1995.
66. **Finkel T, and Holbrook NJ**. Oxidants, oxidative stress and the biology of ageing. *Nature* 408: 239-247, 2000.
67. **Fisher ND, Hughes M, Gerhard-Herman M, and Hollenberg NK**. Flavanol-rich cocoa induces nitric-oxide-dependent vasodilation in healthy humans. *J Hypertens* 21: 2281-2286, 2003.
68. **Fleming I, Fisslthaler B, Dimmeler S, Kemp BE, and Busse R**. Phosphorylation of Thr(495) regulates Ca(2+)/calmodulin-dependent endothelial nitric oxide synthase activity. *Circ Res* 88: E68-75, 2001.
69. **Foreman KE, and Tang J**. Molecular mechanisms of replicative senescence in endothelial cells. *Exp Gerontol* 38: 1251-1257, 2003.

70. **Forstermann U, Schmidt HH, Pollock JS, Sheng H, Mitchell JA, Warner TD, Nakane M, and Murad F**. Isoforms of nitric oxide synthase. Characterization and purification from different cell types. *Biochem Pharmacol* 42: 1849-1857, 1991.
71. **Fraga CG, Litterio MC, Prince PD, Calabro V, Piotrkowski B, and Galleano M**. Cocoa flavanols: effects on vascular nitric oxide and blood pressure. *J Clin Biochem Nutr* 48: 63-67, 2010.
72. **Frantz J, Engelberger RP, Liaudet L, Mazzolai L, Waeber B, and Feihl F**. Desensitization of thermal hyperemia in the skin is reproducible. *Microcirculation* 19: 78-85.
73. **Furchgott RF, and Zawadzki JV**. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288: 373-376, 1980.
74. **Ganji SH, Qin S, Zhang L, Kamanna VS, and Kashyap ML**. Niacin inhibits vascular oxidative stress, redox-sensitive genes, and monocyte adhesion to human aortic endothelial cells. *Atherosclerosis* 202: 68-75, 2009.
75. **Garg UC, and Hassid A**. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest* 83: 1774-1777, 1989.
76. **Geary GG, and Buchholz JN**. Selected contribution: Effects of aging on cerebrovascular tone and [Ca2+]i. *J Appl Physiol* 95: 1746-1754, 2003.
77. **Giller CA, Bowman G, Dyer H, Mootz L, and Krippner W**. Cerebral arterial diameters during changes in blood pressure and carbon dioxide during craniotomy. *Neurosurgery* 32: 737-741; discussion 741-732, 1993.
78. **Gordon GB, Bush DE, and Weisman HF**. Reduction of atherosclerosis by administration of dehydroepiandrosterone. A study in the hypercholesterolemic New Zealand white rabbit with aortic intimal injury. *J Clin Invest* 82: 712-720, 1988.
79. **Gorlach A, Brandes RP, Nguyen K, Amidi M, Dehghani F, and Busse R**. A gp91phox containing NADPH oxidase selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall. *Circ Res* 87: 26-32, 2000.
80. **Halcox JP, Schenke WH, Zalos G, Mincemoyer R, Prasad A, Waclawiw MA, Nour KR, and Quyyumi AA**. Prognostic value of coronary vascular endothelial dysfunction. *Circulation* 106: 653-658, 2002.
81. **Hamilton CA, Brosnan MJ, McIntyre M, Graham D, and Dominiczak AF**. Superoxide excess in hypertension and aging: a common cause of endothelial dysfunction. *Hypertension* 37: 529-534, 2001.
82. **Harman D**. Free radical theory of aging: an update: increasing the functional life span. *Ann N Y Acad Sci* 1067: 10-21, 2006.
83. **Harris JA, and Benedict FG**. A Biometric Study of Human Basal Metabolism. *Proc Natl Acad Sci U S A* 4: 370-373, 1918.
84. **Hayashi T, Esaki T, Muto E, Kano H, Asai Y, Thakur NK, Sumi D, Jayachandran M, and Iguchi A**. Dehydroepiandrosterone retards atherosclerosis formation through its conversion to estrogen: the possible role of nitric oxide. *Arterioscler Thromb Vasc Biol* 20: 782-792, 2000.

85. **He FJ, Nowson CA, Lucas M, and MacGregor GA**. Increased consumption of fruit and vegetables is related to a reduced risk of coronary heart disease: meta-analysis of cohort studies. *J Hum Hypertens* 21: 717-728, 2007.
86. **Hecker M, Nematollahi H, Hey C, Busse R, and Racke K**. Inhibition of arginase by NG-hydroxy-L-arginine in alveolar macrophages: implications for the utilization of L-arginine for nitric oxide synthesis. *FEBS Lett* 359: 251-254, 1995.
87. **Heiss C, Dejam A, Kleinbongard P, Schewe T, Sies H, and Kelm M**. Vascular effects of cocoa rich in flavan-3-ols. *JAMA* 290: 1030-1031, 2003.
88. **Heiss C, Finis D, Kleinbongard P, Hoffmann A, Rassaf T, Kelm M, and Sies H**. Sustained increase in flow-mediated dilation after daily intake of high-flavanol cocoa drink over 1 week. *J Cardiovasc Pharmacol* 49: 74-80, 2007.
89. **Heiss C, Keen CL, and Kelm M**. Flavanols and cardiovascular disease prevention. *Eur Heart J* 31: 2583-2592, 2010.
90. **Heiss C, Kleinbongard P, Dejam A, Perre S, Schroeter H, Sies H, and Kelm M**. Acute consumption of flavanol-rich cocoa and the reversal of endothelial dysfunction in smokers. *J Am Coll Cardiol* 46: 1276-1283, 2005.
91. **Heitzer T, Brockhoff C, Mayer B, Warnholtz A, Mollnau H, Henne S, Meinertz T, and Munzel T**. Tetrahydrobiopterin improves endothelium-dependent vasodilation in chronic smokers : evidence for a dysfunctional nitric oxide synthase. *Circ Res* 86: E36-41, 2000.
92. **Hoffmann J, Haendeler J, Aicher A, Rossig L, Vasa M, Zeiher AM, and Dimmeler S**. Aging enhances the sensitivity of endothelial cells toward apoptotic stimuli: important role of nitric oxide. *Circ Res* 89: 709-715, 2001.
93. **Hollman PC, Cassidy A, Comte B, Heinonen M, Richelle M, Richling E, Serafini M, Scalbert A, Sies H, and Vidry S**. The biological relevance of direct antioxidant effects of polyphenols for cardiovascular health in humans is not established. *J Nutr* 141: 989S-1009S, 2011.
94. **Holowatz LA, Thompson-Torgerson CS, and Kenney WL**. The human cutaneous circulation as a model of generalized microvascular function. *J Appl Physiol* 105: 370-372, 2008.
95. **Holowatz LA, Thompson CS, and Kenney WL**. Acute ascorbate supplementation alone or combined with arginase inhibition augments reflex cutaneous vasodilation in aged human skin. *Am J Physiol Heart Circ Physiol* 291: H2965-2970, 2006.
96. **Holowatz LA, Thompson CS, and Kenney WL**. L-Arginine supplementation or arginase inhibition augments reflex cutaneous vasodilatation in aged human skin. *J Physiol* 574: 573-581, 2006.
97. **Hongo K, Nakagomi T, Kassell NF, Sasaki T, Lehman M, Vollmer DG, Tsukahara T, Ogawa H, and Torner J**. Effects of aging and hypertension on endothelium-dependent vascular relaxation in rat carotid artery. *Stroke* 19: 892-897, 1988.
98. **Hu FB, and Willett WC**. Optimal diets for prevention of coronary heart disease. *JAMA* 288: 2569-2578, 2002.

99. **Iadecola C, Pelligrino DA, Moskowitz MA, and Lassen NA**. Nitric oxide synthase inhibition and cerebrovascular regulation. *J Cereb Blood Flow Metab* 14: 175-192, 1994.
100. **Ignarro LJ, Buga GM, Wood KS, Byrns RE, and Chaudhuri G**. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A* 84: 9265-9269, 1987.
101. **Ito S, Mardimae A, Han J, Duffin J, Wells G, Fedorko L, Minkovich L, Katznelson R, Meineri M, Arenovich T, Kessler C, and Fisher JA**. Non-invasive prospective targeting of arterial P(CO2) in subjects at rest. *J Physiol* 586: 3675-3682, 2008.
102. **Joshi S, Young WL, Duong DH, Ostapkovich ND, Aagaard BD, Hashimoto T, and Pile-Spellman J**. Intracarotid infusion of the nitric oxide synthase inhibitor, L-NMMA, modestly decreases cerebral blood flow in human subjects. *Anesthesiology* 93: 699-707, 2000.
103. **Jung O, Schreiber JG, Geiger H, Pedrazzini T, Busse R, and Brandes RP**. gp91phox-containing NADPH oxidase mediates endothelial dysfunction in renovascular hypertension. *Circulation* 109: 1795-1801, 2004.
104. **Kang LS, Reyes RA, and Muller-Delp JM**. Aging impairs flow-induced dilation in coronary arterioles: role of NO and H(2)O(2). *Am J Physiol Heart Circ Physiol* 297: H1087-1095, 2009.
105. **Keen CL, Holt RR, Oteiza PI, Fraga CG, and Schmitz HH**. Cocoa antioxidants and cardiovascular health. *Am J Clin Nutr* 81: 298S-303S, 2005.
106. **Kellogg DL, Jr., Liu Y, Kosiba IF, and O'Donnell D**. Role of nitric oxide in the vascular effects of local warming of the skin in humans. *J Appl Physiol* 86: 1185-1190, 1999.
107. **Kellogg DL, Jr., Zhao JL, and Wu Y**. Endothelial nitric oxide synthase control mechanisms in the cutaneous vasculature of humans in vivo. *Am J Physiol Heart Circ Physiol* 295: H123-129, 2008.
108. **Khan F, Patterson D, Belch JJ, Hirata K, and Lang CC**. Relationship between peripheral and coronary function using laser Doppler imaging and transthoracic echocardiography. *Clin Sci (Lond)* 115: 295-300, 2008.
109. **Kleinert H, Wallerath T, Euchenhofer C, Ihrig-Biedert I, Li H, and Forstermann U**. Estrogens increase transcription of the human endothelial NO synthase gene: analysis of the transcription factors involved. *Hypertension* 31: 582-588, 1998.
110. **Kris-Etherton PM, Hecker KD, Bonanome A, Coval SM, Binkoski AE, Hilpert KF, Griel AE, and Etherton TD**. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *Am J Med* 113 Suppl 9B: 71S-88S, 2002.
111. **Kubes P, Suzuki M, and Granger DN**. Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci U S A* 88: 4651-4655, 1991.
112. **Kung CF, and Luscher TF**. Different mechanisms of endothelial dysfunction with aging and hypertension in rat aorta. *Hypertension* 25: 194-200, 1995.

113. **Kurose I, Wolf R, Grisham MB, and Granger DN**. Effects of an endogenous inhibitor of nitric oxide synthesis on postcapillary venules. *Am J Physiol* 268: H2224-2231, 1995.
114. **Lakatta EG, and Levy D**. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part I: aging arteries: a "set up" for vascular disease. *Circulation* 107: 139-146, 2003.
115. **Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE, and Harrison DG**. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest* 111: 1201-1209, 2003.
116. **Lavi S, Egbarya R, Lavi R, and Jacob G**. Role of nitric oxide in the regulation of cerebral blood flow in humans: chemoregulation versus mechanoregulation. *Circulation* 107: 1901-1905, 2003.
117. **Lavi S, Gaitini D, Milloul V, and Jacob G**. Impaired cerebral CO2 vasoreactivity: association with endothelial dysfunction. *Am J Physiol Heart Circ Physiol* 291: H1856-1861, 2006.
118. **Lichtenstein AH, Appel LJ, Brands M, Carnethon M, Daniels S, Franch HA, Franklin B, Kris-Etherton P, Harris WS, Howard B, Karanja N, Lefevre M, Rudel L, Sacks F, Van Horn L, Winston M, and Wylie-Rosett J**. Diet and lifestyle recommendations revision 2006: a scientific statement from the American Heart Association Nutrition Committee. *Circulation* 114: 82-96, 2006.
119. **Loscalzo J**. Adverse effects of supplemental L-arginine in atherosclerosis: consequences of methylation stress in a complex catabolism? *Arterioscler Thromb Vasc Biol* 23: 3-5, 2003.
120. **Luscher TF, and Barton M**. Biology of the endothelium. *Clin Cardiol* 20: II-3-10, 1997.
121. **Lyons D, Roy S, Patel M, Benjamin N, and Swift CG**. Impaired nitric oxide-mediated vasodilatation and total body nitric oxide production in healthy old age. *Clin Sci (Lond)* 93: 519-525, 1997.
122. **MacAllister RJ, Fickling SA, Whitley GS, and Vallance P**. Metabolism of methylarginines by human vasculature; implications for the regulation of nitric oxide synthesis. *Br J Pharmacol* 112: 43-48, 1994.
123. **Malek AM, Alper SL, and Izumo S**. Hemodynamic shear stress and its role in atherosclerosis. *JAMA* 282: 2035-2042, 1999.
124. **Malek AM, Izumo S, and Alper SL**. Modulation by pathophysiological stimuli of the shear stress-induced up-regulation of endothelial nitric oxide synthase expression in endothelial cells. *Neurosurgery* 45: 334-344; discussion 344-335, 1999.
125. **Manach C, Scalbert A, Morand C, Remesy C, and Jimenez L**. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 79: 727-747, 2004.
126. **Manach C, Williamson G, Morand C, Scalbert A, and Remesy C**. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr* 81: 230S-242S, 2005.

127. **Martin HL, Loomis JL, and Kenney WL**. Maximal skin vascular conductance in subjects aged 5-85 yr. *J Appl Physiol* 79: 297-301, 1995.
128. **Matsushita H, Chang E, Glassford AJ, Cooke JP, Chiu CP, and Tsao PS**. eNOS activity is reduced in senescent human endothelial cells: Preservation by hTERT immortalization. *Circ Res* 89: 793-798, 2001.
129. **Matsuura E, Hughes GR, and Khamashta MA**. Oxidation of LDL and its clinical implication. *Autoimmun Rev* 7: 558-566, 2008.
130. **Matteis M, Troisi E, Monaldo BC, Caltagirone C, and Silvestrini M**. Age and sex differences in cerebral hemodynamics: a transcranial Doppler study. *Stroke* 29: 963-967, 1998.
131. **Mazza F, Goodman A, Lombardo G, Vanella A, and Abraham NG**. Heme oxygenase-1 gene expression attenuates angiotensin II-mediated DNA damage in endothelial cells. *Exp Biol Med (Maywood)* 228: 576-583, 2003.
132. **McCabe TJ, Fulton D, Roman LJ, and Sessa WC**. Enhanced electron flux and reduced calmodulin dissociation may explain "calcium-independent" eNOS activation by phosphorylation. *J Biol Chem* 275: 6123-6128, 2000.
133. **McCullough ML, Chevaux K, Jackson L, Preston M, Martinez G, Schmitz HH, Coletti C, Campos H, and Hollenberg NK**. Hypertension, the Kuna, and the epidemiology of flavanols. *J Cardiovasc Pharmacol* 47 Suppl 2: S103-109; discussion 119-121, 2006.
134. **McDermott JR**. Studies on the catabolism of Ng-methylarginine, Ng, Ng-dimethylarginine and Ng, Ng-dimethylarginine in the rabbit. *Biochem J* 154: 179-184, 1976.
135. **McGill HC, Jr.** George Lyman Duff memorial lecture. Persistent problems in the pathogenesis of atherosclerosis. *Arteriosclerosis* 4: 443-451, 1984.
136. **Medow MS, Minson CT, and Stewart JM**. Decreased microvascular nitric oxide-dependent vasodilation in postural tachycardia syndrome. *Circulation* 112: 2611-2618, 2005.
137. **Mellion BT, Ignarro LJ, Ohlstein EH, Pontecorvo EG, Hyman AL, and Kadowitz PJ**. Evidence for the inhibitory role of guanosine 3', 5'-monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related vasodilators. *Blood* 57: 946-955, 1981.
138. **Michell BJ, Chen Z, Tiganis T, Stapleton D, Katsis F, Power DA, Sim AT, and Kemp BE**. Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. *J Biol Chem* 276: 17625-17628, 2001.
139. **Milstien S, and Katusic Z**. Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. *Biochem Biophys Res Commun* 263: 681-684, 1999.
140. **Minamino T, and Komuro I**. Vascular cell senescence: contribution to atherosclerosis. *Circ Res* 100: 15-26, 2007.

141. **Minamino T, Miyauchi H, Yoshida T, Ishida Y, Yoshida H, and Komuro I**. Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction. *Circulation* 105: 1541-1544, 2002.
142. **Ming XF, Barandier C, Viswambharan H, Kwak BR, Mach F, Mazzolai L, Hayoz D, Ruffieux J, Rusconi S, Montani JP, and Yang Z**. Thrombin stimulates human endothelial arginase enzymatic activity via RhoA/ROCK pathway: implications for atherosclerotic endothelial dysfunction. *Circulation* 110: 3708-3714, 2004.
143. **Mink PJ, Scrafford CG, Barraj LM, Harnack L, Hong CP, Nettleton JA, and Jacobs DR, Jr.** Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women. *Am J Clin Nutr* 85: 895-909, 2007.
144. **Minson CT, Berry LT, and Joyner MJ**. Nitric oxide and neurally mediated regulation of skin blood flow during local heating. *J Appl Physiol* 91: 1619-1626, 2001.
145. **Minson CT, Holowatz LA, Wong BJ, Kenney WL, and Wilkins BW**. Decreased nitric oxide- and axon reflex-mediated cutaneous vasodilation with age during local heating. *J Appl Physiol* 93: 1644-1649, 2002.
146. **Miyazaki H, Matsuoka H, Cooke JP, Usui M, Ueda S, Okuda S, and Imaizumi T**. Endogenous nitric oxide synthase inhibitor: a novel marker of atherosclerosis. *Circulation* 99: 1141-1146, 1999.
147. **Monahan KD, Feehan RP, Kunselman AR, Preston AG, Miller DL, and Lott ME**. Dose-dependent increases in flow-mediated dilation following acute cocoa ingestion in healthy older adults. *J Appl Physiol* 111: 1568-1574, 2011.
148. **Moncada S, Palmer RM, and Higgs EA**. The discovery of nitric oxide as the endogenous nitrovasodilator. *Hypertension* 12: 365-372, 1988.
149. **Mount PF, Kemp BE, and Power DA**. Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation. *J Mol Cell Cardiol* 42: 271-279, 2007.
150. **Muller-Delp JM, Spier SA, Ramsey MW, and Delp MD**. Aging impairs endothelium-dependent vasodilation in rat skeletal muscle arterioles. *Am J Physiol Heart Circ Physiol* 283: H1662-1672, 2002.
151. **Mulvihill EE, and Huff MW**. Antiatherogenic properties of flavonoids: implications for cardiovascular health. *Can J Cardiol* 26 Suppl A: 17A-21A, 2010.
152. **Naseem KM**. The role of nitric oxide in cardiovascular diseases. *Mol Aspects Med* 26: 33-65, 2005.
153. **Natsume M, Osakabe N, Oyama M, Sasaki M, Baba S, Nakamura Y, Osawa T, and Terao J**. Structures of (-)-epicatechin glucuronide identified from plasma and urine after oral ingestion of (-)-epicatechin: differences between human and rat. *Free Radic Biol Med* 34: 840-849, 2003.
154. **Neveu V, Perez-Jimenez J, Vos F, Crespy V, du Chaffaut L, Mennen L, Knox C, Eisner R, Cruz J, Wishart D, and Scalbert A**. Phenol-Explorer: an online comprehensive database on polyphenol contents in foods. *Database (Oxford)* 2010: bap024, 2010.
155. **North BJ, and Sinclair DA**. The intersection between aging and cardiovascular disease. *Circ Res* 110: 1097-1108.

156. **O'Toole TE, Conklin DJ, and Bhatnagar A**. Environmental risk factors for heart disease. *Rev Environ Health* 23: 167-202, 2008.
157. **Ottaviani JI, Kwik-Uribe C, Keen CL, and Schroeter H**. Intake of dietary procyanidins does not contribute to the pool of circulating flavanols in humans. *Am J Clin Nutr* 95: 851-858, 2012a.
158. **Ottaviani JI, Momma TY, Kuhnle GK, Keen CL, and Schroeter H**. Structurally related (-)-epicatechin metabolites in humans: Assessment using de novo chemically synthesized authentic standards. *Free Radic Biol Med* 52: 1403-1412, 2012b.
159. **Palmer RM, Ashton DS, and Moncada S**. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 333: 664-666, 1988.
160. **Pritchard KA, Jr., Groszek L, Smalley DM, Sessa WC, Wu M, Villalon P, Wolin MS, and Stemerman MB**. Native low-density lipoprotein increases endothelial cell nitric oxide synthase generation of superoxide anion. *Circ Res* 77: 510-518, 1995.
161. **Ramirez-Sanchez I, Maya L, Ceballos G, and Villarreal F**. (-)-epicatechin activation of endothelial cell endothelial nitric oxide synthase, nitric oxide, and related signaling pathways. *Hypertension* 55: 1398-1405, 2010.
162. **Rees DD, Palmer RM, Schulz R, Hodson HF, and Moncada S**. Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br J Pharmacol* 101: 746-752, 1990.
163. **Reich T, and Rusinek H**. Cerebral cortical and white matter reactivity to carbon dioxide. *Stroke* 20: 453-457, 1989.
164. **Ried K, Sullivan T, Fakler P, Frank OR, and Stocks NP**. Does chocolate reduce blood pressure? A meta-analysis. *BMC Med* 8: 39, 2010.
165. **Ringelstein EB, Sievers C, Ecker S, Schneider PA, and Otis SM**. Noninvasive assessment of CO2-induced cerebral vasomotor response in normal individuals and patients with internal carotid artery occlusions. *Stroke* 19: 963-969, 1988.
166. **Rivard A, Fabre JE, Silver M, Chen D, Murohara T, Kearney M, Magner M, Asahara T, and Isner JM**. Age-dependent impairment of angiogenesis. *Circulation* 99: 111-120, 1999.
167. **Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Soliman EZ, Sorlie PD, Sotoodehnia N, Turan TN, Virani SS, Wong ND, Woo D, and Turner MB**. Heart Disease and Stroke Statistics--2012 Update: A Report From the American Heart Association. *Circulation* 2012.
168. **Rogers RL, Meyer JS, Mortel KF, Mahurin RK, and Thornby J**. Age-related reductions in cerebral vasomotor reactivity and the law of initial value: a 4-year prospective longitudinal study. *J Cereb Blood Flow Metab* 5: 79-85, 1985.
169. **Ross R**. Atherosclerosis--an inflammatory disease. *N Engl J Med* 340: 115-126, 1999.

170. **Ross R**. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362: 801-809, 1993.
171. **Rubbo H, Trostchansky A, Botti H, and Batthyany C**. Interactions of nitric oxide and peroxynitrite with low-density lipoprotein. *Biol Chem* 383: 547-552, 2002.
172. **Sakai Y, Masuda H, Kihara K, Kurosaki E, Yamauchi Y, and Azuma H**. Involvement of increased arginase activity in impaired cavernous relaxation with aging in the rabbit. *J Urol* 172: 369-373, 2004.
173. **Santhanam L, Christianson DW, Nyhan D, and Berkowitz DE**. Arginase and vascular aging. *J Appl Physiol* 105: 1632-1642, 2008.
174. **Santhanam L, Lim HK, Miriel V, Brown T, Patel M, Balanson S, Ryoo S, Anderson M, Irani K, Khanday F, Di Costanzo L, Nyhan D, Hare JM, Christianson DW, Rivers R, Shoukas A, and Berkowitz DE**. Inducible NO synthase dependent S-nitrosylation and activation of arginase1 contribute to age-related endothelial dysfunction. *Circ Res* 101: 692-702, 2007.
175. **Sax FL, Cannon RO, 3rd, Hanson C, and Epstein SE**. Impaired forearm vasodilator reserve in patients with microvascular angina. Evidence of a generalized disorder of vascular function? *N Engl J Med* 317: 1366-1370, 1987.
176. **Schachinger V, Britten MB, Elsner M, Walter DH, Scharrer I, and Zeiher AM**. A positive family history of premature coronary artery disease is associated with impaired endothelium-dependent coronary blood flow regulation. *Circulation* 100: 1502-1508, 1999.
177. **Schewe T, Steffen Y, and Sies H**. How do dietary flavanols improve vascular function? A position paper. *Arch Biochem Biophys* 476: 102-106, 2008.
178. **Schnorr O, Brossette T, Momma TY, Kleinbongard P, Keen CL, Schroeter H, and Sies H**. Cocoa flavanols lower vascular arginase activity in human endothelial cells in vitro and in erythrocytes in vivo. *Arch Biochem Biophys* 476: 211-215, 2008.
179. **Schroeder P, Klotz LO, and Sies H**. Amphiphilic properties of (-)-epicatechin and their significance for protection of cells against peroxynitrite. *Biochem Biophys Res Commun* 307: 69-73, 2003.
180. **Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK, Sies H, Kwik-Uribe C, Schmitz HH, and Kelm M**. (-)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proc Natl Acad Sci U S A* 103: 1024-1029, 2006.
181. **Segarra G, Medina P, Ballester RM, Lluch P, Aldasoro M, Vila JM, Lluch S, and Pelligrino DA**. Effects of some guanidino compounds on human cerebral arteries. *Stroke* 30: 2206-2210; discussion 2210-2211, 1999.
182. **Serrador JM, Picot PA, Rutt BK, Shoemaker JK, and Bondar RL**. MRI measures of middle cerebral artery diameter in conscious humans during simulated orthostasis. *Stroke* 31: 1672-1678, 2000.
183. **Shastry S, and Joyner MJ**. Geldanamycin attenuates NO-mediated dilation in human skin. *Am J Physiol Heart Circ Physiol* 282: H232-236, 2002.
184. **Shock NW, R. C. Greulich, R. A. Andres, D. Arenberg, P. T. Costa, E. G. Lakatta, and J. D. Toblin.** Normal Human Aging: The Baltimore Longitudinal Study of

Aging. In: *84-2450*, edited by Health NI. Washington, D. C.: US Govt. Printing Office, 1984, p. 45-57.
185. **Silvestrini M, Vernieri F, Pasqualetti P, Matteis M, Passarelli F, Troisi E, and Caltagirone C**. Impaired cerebral vasoreactivity and risk of stroke in patients with asymptomatic carotid artery stenosis. *JAMA* 283: 2122-2127, 2000.
186. **Singer HA, and Peach MJ**. Calcium- and endothelial-mediated vascular smooth muscle relaxation in rabbit aorta. *Hypertension* 4: 19-25, 1982.
187. **Singh N, Prasad S, Singer DR, and MacAllister RJ**. Ageing is associated with impairment of nitric oxide and prostanoid dilator pathways in the human forearm. *Clin Sci (Lond)* 102: 595-600, 2002.
188. **Smith AR, and Hagen TM**. Vascular endothelial dysfunction in aging: loss of Akt-dependent endothelial nitric oxide synthase phosphorylation and partial restoration by (R)-alpha-lipoic acid. *Biochem Soc Trans* 31: 1447-1449, 2003.
189. **Soucy KG, Ryoo S, Benjo A, Lim HK, Gupta G, Sohi JS, Elser J, Aon MA, Nyhan D, Shoukas AA, and Berkowitz DE**. Impaired shear stress-induced nitric oxide production through decreased NOS phosphorylation contributes to age-related vascular stiffness. *J Appl Physiol* 101: 1751-1759, 2006.
190. **Stary HC**. Evolution and progression of atherosclerotic lesions in coronary arteries of children and young adults. *Arteriosclerosis* 9: I19-32, 1989.
191. **Steffen Y, Gruber C, Schewe T, and Sies H**. Mono-O-methylated flavanols and other flavonoids as inhibitors of endothelial NADPH oxidase. *Arch Biochem Biophys* 469: 209-219, 2008.
192. **Steffen Y, Jung T, Klotz LO, Schewe T, Grune T, and Sies H**. Protein modification elicited by oxidized low-density lipoprotein (LDL) in endothelial cells: protection by (-)-epicatechin. *Free Radic Biol Med* 42: 955-970, 2007.
193. **Steffen Y, Schewe T, and Sies H**. (-)-Epicatechin elevates nitric oxide in endothelial cells via inhibition of NADPH oxidase. *Biochem Biophys Res Commun* 359: 828-833, 2007a.
194. **Steffen Y, Schewe T, and Sies H**. Epicatechin protects endothelial cells against oxidized LDL and maintains NO synthase. *Biochem Biophys Res Commun* 331: 1277-1283, 2005.
195. **Steinbrecher UP, Zhang HF, and Lougheed M**. Role of oxidatively modified LDL in atherosclerosis. *Free Radic Biol Med* 9: 155-168, 1990.
196. **Surdacki A, Nowicki M, Sandmann J, Tsikas D, Boeger RH, Bode-Boeger SM, Kruszelnicka-Kwiatkowska O, Kokot F, Dubiel JS, and Froelich JC**. Reduced urinary excretion of nitric oxide metabolites and increased plasma levels of asymmetric dimethylarginine in men with essential hypertension. *J Cardiovasc Pharmacol* 33: 652-658, 1999.
197. **Suwaidi JA, Hamasaki S, Higano ST, Nishimura RA, Holmes DR, Jr., and Lerman A**. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. *Circulation* 101: 948-954, 2000.

198. **Taddei S, Virdis A, Mattei P, Ghiadoni L, Gennari A, Fasolo CB, Sudano I, and Salvetti A**. Aging and endothelial function in normotensive subjects and patients with essential hypertension. *Circulation* 91: 1981-1987, 1995.
199. **Tanabe T, Maeda S, Miyauchi T, Iemitsu M, Takanashi M, Irukayama-Tomobe Y, Yokota T, Ohmori H, and Matsuda M**. Exercise training improves ageing-induced decrease in eNOS expression of the aorta. *Acta Physiol Scand* 178: 3-10, 2003.
200. **Taubert D, Roesen R, and Schomig E**. Effect of cocoa and tea intake on blood pressure: a meta-analysis. *Arch Intern Med* 167: 626-634, 2007.
201. **Tschudi MR, Barton M, Bersinger NA, Moreau P, Cosentino F, Noll G, Malinski T, and Luscher TF**. Effect of age on kinetics of nitric oxide release in rat aorta and pulmonary artery. *J Clin Invest* 98: 899-905, 1996.
202. **Tsuda Y, and Hartmann A**. Changes in hyperfrontality of cerebral blood flow and carbon dioxide reactivity with age. *Stroke* 20: 1667-1673, 1989.
203. **Turner J, Belch JJ, and Khan F**. Current concepts in assessment of microvascular endothelial function using laser Doppler imaging and iontophoresis. *Trends Cardiovasc Med* 18: 109-116, 2008.
204. **Turrens JF**. Superoxide production by the mitochondrial respiratory chain. *Biosci Rep* 17: 3-8, 1997.
205. **Ushio-Fukai M**. Redox signaling in angiogenesis: role of NADPH oxidase. *Cardiovasc Res* 71: 226-235, 2006.
206. **Valkonen VP, Paiva H, Salonen JT, Lakka TA, Lehtimaki T, Laakso J, and Laaksonen R**. Risk of acute coronary events and serum concentration of asymmetrical dimethylarginine. *Lancet* 358: 2127-2128, 2001.
207. **Vallance P, Leone A, Calver A, Collier J, and Moncada S**. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet* 339: 572-575, 1992.
208. **Vasa M, Breitschopf K, Zeiher AM, and Dimmeler S**. Nitric oxide activates telomerase and delays endothelial cell senescence. *Circ Res* 87: 540-542, 2000.
209. **Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, and Pritchard KA, Jr.** Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci U S A* 95: 9220-9225, 1998.
210. **Verma S, Buchanan MR, and Anderson TJ**. Endothelial function testing as a biomarker of vascular disease. *Circulation* 108: 2054-2059, 2003.
211. **Vicenzini E, Ricciardi MC, Altieri M, Puccinelli F, Bonaffini N, Di Piero V, and Lenzi GL**. Cerebrovascular reactivity in degenerative and vascular dementia: a transcranial Doppler study. *Eur Neurol* 58: 84-89, 2007.
212. **Vincent GK, and Velkoff VA**. *The next four decades: The older population in the United States: 2010 to 2050*. US Department of Commerce, Economics and Statistics Administration, US Census Bureau, 2010.
213. **Vita JA, Treasure CB, Nabel EG, McLenachan JM, Fish RD, Yeung AC, Vekshtein VI, Selwyn AP, and Ganz P**. Coronary vasomotor response to acetylcholine relates to risk factors for coronary artery disease. *Circulation* 81: 491-497, 1990.

214. **White AR, Ryoo S, Li D, Champion HC, Steppan J, Wang D, Nyhan D, Shoukas AA, Hare JM, and Berkowitz DE**. Knockdown of arginase I restores NO signaling in the vasculature of old rats. *Hypertension* 47: 245-251, 2006.
215. **Xin MG, Zhang J, Block ER, and Patel JM**. Senescence-enhanced oxidative stress is associated with deficiency of mitochondrial cytochrome c oxidase in vascular endothelial cells. *Mech Ageing Dev* 124: 911-919, 2003.
216. **Yang Z, and Ming XF**. Endothelial arginase: a new target in atherosclerosis. *Curr Hypertens Rep* 8: 54-59, 2006.
217. **Yasue H, Matsuyama K, Okumura K, Morikami Y, and Ogawa H**. Responses of angiographically normal human coronary arteries to intracoronary injection of acetylcholine by age and segment. Possible role of early coronary atherosclerosis. *Circulation* 81: 482-490, 1990.
218. **Zimmermann C, and Haberl RL**. L-arginine improves diminished cerebral CO2 reactivity in patients. *Stroke* 34: 643-647, 2003.