OXIDATIVE STRESS AND MICROVASCULAR FUNCTION IN CHRONIC KIDNEY DISEASE

by

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My family and friends for their love and support.

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ABSTRACT

Patients with chronic kidney disease (CKD) are at risk of progressing towards end stage renal disease (ESRD), however, they are more likely to die from cardiovascular disease (CVD) before reaching ESRD. Endothelial dysfunction has been shown to play a role in the progression of CKD as well as the development of atherosclerosis however, the mechanisms by which endothelial dysfunction occurs in the CKD population are not clear. Therefore, the specific aims of this study were to determine whether oxidative stress or a relative deficit in L-arginine plays a mechanistic role in reducing NO-mediated cutaneous vasodilation in response to local heating in patients with moderate to severe CKD. **Methods:** We measured red blood cell (RBC) flux via laser Doppler flowmetry in conjunction with intradermal microdialysis of 1) Ringer's solution, 2) 20 mM ascorbic acid, 3) 10 mM L-arginine, and 4) 10 mM *N*G-nitro-L-arginine methyl ester (L-NAME). We hypothesized that nitric-oxide mediated cutaneous vasodilation would be impaired in the CKD population but normalized by the intradermal microdialysis of ascorbic acid and L-arginine. Eight healthy and eight stage 3-4 CKD patients were instrumented with four microdialysis (MD) fibers in the ventral side of the non-dominant forearm. A standardized non-painful local heating protocol (42˚C) was used to elicit cutaneous vasodilation. 28 mM sodium nitroprusside was perfused into all four MD sites and local temperature was increased to 43˚C in order to achieve a maximal dilation in all sites. Cutaneous vascular conductance (CVC) was calculated at RBC flux/MAP. All

data are expressed as a percentage of the maximum CVC achieved in each MD site. **Results:** There were no differences in baseline %CVCmax among group and treatment; Ringer: CKD; 12 ± 1, HC; 9 ± 1, AA: CKD; 13 ± 3, HC; 11 ± 1, L-arg: CKD; 11 ± 2, HC; 13 ± 2, L-NAME: CKD; 11 ± 2, HC; 12 ± 1; ($p > 0.05$). The NO-mediated plateau was attenuated in CKD compared to HC's; CKD: 76 ± 4, HC: 91 ± 2, ($p < 0.05$). The attenuation was significantly improved by AA and L-arg in the CKD group; AA: 89 ± 2, L-arg: 90 ± 1, ($p < 0.05$). There was a significant difference in the initial peak between the HC and CKD groups at the control site (R) and L-arg site; R: HC; 71 ± 4, CKD; 53 ± 5 ($p <0.05$), L-arg: HC: 68 ± 3, CKD; 47 ± 4 ($p < 0.01$). **Conclusion:** NO-mediated cutaneous vasodilation and the axon-reflex are impaired in the CKD population compared to healthy age and gender matched controls. The impairment in NO-mediated cutaneous vasodilation was normalized by the perfusion of ascorbic acid and L-arginine, indicating that oxidative stress and a relative deficit of L-arginine are mechanisms by which endothelial dysfunction occurs in stage 3-4 CKD.

Chapter 1

INTRODUCTION

Cardiovascular disease (CVD) is the major cause of death among chronic kidney disease (CKD) patients (1, 2). Patients with a glomerular filtration rate (GFR) of less than 60 mL/min/1.73m2 are classified as stage 3-5 CKD. These patients are at risk of progressing towards end stage renal disease (ESRD), however, they are more likely to die of CVD before reaching ESRD (3). Cardiovascular related causes of death in CKD patients are 10-20 times higher than in the general population (1, 2). The high incidence of CVD in CKD patients cannot solely be due to conventional risk factors such as hypertension, dyslipidemia, diabetes mellitus, and aging, which have all been shown to be present in CKD patients (4, 5).

Endothelial dysfunction has been shown to play a role in the progression of CKD as well as the development of atherosclerosis (1). The mechanisms by which endothelial dysfunction occurs in the CKD population are less clear, supporting the need for further research to understand the mechanisms of endothelial dysfunction in moderate to severe CKD patients.

The endothelium is responsible for maintaining vascular homeostasis, vascular tone and structure as well as mediating the vascular response to hemodynamic forces, humoral stimulation, and neuronal activation (6). One of the most important endothelium-derived vasoactive substances is nitric oxide (NO). NO is formed from its amino acid precursor L-arginine by nitric oxide synthase (NOS). Endothelial dysfunction is characterized by a decreased bioavailability of NO. The decrease in NO

may, in part, be due to increased oxidative stress as well as the actions of asymmetric dimethylarginine (ADMA), which was first shown in 1992 to be an endogenous inhibitor of NOS (7). Plasma levels of ADMA have been associated with a variety of cardiovascular risk factors and CKD (8).

Oxidative stress has been shown to decrease NO availability through the reaction of the reactive oxygen species (ROS) superoxide and NO, resulting in the formation of peroxynitrite, a powerful oxidant (9). ROS also promote the oxidation of NOS cofactor tetrahydrobiopterin (BH4) (9). This leads to endothelial NOS uncoupling, decreased NO production, and increased superoxide production (9).

Acute intra-arterial administration of vitamin C into the forearm increases endothelium-dependent dilation in the resistance vasculature of pre-dialysis renal failure patients, suggesting that oxidative stress is a mechanism of endothelial dysfunction in renal disease but it is unclear whether this occurs earlier in the progression of CKD (10).

Methylarginines, including ADMA, are eliminated partly by renal excretion (11). In CKD, ADMA levels are elevated due to diminished renal excretion. The enzymes involved in the metabolism of ADMA, protein arginine N-methyltransferases (PRMTs), as well as dimethylarginine dimethylaminohydrolase (DDAH) are redox sensitive (11, 12) with an increase in oxidative stress leading to an increased synthesis and decreased degradation of ADMA. ADMA inhibits endogenous NOS by competing with L-arginine, leading to eNOS uncoupling. Therefore, elevated ADMA levels may result in a relative deficit of L-arginine through competition for eNOS; Hence, both oxidative stress and a relative deficit in L-arginine may be mechanisms of endothelial dysfunction in early stages of CKD. The aims of this study

were 1) to determine whether oxidative stress plays a mechanistic role in nitric oxide mediated cutaneous vasodilation in response to local heating in patients with moderate to severe CKD and 2) to determine whether a relative deficit of L-arginine contributes to endothelial dysfunction in CKD. We hypothesized that nitric oxide mediated cutaneous vasodilation in response to local heating will be lower in CKD patients compared to healthy control subjects but will be improved by intradermal microdialysis of ascorbic acid and L-arginine.

Chapter 2

METHODS

Participants

Eight individuals (5 male, 3 female), age 26 – 74 years, with stage 3-4 chronic kidney disease (CKD) were recruited for this study. Stage 3-4 CKD is characterized by a glomerular filtration rate (GFR) of <60 and >15 ml/min^{-1}/1.73m^2 (13). GFR was estimated (eGFR) using the Modification of Diet in Renal Disease equation based on serum Creatinine, age, gender, and race (14).

The exclusion criteria for the CKD patients included the following:

- History of angina, myocardial infarction, or heart failure
- Screening lab indication of hepatic disease
- Current drug treatment for pulmonary, autoimmune, or HIV diseases
- Current tobacco use

Eight age and sex matched apparently healthy individuals were also recruited to serve as healthy controls (HC). The healthy participants were required to have an eGFR of >60 ml/min as well as being free from disease as assessed by medical history, resting EKG, and screening labs.

Premenopausal women were studied during the early follicular phase of the menstrual cycle to control for any effects of the menstrual cycle on vascular function. The study was approved by the Human Subjects Review Board and all subjects gave oral and written consent prior to participation.

Procedures

Blood and urine samples were collected at a screening visit and sent to the local clinical laboratory to asses liver enzymes, lipid profile, renal function, complete blood count, hemoglobin, hematocrit, glucose, and urinary albumin to creatinine ratio. Medical history forms and resting EKG data were also collected.

The testing visit consisted of microdialysis fiber insertion along with measurements of cutaneous red blood cell flux in response to local heating of the skin to 42 degrees Celsius.

Microdialysis Fiber Insertion & Measurements

Four intradermal microdialysis fibers (MD 2000, Bioanalytical Systems) were inserted into the ventral forearm skin of the non-dominant arm of each subject. Each MD fiber was placed by the insertion of a 25-gauge needle through the dermis with a sterile technique after a 10 minute application of ice to the skin surface to achieve short-term local anesthesia. The entry and exit points of the needle were approximately 2.5 cm apart. The MD fibers were then threaded through the lumen of the needle, which was then removed, leaving the fibers in place. The fibers were taped down in place.

Cutaneous red blood cell (RBC) flux (a measurement of skin blood flow) was measured with a laser-Doppler flowmeter probe placed in a local heater (MoorLab, Temperature Monitor SH02, Moor instruments, Devon, UK) directly on the skin above each microdialysis site. Blood pressure was measured every 10 minutes by an automated oscillometric sphygmomanometer (Dinamap Dash 2000, GE Medical Systems).

Protocol

A standardized non painful local heating protocol was used (15). After the fibers were placed, Ringer's solution was infused at a rate of 2µl/min and RBC flux was monitored to verify that initial hyperemia from insertion trauma had subsided before starting the study protocol. Following, the local heaters were set to 33˚C. The four MD sites were randomly assigned to receive either 1) Ringer's solution (control site), 2) 10 mM NG-nitro-L-arginine methyl ester (L-NAME; Calbiochem) dissolved in Ringer's solution, serving as the NOS inhibition site, 3) 20 mM ascorbic acid dissolved in Ringer solution to serve as a treatment site or 4) 10 mM 2-Amino-5-guanidinopentanoic acid (L-Arginine) to serve as another treatment site. Each site was infused at a rate of 2µl/min for at least 30 minutes. After the baseline period of 33˚C, the local temperature was increased 0.5˚C every 5 seconds until it reached 42˚C, which was held constant for the local heating protocol. Local heating of the skin to 42˚C results in vasodilation that is due to at least two independent mechanisms. An initial peak in cutaneous blood flow occurs in the first 10 minutes and is due to an axon reflex (15). Following, a nadir and a secondary plateau in cutaneous blood flow occurs after approximately 30 minutes of heating and is largely mediated by NO (15, 16). Once the RBC flux reached a stable plateau, around 40 minutes, the local heaters were set to 43˚C and 28 mM sodium nitroprusside (SNP) (Nitropress; Hospira Inc.) was infused into all four MD sites in order to determine a maximum cutaneous vascular conductance (CVC). The doses of L-NAME and SNP were chosen because they have been shown to sufficiently inhibit nitric oxide synthase and maximally vasodilate the skin, respectively (17).

Data Processing

Data were collected at 40 Hz and compressed into one minute averages. CVC was calculated as RBC flux divided by mean arterial pressure. %CVCmax was calculated as CVC/CVCmax*100. Each site was normalized to its own maximum CVC value. Baseline and plateau CVC were collected over a stable 10 minute period. Initial peak and nadir CVC were calculated by averaging the highest and lowest values over a stable 60 second period. In order to assess the contribution of NO to cutaneous vasodilation, the plateau %CVCmax at the L-NAME (NOS inhibition) site was subtracted from the control site (Ringer's) and compared using an unpaired t-test. Data were expressed as a percentage of maximal CVC measured during SNP infusion.

Statistical Analysis

A 2 x 4 ANOVA was used to determine the effect of group, treatment, and group x treatment interaction on %CVCmax. Group is a between subjects factor while treatment is a within subjects factor. A Tukey post hoc was performed in order to determine between and within group differences when appropriate. Unpaired t-tests were used to compare group demographic information. Alpha level was set at 0.05 for all statistical tests. Data are presented as means ± SE.

Chapter 3

RESULTS

Subject characteristics. The characteristics of the CKD and healthy control groups can be found in table 1. A total of 16 subjects were recruited for study participation including 8 chronic kidney disease patients in stage 3-4 CKD with an average eGFR of 37.1 ± 5.2 (ml/min/1.73m²) and 8 apparently healthy individuals with an eGFR > 60 ml/min/1.73m². As expected, CKD patients also had significantly higher blood urea nitrogen and serum creatinine ($p<0.01$). None of the healthy control subjects were taking any medications. However, all of the CKD patients were taking antihypertensive medications as well as a variety of other medications. CKD patients had significantly higher systolic blood pressure and mean arterial pressure than controls ($p< 0.05$).

Cutaneous vascular conductance. There were no differences in baseline %CVCmax between groups or across MD sites; Ringer's: CKD; 12 ± 1, HC; 9 ± 1, AA: CKD; 13 ± 3, HC; 12 ± 1, L-arg: CKD; 11 ± 2, HC; 13 ± 2, L-NAME: CKD; 11 ± 2, HC; 12 ± 1 %CVCmax; ($p > 0.05$).

Table 1 ***Subject characteristics.*** **Values are means ± SE. n, no of subjects; CKD, chronic kidney disease; BMI, body mass index; ACE, angiotensin converting enzyme; ANG, angiotensin; * p < 0.05, ** p < 0.01**

	Control	CKD
	n= 8	n = 8
Demographic Information		
Age (yr)	48 ± 5	52 ± 6
Height (cm)	168 ± 3	170 ± 4
Weight (kg)	72 ± 4	81 + 6
BMI (kg/m²)	25 ± 1	28 ± 1
Hemodynamic Measurements		
Heart rate (b/min)	63 ± 3	64 ± 3
Systolic blood pressure (mmHg)	112 ± 4	139 ± 5**
Diastolic blood pressure (mmHg)	69 ± 3	78 ± 5
MAP (mmHg)	83 ± 3	95 ± 4*
Renal Function		
Blood urea nitrogen (mg/dl)	17 ± 1.3	40 ± 7.4**
Serum creatinine (mg/dl)	0.9 ± 0.03	2.1 ± 0.3**
eGFR (ml/min/1.73m²)	> 60	37.1 ± 5.2
Blood Lipids, Cells, and Glucose		
Total Cholesterol (mg/dl)	195.7 ± 12.5	193.6 ± 12.9
High-Density Lipoprotein (mg/dl)	58.9 ± 4	49.9 ± 7.4
Low-Density Lipoprotein (mg/dl)	118.9 ± 11.9	114.6 ± 9.9
Triglycerides (mg/dl)	90.1 ± 13.5	147.1 ± 24.8
Hemoglobin (mg/dl)	13.8 ± 0.2	12.5 ± 0.8
Hematocrit (%)	40.9 ± 0.5	38.3 ± 2
Glucose (mg/dl)	86.9 ± 2.5	90.3 ± 8
Medications (Number of Patients)		
Antihypertensives	0	8
ACE Inhibitors	0	4
ANG II-receptor Antagonists	0	2
Beta-Blockers	0	2
Diuretics	0	2
Calcium Antagonists	0	6
Statins	0	5
Insulin	0	3
Erythropoiesis-Stimulating Agents	0	2
Allopurinol	0	2

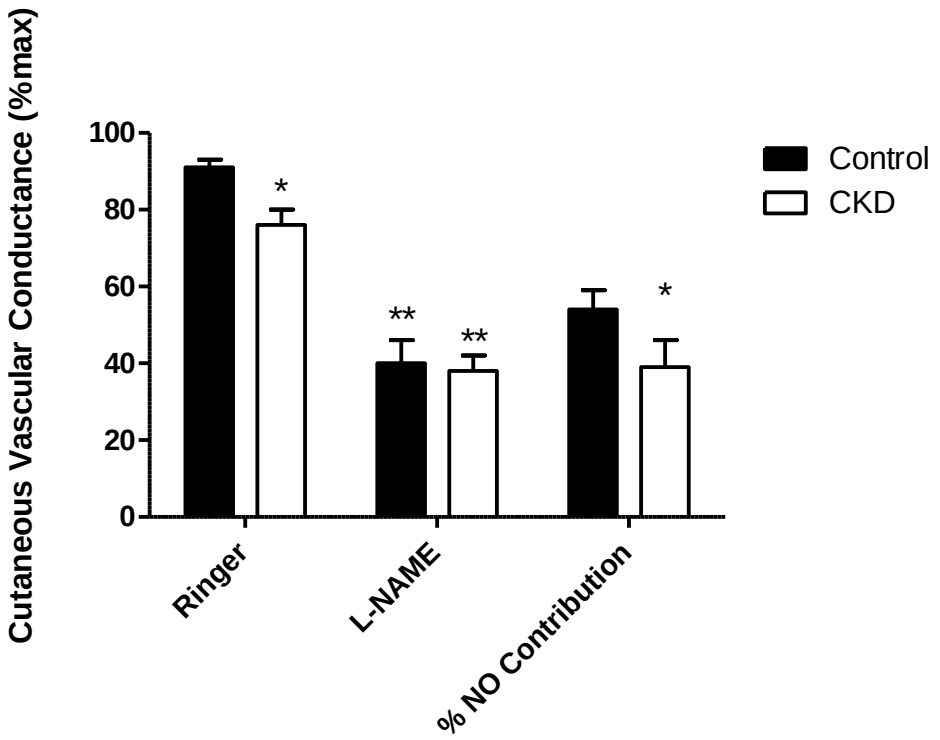

Figure 1 **%NO contribution in Control and CKD. * p < 0.05 from Control Ringer and %NO contribution. **p < 0.05 from CKD and Control Ringer.**

Figure 1 displays the %NO contribution to the plateau in cutaneous vasodilation. %NO contribution was significantly decreased in the CKD group; HC: 54 ± 5, CKD: 39 ± 7%, ($p < 0.05$). There was a significantly lower plateau %CVCmax at the Ringer's site in the CKD group; 76 ± 4 vs. 91 ± 2 %CVCmax, ($p < 0.05$). L-NAME significantly attenuated the plateau in the CKD and control groups; 38 ± 4, 40 ± 6 %CVCmax, respectively. Figure 2 displays the plateau %CVCmax in the ascorbic acid site and L-arginine site of both groups. There were no differences in %CVCmax

between the groups at the two treatment sites; ascorbic acid and L-arginine. %CVCmax was significantly greater at the ascorbic acid and L-arginine sites compared to Ringer in the CKD group; 89 ± 2, 90 ± 1 %CVCmax ($p < 0.05$), respectively and not different from HC ($p > 0.05$) (Figure 2).

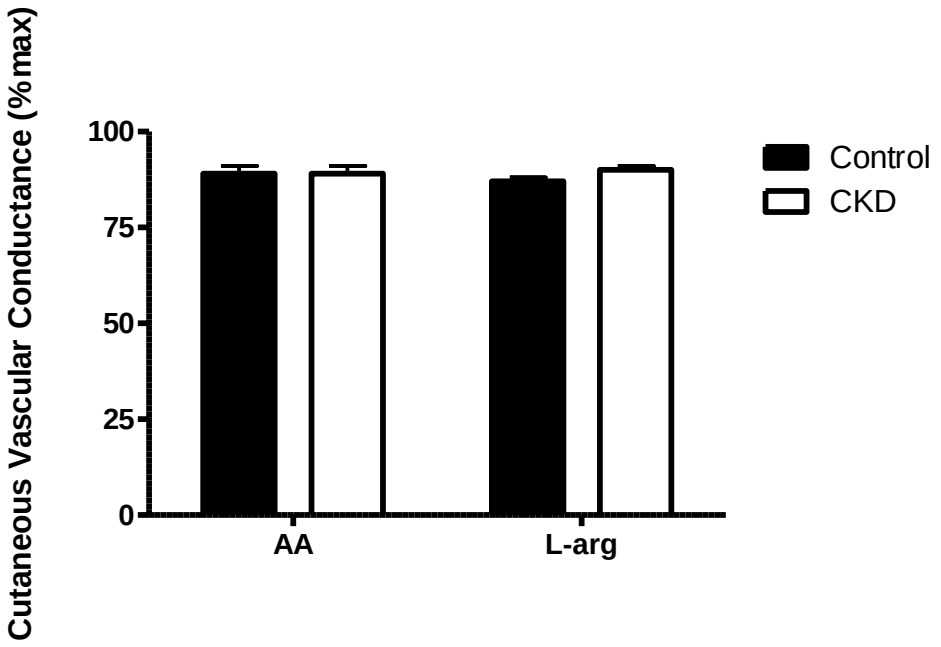

Figure 2. **Figure 2. AA and L-arginine plateau responses in both groups. No differences between groups among the two treatments ($p > 0.05$).**

Figure 3 displays the initial peak %CVCmax values. Initial peak %CVCmax was significantly attenuated at the Ringer's and L-arginine sites in the CKD group compared to HC; Ringer: CKD; 53 ± 5 vs. HC; 71 ± 4, L-arginine: CKD; 47 ± 4 vs. HC; 68 ± 3 %CVCmax ($p < 0.05$). However, initial peak %CVCmax did not differ between groups at the ascorbic acid site; CKD: 59 ± 5, HC: 66 ± 5 %CVCmax.

There was no difference in initial peak %CVCmax between groups at the L-NAME site ($p > 0.05$). There were no differences in nadir between groups or across all four MD sites ($p > 0.05$).

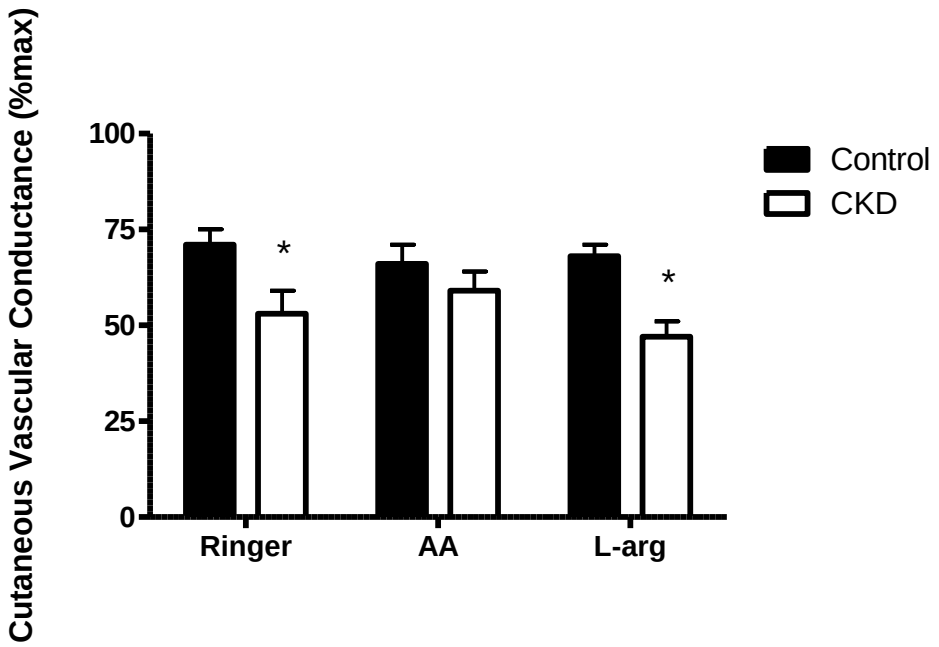

Figure 3 **Initial peak %CVCmax in healthy (n = 8) control and CKD (n = 8) subjects. Initial peak %CVCmax was significantly reduced in the CKD group compared to healthy controls at the Ringer and L-arginine sites. * $p < 0.05$ from Control Ringer and Control L-arginine.**

Chapter 4

DISCUSSION

The primary findings of this investigation are 1) NO-mediated cutaneous vasodilation in response to local heating is impaired in stages 3-4 CKD compared to age and sex matched apparently healthy individuals but improved with the intradermal microdialysis of ascorbic acid and L-arginine and 2) the axon reflex-mediated cutaneous vasodilation in response to local heating is also impaired in stages 3-4 CKD compared to age and sex matched apparently healthy individuals. These findings indicate that oxidative stress and a relative deficit of L-arginine are mechanisms by which microvascular NO dependent vascular dysfunction occurs in CKD. Additionally, the impaired axon reflex indicates neurovascular dysfunction which may indicate early peripheral neuropathy. Importantly, none of the CKD patients in the present study presented with symptomatic neuropathy, indicating that declines in neurovascular function may precede overt peripheral neuropathy in early stages of CKD.

Previous literature has shown impaired endothelial function in CKD patients. Ghiadoni et al observed an impairment in flow-mediated dilation (FMD) of the brachial artery in stages 3-5 CKD as well as hemodialysis patients compared to healthy control subjects (18). Impaired relaxation responses to acetylcholine, an endothelial-dependent dilator, were observed in subcutaneous resistance vessels of chronic renal failure patients. Notably, the dilator response to sodium nitroprusside, an endothelim-independent dilator, was normal, indicating that a reduction in endothelial

derived bioavailability of NO was most likely responsible for the impaired response to acetylcholine (19). Impaired forearm blood flow responses to methacholine were also observed in moderate chronic renal failure patients (20). We found a reduced NO mediated cutaneous vasodilation in response to local heating in stage 3-4 CKD. Our findings indicate that NO mediated dilation is impaired early in the progression of CKD.

NO-mediated cutaneous vasodilation was improved by the intradermal microdialysis of ascorbic acid in CKD, indicating that oxidative stress is a mechanism by which endothelial dysfunction occurs in these patients. This finding is consistent with those of previous studies in pre-dialysis renal failure patients who are more advanced than the stage 3-4 CKD studied in the present investigation. Acute administration of ascorbic acid in pre-dialysis patients increased acetylcholine-induced forearm blood flow (10). Similar findings have been reported in patients on hemodialysis (18). Ghiadoni et al showed that endothelium-dependent dilation, as assessed by brachial artery flow-mediated dilation, was impaired in hemodialysis patients compared to healthy control subjects but improved with an oral supplementation of vitamin C (18). Our results indicate that oxidative stress results in impaired NO-mediated dilation prior to ESRD and hemodialysis.

An increase in reactive oxygen species (ROS) production most likely results in a reduction of NO bioactivity via superoxide and oxidation of NOS cofactor tetrahydrobiopterin (BH4). The reaction of superoxide and NO results in the formation of peroxynitrite (ONOO-), a powerful oxidant capable of cellular damage (9). Additionally, ROS promote the breakdown of BH4. The degradation and oxidation of BH4 leads to the uncoupling of eNOS, decreased NO production, and increased

superoxide production (1, 21). ONOO- along with free radicals can modify arachidonic acid and low-density lipoprotein to generate isoprostanes, which have been used as markers of oxidative stress (9). Isoprostanes also exhibit vasoconstrictor effects on vascular smooth muscle cells. Specifically, hydroxyl radicals and oxidized low-density lipoproteins (OxLDL) contribute to endothelial dysfunction with proatherogenic effects; the production of foam cells and secretion of inflammatory cytokines, which stimulate platelet activation (22). Several studies report elevated markers of oxidative stress in the CKD population. In stages 3-5 CKD, plasma protein carbonyl, F2-isoprostanes, and reduced plasma protein thiols were found to be significantly higher compared to healthy control subjects (23). Additionally, oxLDL was shown to be an independent predictor of endothelium-dependent dilation in all stages of CKD, indicating that oxidative stress likely plays a mechanistic role in endothelial dysfunction of CKD (1). Our results using ascorbic acid to scavenge ROS indicate functional impairment in the microvasculature in CKD as a result of oxidative stress.

NO-mediated cutaneous vasodilation in the CKD group was normalized by the intradermal microdialysis of L-arginine, suggesting that a deficit of L-arginine contributes to endothelial dysfunction in this patient population. ADMA is a naturally occurring amino acid as well as an endogenous inhibitor of eNOS through competing with L-arginine for eNOS (7). Plasma ADMA levels increase due to diminished renal excretory abilities in CKD (6), therefore an increase in ADMA may ultimately lead to increased eNOS uncoupling and decreased NO bioavailability. Elevated levels of ADMA have also been shown to be an independent determinant of brachial artery flow-mediated dilation, a measurement of endothelium-dependent dilation in all stages

of CKD (1). It has also recently been shown that in primarily non-diabetic, stages 3- 4 CKD patients, ADMA was strongly associated with the prevalence of CVD and moderately associated with all-cause and CVD mortality (24).

ADMA becomes synthesized when arginine residues in proteins are methylated by protein arginine methyltransferases (PRMTs) (11). The amount of ADMA synthesized within a cell depends on the amount of arginine methylation in proteins and the rates of protein turnover. It is unclear whether ADMA production is relatively constant, if it changes with PRMT activity or if the rates of protein turnover are the most influential (11). However, it has been shown that there is a relationship between PRMT expression levels and free ADMA production (11). Furthermore, increased free radicals lead to an increase in PRMT activity, ultimately increasing the production of ADMA (23). Renal failure and uremia-related oxidative stress and uremic toxins such as homocysteine and advanced glycation end-products decrease by dimethylarginine dimethylaminohydrolase (DDAH) activity and may also contribute to the increased levels of ADMA. (8). Therefore, oxidative stress is a potential mechanism by which PRMT activity is increased and DDAH activity is decreased, leading to increases in ADMA levels in CKD.

In addition to competition with ADMA, L-arginine availability may be reduced by increased arginase activity. A majority of L-arginine is synthesized in the liver but also metabolized by Arginase I. Arginase I and II compete with NOS for L-arginine, thus they possess the ability to limit NO production as NO production has been attenuated in endothelial cells expressing Arginase I and II (25). Inhibition of arginase has also been shown to protect the kidney from structural damage in the

animal model of CKD, suggesting that arginase inhibition may be a novel approach in slowing renal damage (26).

Our unexpected finding of an attenuated axon-reflex in the CKD group compared to healthy controls presents the need for further investigation into the mechanism. The bimodal skin blood flow response to local warming consists of the initial axon-reflex mediated peak followed by a sustained NO-mediated plateau. The initial peak is primarily mediated by the release of neurotransmitters as well as a small NO component (15). The neurotransmitters stimulate a small amount of NO release via a shear stress mechanism. The most plausible neurotransmitters involved in this response are calcitonin gene-related peptide (CGRP) and substance P (15). Interestingly, 4 of the 8 CKD patients studied in this investigation were diabetic, although none of them had peripheral neuropathy. A recent investigation by Strom et al found no difference in the axon-reflex mechanism in a group of type 2 diabetic patients compared to healthy controls (27). Our data suggests that perhaps an early onset of neurovascular dysfunction occurs in CKD, independent of overt symptoms of peripheral neuropathy. This is potentially a significant clinical finding and requires further study.

In summary, this investigation found that NO-mediated cutaneous vasodilation is impaired in stages 3-4 CKD compared to healthy individuals. This impairment was normalized by the intradermal microdialysis of ascorbic acid and L-arginine in CKD. Therefore, our findings suggest that oxidative stress and a relative deficit of L-arginine are mechanisms of endothelial dysfunction in stages 3-4 CKD. Clinically, this investigation represents possible future antioxidant therapies. Chronic antioxidant therapy may potentially combat increases in oxidative stress, ultimately

slowing the progression of CKD and decreasing cardiovascular events. Future studies should also attempt to determine the source of ROS, for example superoxide production could be the result of increased NAD(P)H oxidase or xanthine oxidase activity. In addition to antioxidants, BH4 may offer another therapeutic strategy. The critical eNOS cofactor can become oxidized, which uncouples eNOS, resulting in the generation of superoxide rather than NO. Therefore, BH4 supplementation may increase the production and availability of NO. L-arginine supplementation may also be another potential intervention aimed at increasing the bioavailability of NO. A combination of antioxidants and L-arginine therapy could also provide potential benefits to CKD patients.

Appendix A

REVIEW OF LITERATURE

19.2 million individuals suffer from chronic kidney disease (CKD) according to the Third National Health and Nutrition Examination survey (28). More than 8 million of these patients are in stages 3-5 CKD, as classified by glomerular filtration rate (GFR) (13). These patients are more likely to die of cardiovascular disease (CVD) before reaching ESRD (3). CVD related causes of death are 10-20 times higher in the CKD population compared to the general population (3, 29, 30). Traditional CVD risk factors such as hypertension, dyslipidemia, diabetes mellitus, and aging have all been shown to be present in CKD patients, but these risk factors alone cannot account for the high prevalence of CVD in CKD (1, 4, 5). Endothelial dysfunction has been shown to lead to a progression of kidney disease and contribute to the development of atherosclerosis (4, 31). Hence, there is a need for further elucidation of mechanisms of CVD in CKD.

A healthy endothelial lining acts as a defense mechanism, mediating vascular tone, structure, and blood/vessel wall relations (18). In order to direct vascular function, the endothelium integrates vasoconstrictive, proliferative, and thrombotic features, along with vasodilatory, antiproliferative, and antithrombotic mechanisms. Endothelial dysfunction occurs due to a disruption in the equilibrium of these elements (20). Endothelial dysfunction has been shown to play a crucial role in the pathogenesis of CVD (1). The dysfunction of the endothelium is marked by a decreased bioavailability of nitric oxide (NO), a powerful vasodilator substance (1).

Oxidative stress and increased levels of asymmetric dimethyl arginine (ADMA) also contribute to endothelial dysfunction (1). ADMA inhibits endothelial nitric oxide synthase (eNOS) through competition with L-arginine, an amino acid precursor for NO production (32). Increased ADMA, along with reduced systemic NO have also been found in chronic renal disease patients, contributing to CVD such as hypertension and atherosclerosis (34).

Oxidative stress has been shown to reduce NO availability through the reaction of superoxide and NO. This reaction results in the formation of peroxynitrite anion (ONOO-), a powerful oxidant capable of nitrosylation of cellular proteins and lipoproteins (9). Previous literature has shown that increases in superoxide production are responsible for a large proportion of the NO deficit in animal models for vascular diseases such as hypertension (34, 35) and heart failure (36). Additionally, superoxide stimulates mitogenesis in vascular smooth muscle cells along with decreasing NOS activity in endothelial cells (9). NAD(P)H-dependent oxidases, xanthine oxidases, lipooxygenase, mitochondrial oxidases, and NO synthases have been shown to be producers of vascular superoxide (9). Guzik et al found that there is an association between endothelial dysfunction and increased vascular superoxide in human atherosclerosis (9). Superoxide may also alter NO-mediated vascular signaling; reducing eNOS activity, ultimately resulting in hindered NO production (9). Taken together, increased levels of pro-oxidants and decreased antioxidant defense appear to facilitate endothelial dysfunction. Increaed reactive oxygen species (ROS) promote the oxidative breakdown of NOS cofactor tetrahydrobiopterin (BH4) (1, 21). This degradation results in eNOS uncoupling, ultimately decreasing NO production (1, 21). Hydroxyl radicals and oxidized low density lipoproteins (OxLDL) cause direct injury

to the cell membrane and nuclei (22). Additionally, OxLDL can produce foam cells and stimulate the secretion of inflammatory cytokines, which encourage phenotypic changes in vascular smooth muscle cells and stimulate platelet activation (22). ROS can also modify arachidonic acid to generate isoprostanes (37). When arachidonic acid is modified by a radical species, the double bond structure of the acid undergoes a complex series of radical rearrangements, terminating in metabolites with three OH groups (37). These metabolites are called F2-isoprostanes and are useful markers of in vivo assessment of oxidative stress, specifically 8-isoprostane F2α (37). 8-isoprostane F2α has also been shown to have vasoconstrictor effects on vascular smooth muscle cells (38). Additionally, Oberg et al found an elevated level of plasma F2-isoprostanes in stages 3-5 CKD patients, indicating an increase in oxidative stress (23).

Increased levels of ADMA also contribute to endothelial dysfunction via a disruption in the NOS pathway (31). The decrease in NO is in part due to the endogenous inhibitory actions of ADMA (32). Plasma levels of ADMA have been associated with a variety of cardiovascular risk factors and chronic kidney disease (CKD) (33).

ADMA becomes synthesized when arginine residues in proteins are methylated by protein arginine methyltransferases (PRMTs) (11). This process adds 1 or 2 methyl groups to the guanidine nitrogens of the arginine proteins. S-adenosylmethionine (SAM) functions as a methyl donor in the PRMT-mediated reactions (8). There are two main types of PRMTs; type 1 and type 2. Type 1 catalyzes the formation of ADMA, while type 2 methylate both guanidine nitrogens, resulting in the formation of symmetric dimethylarginine (SDMA) (11). Both of these PRMTs can also lead to the formation of NG-monomethyl-L-arginine (L-NMMA). ADMA and L-

NMMA are both inhibitors of NOS, ultimately inhibiting NO production (11). The amount of ADMA synthesized within a cell depends on the amount of arginine methylation in proteins and the rates of protein turnover. It is unclear whether ADMA production is relatively constant, if it changes with PRMT activity or if the rates of protein turnover are the most influential (11). However, it has been shown that there is a relationship between PRMT expression levels and free ADMA production (11).

Within the cardiovascular system, type 1 PRMTs are present in the heart, smooth muscle cells, and endothelial cells. Furthermore, PRMT-1 expression in endothelial cells increases in response to shear stress (11). The response to shear stress can be blocked by I_B kinase or by the peroxisome proliferator-activated receptor (PPAR) activator troglitozone, altering the expression of PRMT-1. The changes in PRMT-1 expression correspond with changes in ADMA release, which leads to the belief that the rates of ADMA generation within the vessel wall may be partly regulated through the change in PRMT expression (11). PRMT-1 expression can also be increased through low density lipoprotein expression (LDL) expression through the upregulation of PRMT-1 expression by endothelial cells, ultimately increasing ADMA production (8).

Methylarginines, including ADMA, are eliminated partly by renal excretion (11). The majority of SDMA is excreted entirely by the kidney, whereas L-NMMA and ADMA are thoroughly metabolized (11). The main metabolic pathway is catalyzed by dimethylarginine dimethylaminohydrolases (DDAHs) (11). DDAH has been shown to hydrolyse ADMA and L-NMMA to yield citrulline, dimethylamine, or monomethylamine. DDAH appears in two isoforms; DDAH1 and DDAH2. DDAH2 is predominately in tissues containing endothelial NOS (8). Endothelial cells express

PRMTs and DDAHs, with the inhibition of DDAH leading to increased production of ADMA. This increase in ADMA leads to changes in endothelial function, perhaps suggesting that ADMA levels increase under certain diseases/conditions (11). This also suggests that the ADMA-DDAH system plays a role in the regulation of NO synthesis (8).

It has been shown that plasma concentrations of SDMA and ADMA increase in patients with renal failure (11). The SDMA levels increase more than the ADMA since ADMA is also metabolized by DDAH (11). In other disease states, SDMA levels do not change with an increase in ADMA levels. This suggests that DDAH dysfunction is the main cause of an increase in ADMA levels (11). The effects of increased ADMA include elevated blood pressure, vasoconstriction, impaired endothelium-dependent relaxation, and increased endothelial cell adhesiveness (11). Long term elevations of ADMA may enhance the effects of atherogenesis and hypertensive damage to organs. ADMA also reduces heart rate and cardiac output. Long term NOS inhibition can also lead to reduced sodium excretion, contributing to hypertension (11).

In renal failure patients, ADMA accumulates as the rate of renal clearance is reduced (6). This is due to the fact that dimethylarginines are excreted through the urine. However, recent evidence has shown that only 5% of ADMA is excreted in the urine, leading researchers to believe that plasma levels may contribute significantly to increased ADMA (8). Increased plasma levels of ADMA have been shown to be present in hypercholesterolaemia, hypertension, diabetes, and in atherosclerosis patients (6). In end stage renal disease (ESRD) patients, it was reported that plasma ADMA was associated with carotid intima-media thickness, left

ventricular hypertrophy, cardiovascular complications, and mortality (8). There was also an inverse correlation between ADMA plasma levels and circulating endothelial progenitor cells, suggesting that the ADMA-DDAH system may contribute to the regulation of angiogenic responses and endothelial repair in vascular diseases (8). Ueda et al found that renal and liver DDAH-1 and DDAH-II expression were significantly decreased in kidney-replacement rats, suggesting that the degradation of ADMA due to reduced DDAH levels may also be a cause of elevated ADMA in disease states (8). Oxidative stress may also play a mechanistic role in the dysfunction of PRMT and DDAH. PRMT-1 expression was increased by oxidized LDL in cultured endothelial cells while DDAH expression in endothelial cells was reduced under high glucose conditions (8). Yilmaz at al reported increased markers of oxidative stress and ADMA in CKD patients with endothelial dysfunction (1). The endothelial dysfunction is most likely due to a decreased availability of nitric oxide. ADMA inhibits NOS by competing with L-arginine, leading to endothelial dysfunction. Increased free radicals lead to an increase in PMRT activity, ultimately increasing the production of ADMA (23). The increased ADMA can then lead to a loss in NO through uncoupling eNOS (23). Uremia-related oxidative stress and uremic toxins such as homocysteine and advanced glycation end-products decrease DDAH activity and may also contribute to the increased levels of ADMA. It was also found that DDAH expression was decreased in the lungs of rats under hypoxic conditions, as well as the observation of chronic tubulointerstitial hypoxia in CKD (8). Therefore, oxidative stress and chronic hypoxia could be possible mechanisms involved in the dysfunction of PRMT and DDAH, leading to increases in ADMA levels.

The early onset of atherosclerosis is a primary cause of death in CKD patients (29). Annuk et al showed that patients with moderate renal disease have impaired endothelium dependent dilation (EDD) compared to healthy control subjects (20). The renal failure patients exhibited considerably less pronounced vasodilation during the infusion of metacholine but not during sodium nitroprusside infusion (20). Therefore, vasodilator function in CKD appears to be limited by the bioavailability of NO (20).

The effects of antioxidants on EDD have been used as a method of assessment of oxidative stress on vascular function (10). Acute administration of vitamin C into the forearm increases EDD in the resistance vasculature of pre-dialysis renal failure patients, which are more advanced than the stages 3-4 CKD patients studied in the current investigation (10). The ability of vitamin C to normalize EDD appears to be through an increased production of NO, release of NO from endogenous thiols, protection of NO from premature deactivation by combining with superoxide, and limiting the oxidation of low density lipoproteins (10). Similarly, the effect of vitamin C on acetylcholine-induced forearm vasodilation has been shown to be an independent predictor of cardiovascular events in patients with coronary artery disease (39). However, it is uncertain whether this occurs in CKD patients. Hence, the administration of ascorbic acid and L-arginine via intradermal microdialysis coupled with laser Doppler flowmetry in this investigation allowed for the assessment of oxidative stress in microvascular function of the stages 3-4 CKD population.

Skin Blood Flow Response to Local Heating

Human skin is innervated by two branches of the sympathetic nervous system; the adrenergic vasoconstrictor system and the cholinergic vasodilator system

(15). The adrenergic vasoconstrictor system contributes to resting cutaneous vascular tone while the cholinergic vasodilator system consists of an unknown neurotransmitter than is simultaneously released with acetylcholine (15). The main mechanism involved in thermoregulatory vasodilation is the cholinergic vasodilator system; however, it is perceived that these nerves do not contribute to vasodilation during local heating (15). There are at least two independent mechanisms that contribute to the increase in skin blood flow (SkBF) when local heating is applied to the skin at temperatures below the pain threshold (15). The initial peak in the SkBF response to heating demonstrates to be primarily mediated by an axon reflex mechanism (15). However, the latter rise in SkBF appears to be mediated by NO. Minson et al described the cutaneous vascular response to local skin warming as biphasic, including an initial rise in flow, proceeded by a decline with another increase to an NO-mediated plateau (15). Hence, NO is required for vasodilatory mechanisms in response to local heating of the skin.

Appendix B

INFORMED CONSENT

Research Study: Microvascular Function in Chronic Kidney Disease
Investigators: David Edwards, PhD, Allen Prettyman MSN, RN, and Michael Stillabower, MD

Participant Name: _____

1. PURPOSE/DESCRIPTION OF THE RESEARCH

You are being asked to participate in a research study conducted by the Department of Health, Nutrition, and Exercise Sciences at the University of Delaware. Your skin makes natural substances when exposed to the heat that cause the skin's blood vessels to get bigger which increases skin blood flow. The purpose of this research is to study the differences in blood vessel function in the skin between apparently healthy individuals and individuals with kidney disease. To do this we will use "microdialysis" (MD). This technique involves placing very thin plastic tubing between the layers of your skin. The largest part of the tubing is about 6 times the diameter of human hair. We pump fluid like that found in your body's tissues through the tubing and the tubing acts like a very small blood vessel in your skin by allowing some substances to pass between the fluid in the tubing. These substances can only reach a 0.4 inches^2, nickel-sized area of the skin at each tube. These substances are like some natural substances found in your body. These substances are:

1. L-arginine HCl – one of the building blocks for proteins found in your body
2. Vitamin C – an antioxidant that helps shut down cell reactions with reactive oxygen
3. L-NAME (NG – nitro-L-arginine methyl ester) – like a natural protein found in your cells. It stops chemical reactions that involve protein.
4. SNP (sodium nitroprusside) – causes blood vessels to get as large as they can.

You will be 1 of 40 participants. Two groups will be recruited and include:

- Apparently healthy participants (20 total participants, age range 18-75 yrs)

- Participants with kidney disease (20 total participants, age range 18-75 yrs)

Apparently healthy participants will be recruited from the university community and surrounding areas. Participants with kidney disease will be recruited from Physician Offices.

Full participation in this study will require 2 visits for a total of approximately 5 hours at the Human Performance Lab, 541 S. College Avenue (the rear section of the Fred Rust Arena) in Newark, DE.

WHAT YOU WILL DO

First visit:

Your first visit will last approximately 30 minutes. You will be asked to not eat food, alcohol, and caffeine for 12 hours and to not exercise for 12 hours. This is a screening visit where:

- You will complete a questionnaire that asks about your current and past health.
- A resting electrocardiogram will be recorded and resting blood pressure, height, and weight will then be measured.
- A blood sample will be collected by inserting a needle into an arm vein (approximately 3 tablespoons of blood will be removed). The blood sample will be used to make an assessment of liver and kidney function, electrolytes such as sodium, a cholesterol profile, red blood cells, glucose (blood sugar). A urine sample will also be collected to assess kidney function. Some of your blood will be stored (frozen) only for measurement of blood markers related to blood vessel function at the completion of the study and will not be used for anything else.

You will not be able to participate if:

- You are not in good overall health other than kidney disease
- You are taking hormone replacement therapy
- You use tobacco products
- You are pregnant (an over-the-counter pregnancy test will be performed and interpreted by the investigators).

If you are taking antioxidant supplements you will be asked to discontinue them during your study participation.

Second Visit:

This visit to the Human Performance Lab will occur within 4 weeks of visit 1 and will be approximately 4 ½ hours in length. You will be asked not to eat food for 8 hours, drink alcohol and caffeine for 12 hours and refrain from exercise f or 24 hours prior to this visit. You will also be asked to delay taking any daily medications until the conclusion of this visit. Please bring your medications so that you can take them at the end of this visit.

The following will occur:

- You will be asked to wear a short sleeve shirt. If you do not bring one, we will provide one for you. You will wash your forearm and pat it dry. We will then prepare the microdialysis (MD) sites on your arm.

- Microdialysis (MD): We will place a tight band around your upper arm so your veins are easy to see. We will make 4 pairs of pen marks on your arm approximately 1 inch apart and away from veins. The MD tubing will enter and exit your skin at the marks. We will then clean your arm with an orange-colored betadine fluid and alcohol. We will place an ice bag on your arm for 5 minutes to numb your skin. Then we will insert a thin needle into your skin at each entry mark. The needle's tip travels between the layers of skin for 1 inch and leaves your skin at the matching exit mark. The MD tubing is threaded through the needle. Next, we will withdraw the needle leaving the tubing in your skin. You will have 4 sets of tubing in your skin. Any redness of your skin subsides in about 60 minutes.

- We tape a thin probe and its holder over each place where there is tubing in your skin. We can control the temperature of the holders. The temperature will start at 91.4˚F. Then we will start to run a salt like solution (Lactated Ringer's solution) through the tubing in your skin. Lactated Ringer's solution is a salt like solution containing sodium, chloride, potassium, calcium, and lactate. When the redness on your arm is gone, the study begins.

- During the experiment we measure:

Skin blood flow: we place pencil-sized probes over the tubing in your skin. The probes use a weak laser light to measure blood flowing in the small vessels in your skin.

Heart rate and blood pressure: you will have 3 electrodes placed on your chest to monitor heart rate. A blood pressure cuff will be placed on your arm that does not have tubing in it.

- When the experiment begins you will rest for 20 minutes. Then we will add the test substances to the plain fluid running through the tubing;

Probe 1 will receive salt like solution only

Probe 2 will receive salt like solution + L-NAME

Probe 3 will receive salt like solution + vitamin C
Probe 4 will receive salt like solution + L-arginine

- After about 30 minutes we will perform another set of measurements. Then we will increase the temperature of the skin under the local heaters to 104˚F. We maintain this temperature for about 40 minutes. When the skin blood flow becomes stable again, we will stop the flow of test substances through the tubing. Lastly, the salt like solution + SNP will flow through all of the tubing and we heat the skin to 108˚F for 30-45 minutes. This creates the greatest amount of blood flow possible.

- The trial then ends. We will clean all the places where the tubing enters and exits the skin with alcohol and the tubing will be pulled from your skin. We place a sterile bandage over the sites where the tubing was in your skin. We place a bag of ice on your skin for 10 minutes to reduce any bruising that may occur. We will check your heart rate and blood pressure again before you leave.

2. CONDITIONS OF PARTICIPATION

Information obtained from this study will be kept strictly confidential. You will not be individually identified, except by a participant number known only to the investigators. All data will be stored in a locked cabinet or password protected computer indefinitely. While the results of this research may be published, neither your name nor your identity will be revealed.

In the event of injury during these research procedures, you will receive first aid. If you require emergency room or other additional medical treatment, you will be responsible for the cost. You are free to discontinue participation at any time without penalty.

3. RISKS AND BENEFITS

There are no known risks associated with obtaining your height, weight, resting electrocardiogram, and resting blood pressure. You may have pain and/or bruising at the site where blood is taken, and there is a small risk of infection. Fainting sometimes occurs during or shortly after blood is drawn.

Microdialysis: you will probably experience some pain and bruising like that from a blood draw. However, we use ice to numb your skin during insertion of the tubing. Also, the small needle reduces pain during placement of the tubing. You will probably not have pain after the tubing is in place. You may feel a little pain when the tubing is removed from your skin. You may become lightheaded or faint. Sometimes the tubing

can break during removal from your skin in which case we will remove it by pulling it out from the other end. The tubing could break so that a small piece is left under your skin. This has not occurred in any of our studies or at any of the laboratories we know that are using this technique. If this happened, we would treat any tubing remaining in your skin like a splinter. The thin layer of skin over the tubing may have to be cut to allow removal. Mild pressure with sterile gauze stops any slight bleeding that may occur. Infection is possible. Sterile techniques and supplies like those used in a hospital keep the risk minimal. We apply a sterile bandage after the experiment and tell you how to take care of the site.
Fluid flowing through the tubing: The substances flowing through the tubing only got to a 0.4 inches^2 area of the skin (nickel size) at each tubing site. The amount that enters the skin is very small. However, there is a chance of you having a bad reaction to the substances. This reaction could produce redness, itching, rash, and/or swelling. Although unlikely, it is possible that a worse reaction could also cause fever, breathing problems, changes in heart rate, convulsions, and/or collapse. If a bad reaction should occur, medical help will be summoned right away.

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

L-NAME, L-arginine, Vitamin C, SNP: Only minute amounts of these substances enter the nickel-sized area of the skin around the tubing. Other researchers have used these substances in human skin and there have been no reports of bad reactions.

Laser Doppler: The laser Doppler will not damage the skin. Lasers can damage the eye if pointed into the eye for a prolonged period of time. We will not turn the laser on until it is taped to the skin surface and will turn it off before removing it.

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

There may be no direct benefit to you for participating in this research study, however you will be provided the results of your screening blood work when you complete the study. These results will not be interpreted but are provided for your information. This study may provide new information regarding blood vessel function in individuals with kidney disease.

4. FINANCIAL CONSIDERATIONS

All participants will receive $100.00 to offset the cost of transportation, etc. for full completion of visits 1 and 2.

5. CONTACTS

Any questions regarding the study can be directed to Dr. David G. Edwards (302-831-3363), Associate Professor, Department of Health, Nutrition, and Exercise Sciences. Questions regarding the rights of individuals who agree to participate in this research may be directed to: Chair, Human Subjects Review Board (302-831-2136), University of Delaware.

6. PARTICIPANT ASSURANCES

I have read the above informed consent document. The nature, demands, risks, and benefits of the project have been explained to me. I knowingly assume the risks involved, and understand that I may withdraw my consent and stop my participation in this study at any time.

7. CONSENT SIGNATURES

Participant's Signature:_____ Date:_____

Participant's Name (printed):_____

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