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Impaired Endothelium-Independent Microvascular Function in Obese Young Adults

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Impaired Endothelium-Independent Microvascular Function in Obese Young Adults

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Thesis

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

Master of Science in Kinesiology

The University of Texas at Austin

August, 2014

Acknowledgements

Most importantly, I would like to thank my wife Amanda for her unwavering support of my academic endeavors. I would like to thank Dr. Brothers for his guidance on this project. Additionally, I would like to thank Dr. Tanaka for agreeing to serve on my committee on short notice. I also would like to express sincere gratitude to my current and former lab mates for their assistance and input on this and other projects. Finally, I would never have made it to this point in my career without the support and encouragement of my family and friends including Roy and Susie Haines, Sam and Marci Ditzell, Seiji Ishii, and Dale and Bonnie Hill.

Abstract

Impaired Microvascular Endothelium-Independent Function in Young Obese Adults

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The University of Texas at Austin, 2014

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Microvascular dysfunction is believed to precede the development and contribute to the progression of obesity related diseases such as insulin resistance, hypertension, and coronary artery disease. Multiple studies have found impaired microvascular endothelium-dependent vasodilation occurs prior to the onset of disease in middle aged adults. In order to test the hypothesis that the cutaneous microvasculature of young obese (BMI>30kg/m^2), but otherwise healthy, adults would exhibit impaired microvascular response, we recruited 12 obese and 12 lean (BMI<25 kg/m^2) individuals. Each group was age-matched and consisted of 5 females and 7 males. Each participant was instrumented with two microdialysis probes inserted in the dermis of the non-dominant forearm for a wide dose range of infusions of either the endothelium-dependent vasodilator methacholine (MCh) or the endothelium-independent vasodilator sodium nitroprusside (SNP). Each microdialysis site was clamped at 33°C with a local heater and affixed with a laser Doppler flux (LDF) probe for determination of local red blood cell flux, an index of blood flow. LDF was recorded continuously while 7 doses of each drug

(MCh: 10^{-3}-10^{3}mM; SNP: $5x10^{-5}$-50mM) were infused at a rate of 2 µl/min for 8 minutes per dose. Both sites finished with heating to 43°C and infusion of 50mM SNP to confirm site specific maximal vasodilation. Blood pressure was recorded in the last minute of each stage and the corresponding LDF was used to calculate cutaneous vascular conductance (CVC). Dose response curves for CVC at each dose, as well as maximal CVC were analyzed. MCh dose response showed a trend toward endothelium–dependent impairment in obese ($p=0.06$) and maximal absolute CVC at the MCh site was attenuated in obese versus lean (2.70 ± 0.73 vs 3.30 ± 0.81 LDF/mmHg, $p=0.027$). Endothelium-independent vasodilation with SNP was impaired at the 4 highest doses of SNP (all $P<0.006$) and maximal CVC was attenuated in obese compared to lean (2.44 ± 0.74 vs 3.31 ± 0.65 LDF/mmHg, $p=0.004$). These results support the hypothesis that microvascular function is impaired in young, healthy obese, individuals; however they suggest the impairment is partially endothelium-independent.

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Chapter 1: Introduction

Background

Cardiovascular disease is the leading cause of morbidity and mortality in obese individuals, especially those with additional risk factors of hypertension, dyslipidemia, microalbuminuria or insulin resistance (Isomaa et al., 2001). The microcirculation has been used as a novel location to detect the initial insult of cardiovascular impairments. Impairments in microvascular endothelial function, including decreased nitric oxide (NO)-dependent vasodilation, vessel structural remodeling, and decreased perfusion due to rarefaction, may precede the development of hypertension (Cohuet & Struijker-Boudier, 2006; Levy, Ambrosio, Pries, & Struijker-Boudier, 2001), atherosclerosis (Davignon & Ganz, 2004; Landmesser, Hornig, & Drexler, 2004) and insulin resistance (Serné, de Jongh, Eringa, IJzerman, & Stehouwer, 2007). It has been well established that obesity (BMI>30 kg/m^2) is related to development of hypertension (Kannel, Brand, Skinner, Dawber, & McNamara, 1967; Stamler, Stamler, Riedlinger, Algera, & Roberts, 1978) and insulin resistance (Mokdad, Ford, Bowman, & et al., 2003). Therefore, it is likely that obesity contributes to microvascular dysfunction, which then influences the development of obesity-related diseases such as type 2 diabetes mellitus and cardiovascular disease. Supporting this hypothesis, capillary recruitment and cutaneous blood flow response to the endothelium-dependent vasodilator acetylcholine (ACh) have been correlated with impaired insulin sensitivity (de Jongh, Serné, IJzerman, de Vries, & Stehouwer, 2004b; Serné et al., 1999). Similarly, slowed response to hyperemia and decreased functional capillary density are associated with both insulinemia and BMI (Panazzolo et al., 2012).

The effect of obesity on endothelial function has been previously examined. Attenuations in forearm blood flow response to intra-arterial infusions of ACh have been shown in some (Higashi et al., 2001; Sivitz, Wayson, Bayless, Sinkey, & Haynes, 2007; Van Guilder, Stauffer, Greiner, & DeSouza, 2008), but not all (Tack, Ong, Lutterman, & Smits, 1998) obese individuals compared to their lean counterparts. Cutaneous blood flow is attenuated during post occlusive reactive hyperemia in obese adults (Rossi et al., 2011) suggesting a potential impairment in NO-production or action, though the NO contribution of this test is debated (Wong, Wilkins, Holowatz, & Minson, 2003). Similarly, impairments in cutaneous blood flow responsiveness to ACh delivered via iontophoresis infusions are present in middle aged obese adults (de Jongh et al., 2004b).

Much less is known about microvascular function in young obese individuals prior to the onset of obesity-related disease and the results of the few studies that have been performed are difficult to interpret due to the variety of techniques used to analyze microvascular function. Lean and obese young adults have shown no difference in brachial blood flow response to intra-arterial infusions of ACh and endothelium-independent vasodilator sodium nitropusside (SNP) (Limberg et al 2013). In contrast, obese young adults demonstrated a lower cutaneous blood flow response to ACh iontophoresis (Al-Tahami, Bee, Ismail, & Rasool, 2011). These data have left the effect of excess body weight on vascular reactivity difficult to interpret and led us to examine another technique to answer the question.

Statement of Purpose

Investigation of the cutaneous microcirculation has been recognized as a suitable model for assessment of mechanisms underlying systemic vascular disease (Debbabi, Bonnin, Ducluzeau, Leftheriotis, & Levy, 2010; Holowatz & Kenney, 2011; Holowatz,

Thompson-Torgerson, & Kenney, 2008b) and with the simultaneous use of microdialysis we have the advantage of being able to systematically examine mechanisms of impairment at the microvascular level without having a systemic effect. Therefore, this study aims to be the first to utilize microdialysis to examine microvascular function in relatively young obese individuals without any concomitant disease.

Confirmation of microvascular dysfunction occurring prior to the onset of obesity related disease would give support to the body of literature that impairments in microvascular function precede the development of insulin resistance and hypertension. Furthermore, future use of microdialysis may allow for identification of the mechanisms by which obesity impairs microvascular function. Knowledge of which may prove invaluable in the prevention or early treatment of obesity-related disease prior to the onset of symptoms.

Hypothesis

The hypothesis of this study is that cutaneous microvascular function, more specifically endothelium dependent and/or independent vasodilation is impaired in young, healthy obese individuals when compared to their age-matched lean counterparts.

Chapter 2: Review of Literature

With approximately one-third of the adult population of the United States classified as obese (BMI>30kg/m^2), obesity and the health complications of excessive body weight are an important area of research. Recently, microvascular function has been identified as a possible early link between disease states and development of further disease. The following review intends to focus on the health consequences of obesity, how microvascular dysfunction may contribute to further complications, and the mechanisms through which obesity might influence these impairments

Obesity and Related Diseases

The health impact of obesity is of the utmost concern to scientists and clinicians. Roughly two thirds of the United States' adult population is overweight and one third is obese (Flegal, Carroll, Ogden, & Curtin, 2010). In addition, about 17% of children and adolescents under the age of 20 are also obese. While some suggest obesity rates are levelling off (Rokholm, Baker, & Sørensen, 2010), others contend that the number of Americans with a BMI over 30kg/m^2 will continue to climb, albeit at a slower rate than we have seen in the 1980s and 1990s (Flegal et al., 2010). One estimate states that nearly 65 million more Americans will be obese by the year 2030 (Wang, McPherson, Marsh, Gortmaker, & Brown, 2011). That number includes a projected 50% obesity rate for males over the age of 20. Large epidemiological studies have shown that obesity relates strongly to diabetes (Chan, Rimm, Colditz, Stampfer, & Willett, 1994; Colditz, Willett, Rotnitzky, & Manson, 1995). These studies showed that diabetic risk is elevated not only by current BMI, but also by BMI at the age of maturity and adult weight gain. Additionally, increased BMI is associated with increased cardiovascular risk factors such as increased blood pressure, impaired fasting glucose, and dyslipidemia (Lamon-Fava, Wilson, & Schaefer,

1996). Taken together, this data suggests the consequences of obesity are dire for both the individual and the healthcare system. Illustrating the latter point, obesity related diseases are estimated to have cost $147 billion in 2008 (Finkelstein, Trogdon, Cohen, & Dietz, 2009) and are expected to increase regularly to the point where additional obesity related spending will reach $48-66 billion per year by 2030 (Wang et al., 2011). While obesity prevention efforts must continue, science must also continue to work to elucidate the mechanisms which link obesity to development of disease thereby allowing the possible development of therapeutic interventions that can counteract the elevated risks associated with obesity.

Microvascular Assessment

Impaired microvascular function is thought to precede the onset of hypertension (Levy et al., 2001), atherosclerosis (Al Suwaidi et al., 2000), and insulin resistance (Jonk et al., 2007; Serné et al., 2007). For this reason, research into the pathogenesis of microvascular dysfunction in various disease states, such as obesity, can provide insight into the mechanisms that lead to further disease. The microvasculature is the level of the vascular system that has the greatest effect on resistance and is the location of nutrient exchange. Therefore, any impairment in the microcirculation could potentially lead to increased resistance, and thus blood pressure, and/or impaired glucose uptake.

Many early studies used venous occlusion plethysmography to measure forearm or leg blood flow after intra-arterial infusions of vasoactive substances; however, this technique requires medical supervision. More recently the use of laser Doppler flowmetry (LDF) has become popular to study the cutaneous circulation. The cutaneous circulation is studied because it is considered to be an excellent model for full body microvascular function due to its ease of accessibility (Holowatz, Thompson-Torgerson, & Kenney,

2008a). Microvascular function is assessed with LDF in response to many different stimuli including post-occlusive reactive hyperemia (PORH), systemic and local thermal reactivity, and iontophoresis of vasoactive substances across the skin via electrical charge, and microdialysis infusion directly into the dermal layer. Using PORH, thermal reactivity and iontophoresis have the advantage of being non-invasive. However, these techniques all have limitations. For instance, PORH has been shown to be independent of NO (Wong et al., 2003). Additionally, thermal reactivity is a complex phenomenon and the contribution of NO and other vasodilatory stimuli are dependent upon temperature and rate of heating (Choi, Brunt, Fujii, & Minson, 2014; Minson, Berry, & Joyner, 2001). Limitations to iontophoresis include: the dose is indirectly estimated as time charge is applied x electrical current strength (Tesselaar & Sjöberg, 2011), the potential for non-specific effects of the current to induce vasodilation (Droog & Sjöberg, 2003; Grossmann et al., 1995), and the inverse relationship between skin electrical resistance and vasodilation (Ramsay, Ferrell, Greer, & Sattar, 2002). On the other hand, microdialysis allows for systematic study of mechanism due to the ability to directly infuse enzyme inhibitors or substrates. Disadvantages include the somewhat invasive nature (Cracowski, Minson, Salvat-Melis, & Halliwill, 2006) and the need to allow for trauma induced hyperemia to subside before measurements can be taken.

Endothelium-Dependent Vasodilation

The endothelium is the single layer of cells lining the lumen of blood vessels and has the ability to release vasoactive substances in response to various stimuli. Endothelial function is a key indicator of overall vascular health and dysfunction influences the development of coronary artery disease, peripheral artery disease and cerebrovascular disease (Beckman, Creager, & Libby, 2002). Depending upon the stimuli, the endothelium can release vasoconstrictor substances like endothelin-1 and angiotensin II or vasodilators

like nitric oxide (NO) and endothelium derived hyperpolarizing factors (EDHF). The endothelium can also release prostaglandins that signal vascular smooth muscle cells (VSMC) to vasoconstrict (via thromboxane) or vasodilate (via prostacyclin). In-vivo, ACh or shear stress will signal the production of NO by endothelial nitric oxide synthase (eNOS). In turn, this NO release diffuses into the adjacent VSMC triggering a cascade beginning with guanylate cyclase (GC), then cyclic guanosine monophosphate (cGMP) and finally protein kinase G (PKG), which ultimately lowers cytosolic calcium and relaxes the smooth muscle. In addition to NO, prostaglandins signal the VSMC in a similar manner as NO and have been shown, via cyclooxygenase (COX) inhibition, to be a contributor to endothelium dependent vasodilation in response to ACh, (Doyle, Duling, McGahren, & Dora, 1997; Durand et al., 2004; Holowatz, Thompson, Minson, & Kenney, 2005; Kellogg, Zhao, Coey, & Green, 2005).

The effect of obesity on endothelium dependent vasodilation is contested. An early study showed no difference in forearm blood flow via plethysmography between lean and obese individuals who had intra-arterial infusions of Ach (Tack et al., 1998). However, another study with a similar protocol showed that obese individual with and without impaired fasting glucose and those with Type 2 diabetes all had impaired forearm blood flow response to infusions of ACh (Sivitz et al., 2007). In agreement with these results, another group examined forearm blood flow in obese versus lean subjects and found the obese individuals to have blunted responses to muscarinic agonists ACh and MCh also to bradykinin, substance P, and isoproterenol, therefore endothelial-impairment is not a muscarinic receptor deficiency (Van Guilder et al., 2008). Further supporting that impairments in endothelial dependent vasodilation are not confined to responsiveness to ACh, a study examining forearm blood flow in young, sedentary lean, obese and metabolic syndrome subjects found that responsiveness to epoprostenol, a prostaglandin, was

impaired in obese and metabolic syndrome subjects compared to lean counterparts (Limberg et al., 2013). Interestingly, they did not demonstrate impairment in ACh responsiveness versus controls.

Similar discrepancies exist in studies using PORH. One study found that obese individuals had increased skin blood flow at baseline compared to age matched controls yet they showed lower skin blood flow following PORH (Rossi et al., 2011). Skin blood flow responses were examined 12 months after bariatric surgery and subsequent weight loss and baseline blood flow was decreased to that of the lean controls while PORH responses were augmented to the level of the lean (Rossi et al., 2011). These results support the hypothesis that excessive adiposity impairs endothelium function, but it can be restored through weight loss. Confounding these results, however, is a study by Schlager et al. in morbidly obese teenagers in which the authors showed an increased skin blood flow response to PORH along with a slowed recovery time (Schlager et al., 2011). These authors speculated that these results infer an impaired myogenic vasoconstrictor response in obese children and adolescents. While these two populations are different, it is interesting that the researchers showed nearly opposite responses and each concluded microvascular dysfunction to be a key component.

Multiple research groups have used iontophoresis to transdermally deliver vasoactive substances via an electric charge to the cutaneous microcirculation which they measure with laser Doppler flowmetry or more recently laser Doppler imaging. Commonly, ACh is used as the endothelium dependent stimulus. Impairments in cutaneous blood flow responsiveness to ACh infusions have been shown in obese individuals (Al-Tahami et al., 2011; de Jongh et al., 2004b). However, obese individuals with insulin resistance or Type II diabetes were able to show improvements in flow mediated dilation but not in cutaneous blood flow responsiveness to iontophoretic administration of ACh after a 6 month exercise

and lifestyle intervention (Hamdy et al., 2003). This result can be explained in a variety of ways. First, the authors hypothesized that their unexpected findings could be explained by an increased sensitivity in conduit arteries compared to the microcirculation. However, a more likely explanation is that iontophoresis is not an ideal method to deliver ACh and other vasoactive drugs to the cutaneous microcirculation. Limitations of iontophoresis include the inability to calculate the exact dose being delivered and the potential for non-specific vasodilation (Tesselaar & Sjöberg, 2011) as well as the individual variability in electrical resistance of the skin, which has been shown to negatively correlate with skin blood flow response (Ramsay et al., 2002). It is entirely likely that these factors could contribute to or hide differences seen between subject groups and therefore results of studies utilizing iontophoresis should be viewed with caution.

Endothelial function must also take into account endothelium dependent vasoconstriction. As discussed previously, obese children and adolescents may have inadequate myogenic vasoconstrictor responses (Schlager et al., 2011), but endothelial dysfunction should be viewed as exhibiting excessive basal vasoconstriction. For instance, when overweight and obese subjects were given arterial infusions of the potent endothelium derived vasoconstrictor endothelin-1 (ET-1), they displayed a blunted vasoconstrictor response (Weil et al., 2011). Interestingly, when given an endothelin receptor blockade (BQ-123), the overweight and obese subjects had a vasodilatory response while blood flow was unaffected in lean subjects. This indicates that overweight and obese have either greater basal ET-1 production or increased sensitivity leading to increased vasoconstriction. Similar results were seen in overweight and obese hypertensives in comparison with lean hypertensives (Cardillo, Campia, Iantorno, & Panza, 2004). However, the latter study, Cardillo et al., showed no difference in BQ-123-induced vasodilation between non-hypertensive overweight and obese subjects versus non-hypertensive lean subjects,

suggesting that increased ET-1 activity may not be inherent to obesity. Previously Ferri et al. had shown no difference in circulating ET-1 in obese normotensives and obese hypertensives, yet both obese groups have greater ET-1 versus lean normotensives (Ferri et al., 1995).

The discovery that insulin has nitric oxide-induced vasodilatory actions has made it clear that part of insulin's effect on blood glucose is its ability to direct blood flow to tissues and can leave us speculating at potential mechanisms through which obesity leads to insulin resistance (Laakso, Edelman, Brechtel, & Baron, 1990; Steinberg, Brechtel, Johnson, Fineberg, & Baron, 1994). The direct action of insulin on cutaneous blood flow has been investigated using iontophoresis in obese and lean women, with the obese showing a complete attenuation of insulin-induced vasodilation (de Jongh, Serné, IJzerman, Jørstad, & Stehouwer, 2008). There is an inherent difference in the plasma milieu in obese individuals compared to lean counterparts, but the effect of obesity-related elevated plasma free fatty acids (FFA) on microvascular function has been investigated in obese and lean women (de Jongh, Serné, IJzerman, de Vries, & Stehouwer, 2004a). Lean women with artificially elevated free fatty acids (FFAs) showed impaired capillary recruitment and responsiveness to ACh at rest and during a hyperinsulinemic clamp, suggesting that FFAs play a role in impairment of microvascular function in obese individuals. In contrast, obese women with artificially lowered FFA experienced improved capillary recruitment at rest and during hyperinsulinemia, but did not see any gains in ACh-induced vasodilation in either state. Once again, this latter finding was based on iontophoresis of ACh and therefore may be of limited value.

Any impairment in endothelial function at the microvascular level may manifest later as insulin resistance and/or hypertension. Research has shown that obese individuals are more likely to have impaired endothelium dependent vasodilation or excessive

endothelial dependent vasoconstriction. Taken together, it is reasonable that insulin resistance and hypertension are connected to obesity via the endothelial function of the microvasculature.

Endothelium-Independent Vasodilation

As mentioned previously, endothelium-independent vasodilation is a measure of the VSMC response to NO-donors. Any impairment can be either inactivation/destruction of NO prior smooth muscle signaling, or compromised signaling of the NO-guanylate cyclase-cGMP-PKG cascade.

Endothelium-independent dysfunction has been shown in a variety of disease states, such as rheumatoid arthritis, hypercholesterolemia, and heart disease. In a study of rheumatoid arthritis patients, forearm blood flow was decreased with intra-arterial SNP infusions and correlated with markers of inflammation (Yki-Järvinen, Bergholm, & Leirisalo-Repo, 2003). Hypercholesterolemics also show an attenuated forearm blood flow response to SNP (Creager et al., 1990). In chronic heart failure patients, those with attenuated SNP forearm blood flow responses had a higher rate of hospital admissions for worsening of their disease (Nakamura et al., 2001). Similarly, coronary artery disease patients showed a lower effect of sublingual nitroglycerin as measured by peripheral artery tonometry (Bonetti et al., 2004).

Within the cutaneous circulation, as shown by LDF response to iontophoresis, SNP produces blunted vasodilation in older smokers compared to their age matched non-smokers (Pellaton, Kubli, Feihl, & Waeber, 2002). Interestingly, this impairment is not seen in younger smokers suggesting that it takes time to develop. Young Type 1 diabetics exhibit impaired endothelium-independent vasodilation in the cutaneous microvasculature (Khan, Elhadd, Greene, & Belch, 2000) as do lean and obese, but not overweight, hypertensive

adolescents (Monostori et al., 2010). Healthy aging has shown a decreased maximal response to SNP (Minson et al., 2001).

Endothelial dependent microvascular dysfunction has been fairly well established in obesity, but the question remains if there can also be endothelium independent mechanisms that cause impairments in microvascular function in obesity. Sivitz et al. showed a decreased forearm blood flow response to SNP in obese, impaired fasting glucose and type 2 diabetics compared to insulin sensitive lean individuals (Sivitz et al., 2007). Contrasting that result, Weil et al. have shown no change in forearm blood flow in response to SNP in obese versus lean controls (Weil et al., 2011). Furthermore, with iontophoretic treatment of SNP, differences in responses between obese and lean are not seen (de Jongh et al., 2004a; de Jongh et al., 2004b). However, Al-Tahimi and colleagues had a trend approaching significance ($p=0.053$) showing impaired vasodilation in response to SNP iontophoresis in relatively young obese individuals (Al-Tahami et al., 2011). Another study indicated the potential for impaired endothelium independent vasodilation by examining the brachial artery blood flow response to sublingual nitroglycerin and showed an obesity related impairment, with the subjects with the highest degree of visceral fat having a 35% impairment in vasodilation (Christou et al., 2012). These results suggest the possibility that obesity may also affect the VSMC responsiveness to nitric oxide, independent of endothelial function, but the mechanisms have not been established.

Oxidative stress: A potential mechanism

The contention of this review is that microvascular impairments, particularly endothelial dependent vasodilation, are a consequence of obesity and lead to the development of insulin resistance and hypertension. The connection between obesity and microvascular dysfunction is likely to be multifactorial, however one possible link is oxidative stress seen in obesity. Thorough investigation of this and other potential causes of

obesity induced microvascular function may lead to development of new therapies to combat the progression from obesity to microvascular dysfunction and subsequent disease.

Systemic oxidative stress is highly correlated with obesity independent of insulin resistance with each $5kg/m^2$ increase in BMI equating to a nearly 10% increase in oxidative stress measured in the urine (Keaney et al., 2003). In agreement with that finding, urinary and plasma measures correlated strongly with BMI and waist circumference (Furukawa et al., 2004). Owing to these observations, the influence of increased adiposity on oxidative stress was studied in animal models. In obese, non-diabetic mice, elevated oxidative stress was apparent versus lean control mice (Furukawa et al., 2004). Furthermore, the increased plasma oxidative stress was traced to the white adipose tissue with no differences in lipid oxidation being shown in liver or muscle tissue. This increase in lipid oxidation by the accumulated adipose tissue was linked to increased expression of NADPH-oxidase mRNA, decreased expression and activity of anti-oxidant enzyme mRNA and increased reactive oxygen species production by adipocytes. Though this is an animal model, similar consequences of excess adiposity can be assumed in humans.

NADPH-oxidase is a likely link between obesity and endothelial dysfunction as seen in obesity. NADPH-oxidase reduces nitric oxide bioavailability by increasing production of the free radical superoxide (Landmesser, Harrison, & Drexler, 2006). Superoxide and nitric oxide then quickly react to form perioxynitrite before nitric oxide has a chance to exert its effect on the VSMC thus limiting its bioavailability and subsequent vasodilation (Kojda & Harrison, 1999). If endothelial nitric oxide synthase (eNOS) becomes uncoupled, either due to lack of substrate L-arginine or cofactor tetrahydrobiopterin (BH_4), then it will produce superoxide rather than nitric oxide and further inhibit vasodilation. Whether obesity results in decreased L-arginine or BH_4 is

currently unknown, but it stands to reason that some form of oxidative stress contributes to impaired endothelial dependent vasodilation in obesity.

The pathways that affect microvascular function have been studied recently in other conditions also known to increase risk of insulin resistance and/or cardiovascular disease. For instance, a study in middle aged adults utilizing laser Doppler flowmetry in combination with local infusions of various NOS inhibitors via microdialysis showed that eNOS activity determines cutaneous vasodilatory responsiveness to local heating and ACh infusion (Bruning et al., 2012). This same group then determined that, in aged humans, oral BH_4 supplementation increases cutaneous vascular conductance and that increase is entirely due to improved nitric oxide bioavailability (Stanhewicz, Bruning, Smith, Kenney, & Holowatz, 2012). Taken together, these results indicate the importance of nitric oxide production in vasodilation of an elevated risk population. Additionally, another at-risk group, young smokers, was studied using a single bolus of ACh administered via microdialysis (Fujii, Reinke, Brunt, & Minson, 2013). They found that smokers did see a significantly blunted increase in cutaneous vascular conductance compared to non-smokers, but this eNOS inhibition did not further attenuate the increase in vasodilation in response to a one-minute infusion of ACh, indicating that in young cigarette smokers, eNOS is significantly inhibited likely due to the elevated superoxide production known to occur in cigarette smoking. Lastly, chronic kidney disease is known to cause elevated oxidative stress and thus can be seen as a good model of the effect of oxidative stress on the cutaneous microvasculature (DuPont, Farquhar, Townsend, & Edwards, 2011). In a study of patients with stage 3 and 4 chronic kidney disease compared with aged matched controls, it was observed that the increase in cutaneous vascular conductance seen with local heating was attenuated in the patients and this impairment was due to the nitric oxide contribution. Local microdialysis infusions of the antioxidant ascorbic acid and the eNOS substrate L-

arginine both restored microvascular function to the level of healthy controls. These results further support the role of oxidative stress as a cause of microvascular dysfunction in obesity.

Perspectives

Microvascular dysfunction is thought to be a possible link between obesity, insulin resistance and hypertension. Multiple studies support the idea that microvascular function is impaired in populations with increased risk of insulin resistance, hypertension, and cardiovascular disease. Additionally, multiple studies have demonstrated microvascular dysfunction in obese individuals. However, it is important that this microvascular dysfunction is demonstrated in young obese individuals without signs of further disease in order to verify that impaired microvascular function precedes the development of obesity related disease. Preliminary work in our lab shows impaired endothelium dependent vasodilation in obese college aged individuals. Future research should focus on the mechanisms through which obesity impairs microvascular function, namely increased oxidative stress. While obesity prevention should be the utmost priority, appropriate therapeutic interventions for obese individuals that address endothelial function have the potential to lessen the rates of obesity related diseases such as insulin resistance and hypertension and could possibly ameliorate type II diabetes mellitus and cardiovascular disease in obese individuals.

Chapter 3: Methods

Subjects and Ethical approval

Twenty-four normotensive, non-smoking volunteers participated in this study. Of the 24 subjects, 12 were classified as obese (5 women, 7 men) with a body mass index (BMI) $\geq$ 30 kg/m^2. The other 12 subjects (5 women, 7 men) were classified as lean with a BMI $\leq$ 25 kg/m^2 and were recruited after the obese subjects in order to match age and gender. Participant characteristics are presented in Table 1. All subjects were free of diagnosed cardiovascular, metabolic or neurological disease. Smokers and individuals on prescription medication, including hormonal birth control, were excluded from the study. Women were tested in the early follicular phase of the menstrual cycle (days 2-7). Subjects were asked to avoid alcohol, strenuous exercise, and over-the-counter medications for 24 hours prior to testing. All experimental procedures were approved by the Institutional Review Board of the University of Texas at Austin. All purposes, risks, and procedures of the study were explained to subjects and subjects gave written informed consent prior to participation.

Instrumentation and Measurements

Volunteers reported to the lab in the morning after fasting for 12 hours. Upon arrival to the lab, subjects were weighed to the nearest tenth of a kilogram and measured to the nearest half centimeter on a digital medical scale with platform stadiometer wearing light clothing (Seca 763, Seca, Chino, CA). Subjects were then positioned on a semi-recumbent patient bed and remained still for the remainder of the study. Blood pressure and heart rate were monitored with an electrosphygmomanometer with integrated ECG (Tango+, SunTech Medical, Raleigh, NC). The blood pressure cuff was placed on the dominant arm for auscultation of the brachial artery. All testing occurred in an air conditioned laboratory set to 22°C.

Two microdialysis fibers (CMA 31, CMA Microdialysis, Kista, Sweden) were inserted intradermally in the dorsal side of the non-dominant forearm as described previously (Minson et al., 2001). Briefly, a 25 gauge needle was inserted intradermally with entry and exit points approximately 2.5 cm apart. The microdialysis fiber was threaded through the lumen of the needle with the 1 cm semi-permeable membrane placed within the needle. The needle was then withdrawn leaving the membrane in the dermis and the microdialysis fiber was taped in place. The second site was located a minimum of 5 cm away. Both microdialysis fibers were then perfused with lactated Ringer's solution for 60-90 minutes allowing for resolution of the hyperemic response to the needle-induced trauma. Infusion rate was controlled via a dual syringe pump set to 0.2 $\mu l \cdot min^{-1}$ (Pump 11 Plus, Harvard Apparatus, Holliston, MA).

Blood analysis for fasting glucose and blood lipids was completed using fingertip blood samples in Cholestech LDX system (Alere, Waltham MA). Glycosylated hemoglobin content (HbA1C) was also measured with a fingertip blood sample (in2it II, Bio-Rad Laboratories, Hercules, CA).

Experimental Protocol

Red blood cell flux was measured via laser Doppler flowmeter (LDF) probes (VP2T, Moor Instruments, Wilmington, DE) placed immediately above each microdialysis site and connected to LDF monitor (moorVMS-LDF, Moor Instruments, Wilmington, DE). Each probe was inserted into a local heating element (PF450, Perimed AB, Stockholm, Sweden) that was securely taped to the skin. Local heaters (Periflux 5000, Perimed AB, Stockholm, Sweden) were set at 33° C to normalize skin temperature among participants at baseline.

Endothelium dependent and endothelium independent vasodilation was assessed by response to seven doses of methacholine (MCh) (Acetyl-β-methylcholine chloride, Sigma-

Aldrich, St. Louis, MO) and sodium nitroprusside (SNP) (Nitropress, Sigma-Aldrich, St. Louis, MO), respectively. The microdialysis site for each drug was randomly assigned. MCh was diluted in lactated Ringer's solution from 10^{-3} to 10^{3} mM while SNP doses ranged were similarly diluted from 5 x10^{-5} to 50 mM. Following a 10 minute baseline period, each dose of MCh and SNP was infused for ~8 minutes at 0.2 µl·min^{-1} beginning with the lowest dose. During the first minute of each dose, the infusion rate was set at 5.2 µl·min^{-1} to flush the microdialysis probe of the previous dose. The final dose of each drug was infused for 20 minutes in order to establish a plateau in LDF response. Each site then was heated to 43°C and infused with 50 mM SNP to insure site specific maximal vasodilation had been reached.

Data Analysis and Statistics

LDF (in flux units) and skin temperature data were integrated with a data acquisition system (MP150, BIOPAC Systems, Goleta, CA) and recorded on a laboratory computer for off-line analysis (Acknowledge, BIOPAC, Goleta, CA). Blood pressure measurements were taken in the last minute of each infusion stage. The LDF at each site was averaged over the final minute of each stage and used to calculate cutaneous vascular conductance (CVC) which is defined as LDF/MAP) and expressed in arbitrary units (mV·mmHg^{-1}). A dose response curve for each site was constructed using the CVC calculated at each dose. Statistical analysis software (SigmaPlot12.5, SyStat Software, San Jose, CA) was used to fit individual responses four parameter logistic curves for identification of the dose that elicited one-half of the maximal response (EC_{50}) for each subject at each infusion site. Group mean EC_{50} for MCH and SNP were then compared with independent samples t-tests. CVC values for each dose were analyzed across groups via a two-way repeated measures ANOVA with dose as the repeated factor. Where appropriate, post hoc analyses were performed using Tukey's HSD. Additionally maximal CVC response at each site was

identified, independent of dose, and evaluated with a one-tail independent samples t-test with the hypothesis that the obese group would have an impaired response. All descriptive characteristics were evaluated with two-tail tests, except where directional differences were part of the study design, i.e. weight and BMI. Differences were considered significant at the α level of 0.05. All data are presented as mean ± standard deviation (SD).

Chapter 4: Results

Subjects

As shown in Table 1, subjects were well matched in age, height, sex and blood pressure. By design, the obese group had a greater weight and body mass index(BMI) versus the lean group (95.4 ± 13.1 vs 66.1 ± 11.8 kg, P<0.001; 33.5 ± 3.4 vs 22.3 ± 1.9 kg/m^2, P<0.001). Additionally, the obese group had lower HDL (32 ± 13 vs 54 ± 11mg/dl, P<0.001) and a higher LDL/HDL ratio (3.9 ± 2.0 vs 1.7 ± 0.8, P<0.001). Though no differences in blood glucose concentration after an overnight fast were seen between groups (81 ± 11 vs 82 ± 10 mg/dl, P=0.75), obese did exhibit slightly higher HBA1c (5 ± 0.6 vs 4.5 ± 0.4%, P=0.04).

Endothelium-Dependent Vasodilation

Sensitivity to MCH was assessed by comparing calculated EC50 values as seen in Figure 1. Obese did not differ from lean (-3.60 ± 1.06 vs -3.60 ± 1.53 log-molar [MCH], P=0.99). However, as seen in Figure 2, there was a trend toward attenuated CVC in obese across the dose response curve with a main effect of group (P=0.06). Baseline CVC at the MCH site was 0.29 ± 0.17 $mV \cdot mmHg^{-1}$ for Obese and 0.40 ± 0.19 $mV \cdot mmHg^{-1}$ for lean and was not different between groups (P=0.149). Figure 3 shows that maximum CVC at the MCH site was lower in obese (2.70 ± 0.73 vs 3.34 ± 0.81 $mV \cdot mmHg^{-1}$, p=0.027). Maximal CVC with MCh infusion, however, did not signifcantly correlate to BMI, as seen in Figure 4.

Endothelium-Independent Vasodilation

Three subjects were not included in the SNP analysis due to technical problems with the microdialyisis membrane at this site. For the following data obese consists of 6 males/4 females and lean consists of 6 males and 5 females. As seen in Figure 5, SNP EC_{50} was not different between Obese (-3.05 ± 0.61 log-molar [SNP]) and Lean (-3.66 ± 1.43 log-molar

[SNP]) (P=0.198). Figure 6 shows an interaction effect of group x dose response to SNP (p=0.007) with attenuations in obese CVC at doses ranging from 0.05mM to 50mM (all P<0.006). Baseline CVC was not different at the SNP site between obese and lean groups (0.33 ± 0.21 vs 0.42 ± 0.14 $mV \cdot mmHg^{-1}$, P=0.237). Maximal CVC at the SNP site was attenuated in the obese (2.44 ± 0.74 vs 3.31 ± 0.65 $mV \cdot mmHg^{-1}$, p=0.005). Further supporting this attenuation in endothelium independent vasodilation amongst obese, each site was heated to 43°C and infused with 50mM SNP. Maximal CVC is attenuated in obese at both the MCh and SNPsites as seen in Figures 7 and 8, respectively. Maximal SNP-induced CVC is negatively correlated with BMI (P=0.007), as seen in Figure 10.

Within-Subject Comparisons

As seen in Table 2, there were no differences in baseline or maximal CVC within groups. These results allow for the comparison of absolute, rather than relative, CVC responses across obese and lean groups.

Table 1: Subject Characteristics

	Lean	Obese	P
Sex M, F	7, 5	7, 5	
Age	24.6 ± 4.8	24.6 ± 4.9	0.87
Height (cm)	171.6 ± 11	167.5 ± 8	0.27
Weight (kg)	66.1 ± 11.8	95.4 ± 13.1*	<0.001
BMI ($kg \cdot m^{-2}$)	22.3 ± 1.9	33.7 ± 3.4*	<0.001
Systolic BP (mmHg)	113 ± 11	115 ± 9	0.63
Diastolic BP (mmHg)	68 ± 8	66 ± 9	0.57
Total Cholesterol ($mg \cdot dl^{-1}$)	157 ± 36	165 ± 42	0.60
HDL ($mg \cdot dl^{-1}$)	54 ± 11	33 ± 13*	<0.001
LDL ($mg \cdot dl^{-1}$)	88 ± 29	106 ± 40	0.20
LDL/HDL ratio	1.7 ± 0.8	3.9 ± 2*	<0.001
Triglycerides ($mg \cdot dl^{-1}$)	78 ± 43	135 ± 119	0.14
Fasting Glucose ($mg \cdot dl^{-1}$)	82 ± 10	81 ± 11	0.75
HbA1C (%)	4.5 ± 0.4	5 ± 0.6*	0.04

Values are mean ± SD. M, Male; F, Female; BMI, body mass index; BP, Blood Pressure; HDL, high density lipoprotein; LDL, low density lipoprotein; HbA1C, glycated hemoglobin. * indicates significant difference between obese and lean ($P<0.05$).

Table 2: Maximal CVC within subjects comparison between MCH and SNP sites

	Lean	Obese	Pooled
Max CVC- MCh	3.46 ± 0.67	2.84 ± 0.64	3.17 ± 0.73
Max CVC -SNP	3.31 ± 0.62	2.44 ± 0.70	2.90 ± 0.79
P	0.6	0.22	0.27

Values are mean ± SD. Maximal CVC in $mV \cdot mmHg^{-1}$ for both MCh and SNP are compared within groups and pooled together. Comparisons are made within groups and as pooled data and are assessed with two tail dependent samples t-tests. Subjects who were not used in SNP analysis are not included in this analysis, therefore obese n=10 and lean n=11. These data indicate that the maximal responses for each site are not different.

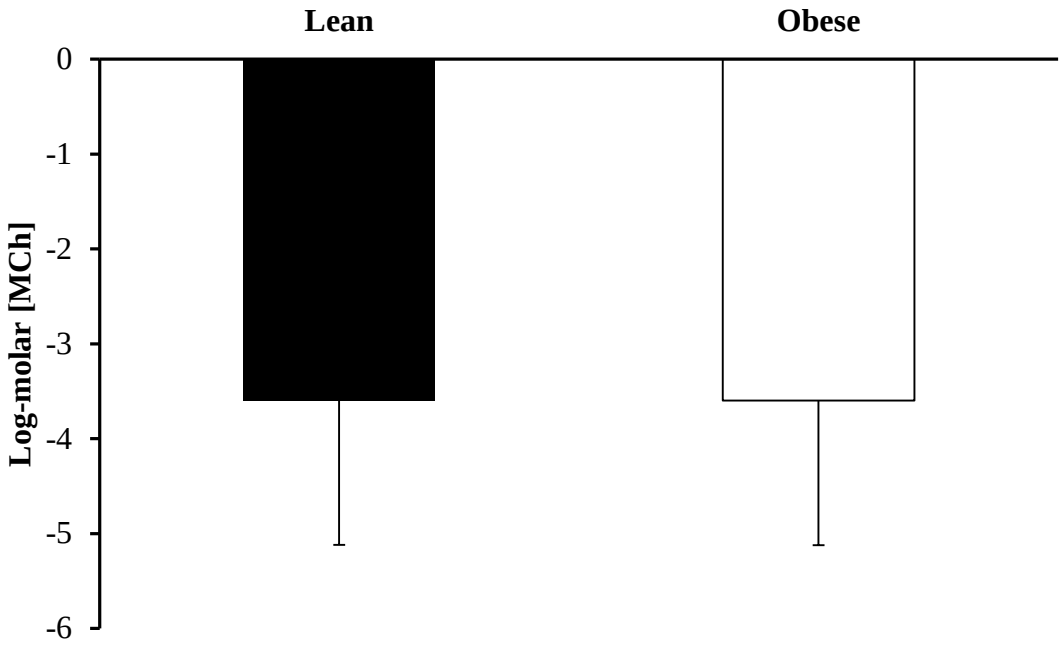

Figure 1: Mean ± SD EC_{50} for MCh in lean and obese as determined by MCh dose response curve (P=0.99).

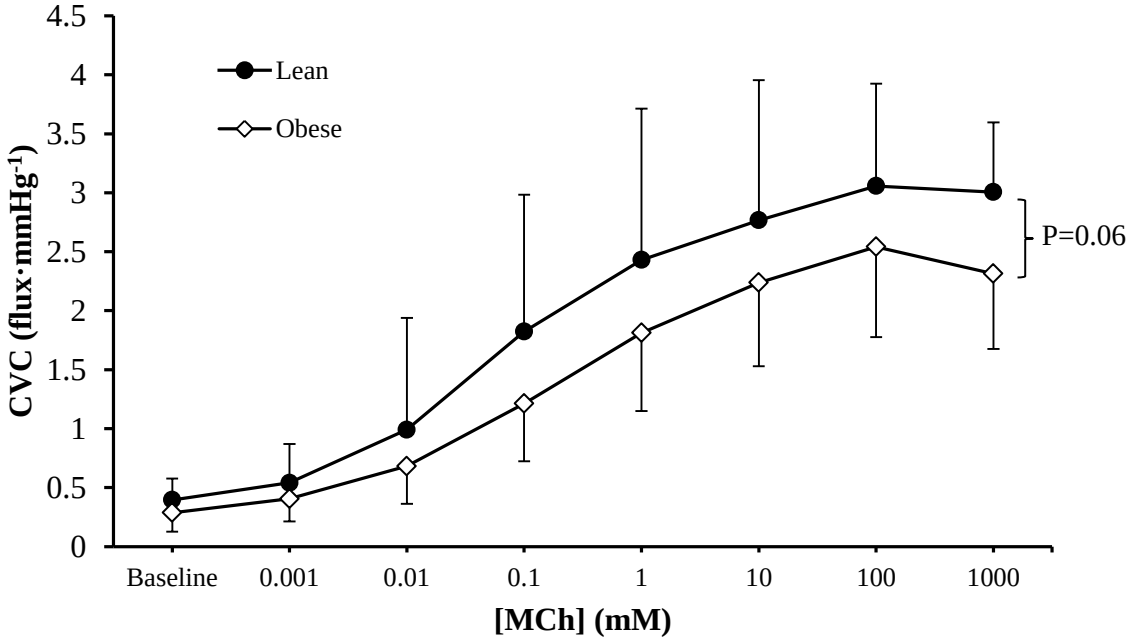

Figure 2: CVC graph of MCh dose response curve. There is a trend toward a main effect of group (P=0.06).

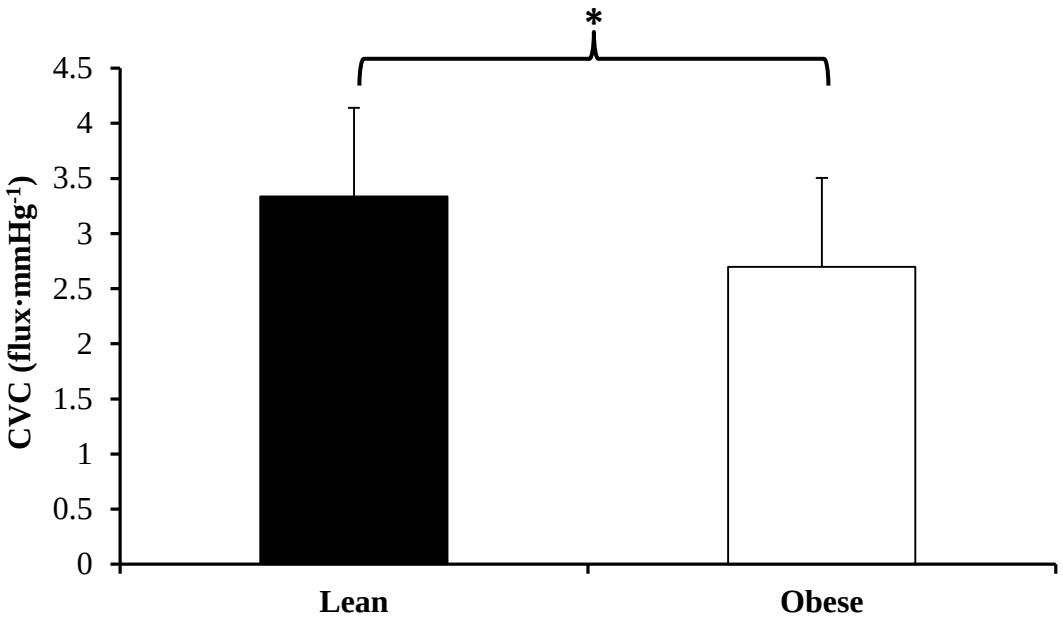

Figure 3: Mean ± SD Maximal CVC response to MCh. There is a significant difference between lean and obese (P=0.027)

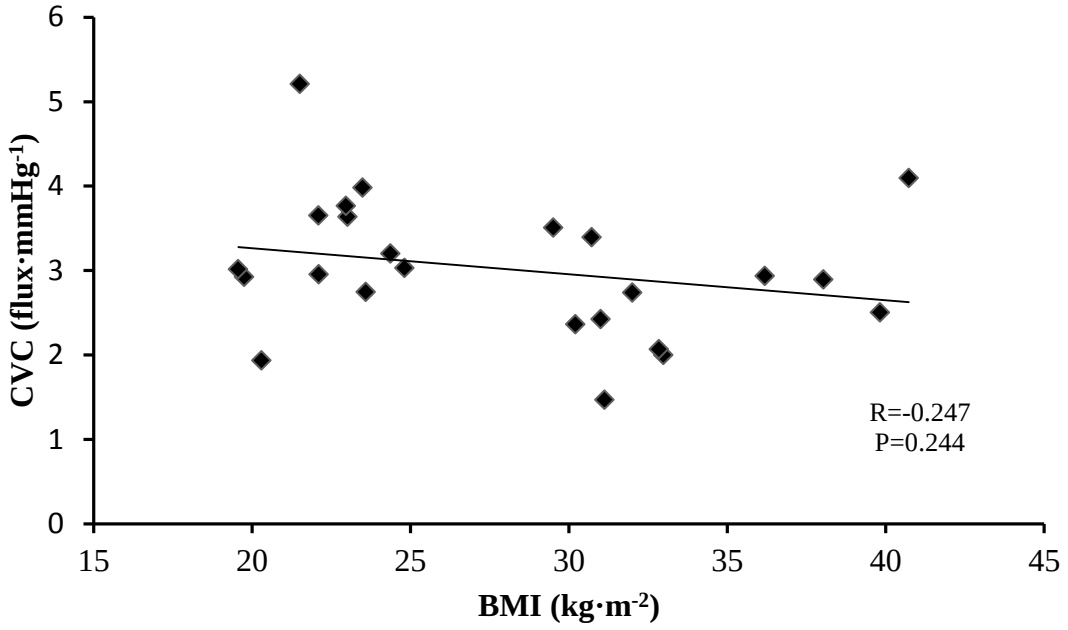

Figure 4: Relationship of maximal MCh CVC values to BMI. There is a not a significant correlation between MCh induced maximal CVC and BMI (P=0.244)

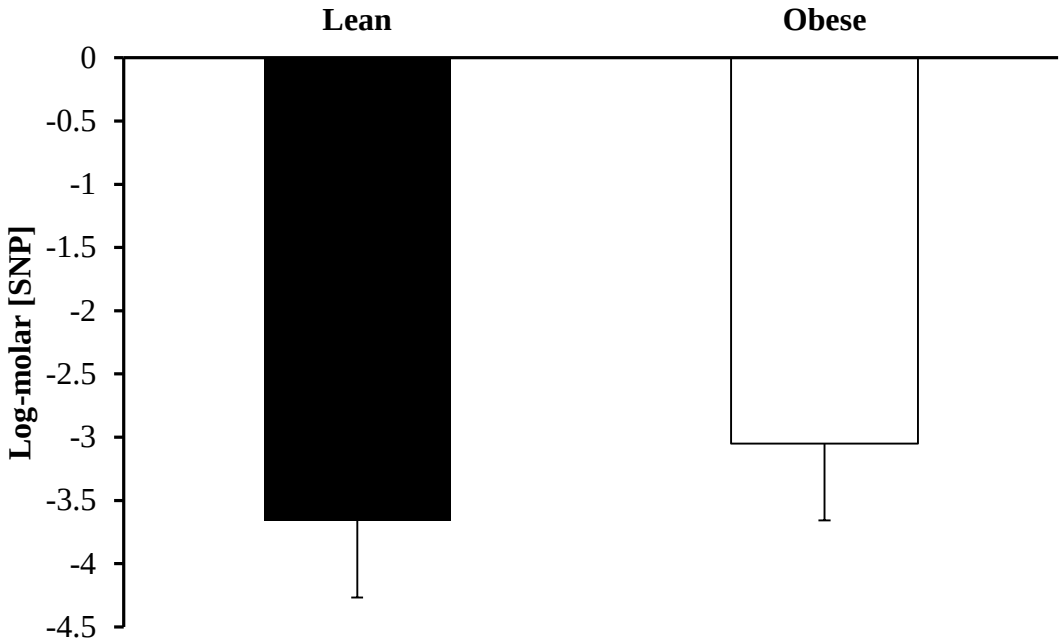

Figure 5: Mean ± SD EC_{50} for SNP in lean and obese as determined by SNP dose response curve. There is no difference between groups (P=0.198).

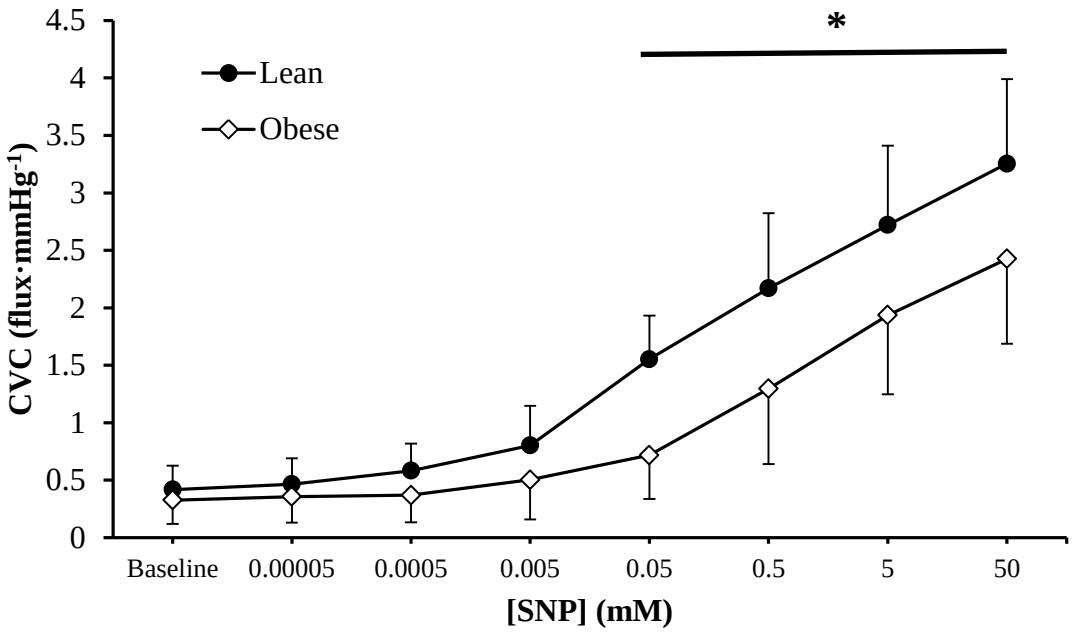

Figure 6: CVC graph of SNP dose response curve. There is a group x dose interaction (P=0.007). CVC is significantly lower in obese from 0.05mM to 50mM [SNP] (* all P<0.006).

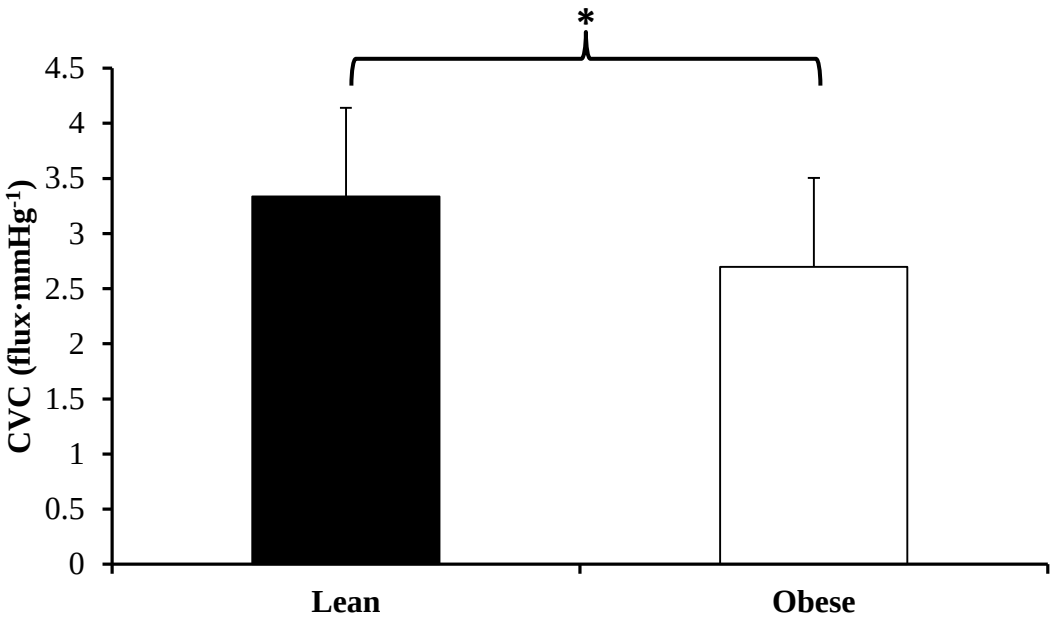

Figure 7: Mean ± SD Maximal CVC response to SNP. There is a significant attenuation in maximal CVC response to SNP in obese (*P=0.005).

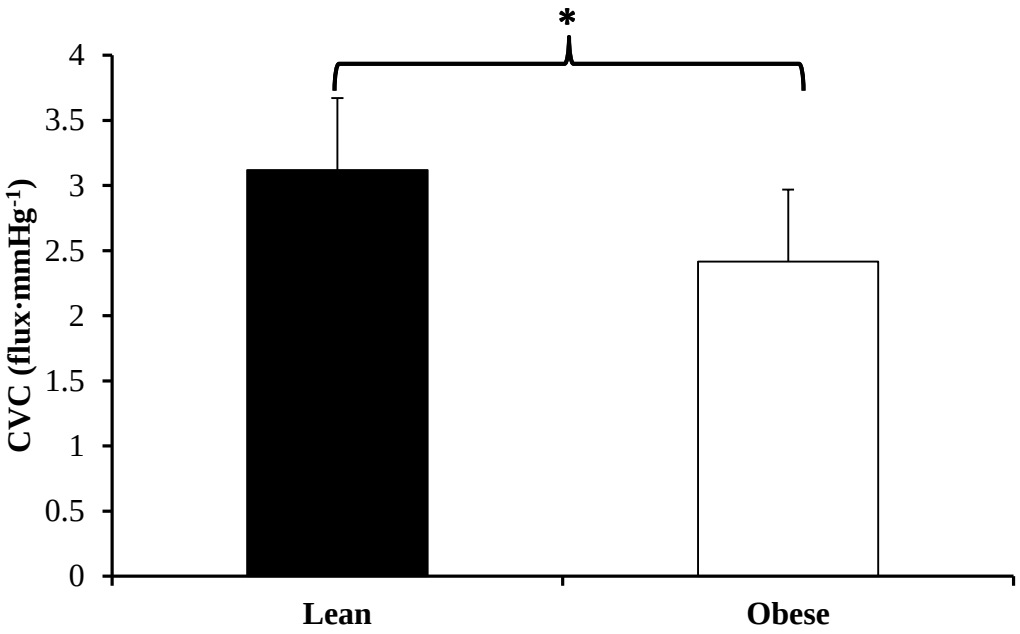

Figure 8: Mean ± SD CVC response to 50mM SNP +43° at MCh site. Obese has a significantly attenuated maximal CVC (P=0.008).

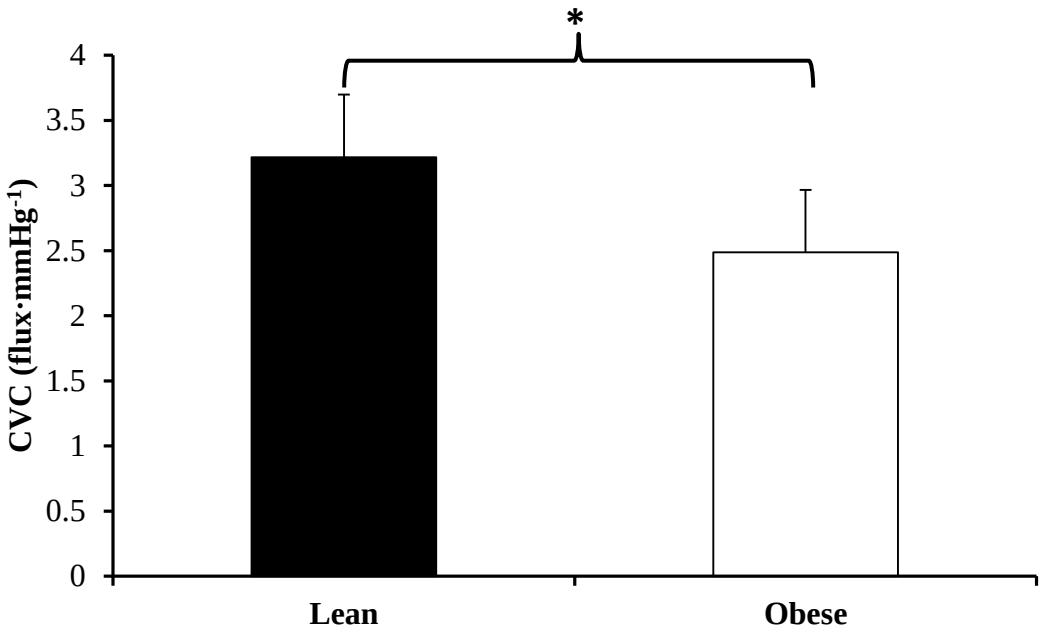

Figure 9: Mean± SD CVC response to 50mM SNP +43° at SNP site. Obese has a significantly attenuated maximal CVC (P=0.027).

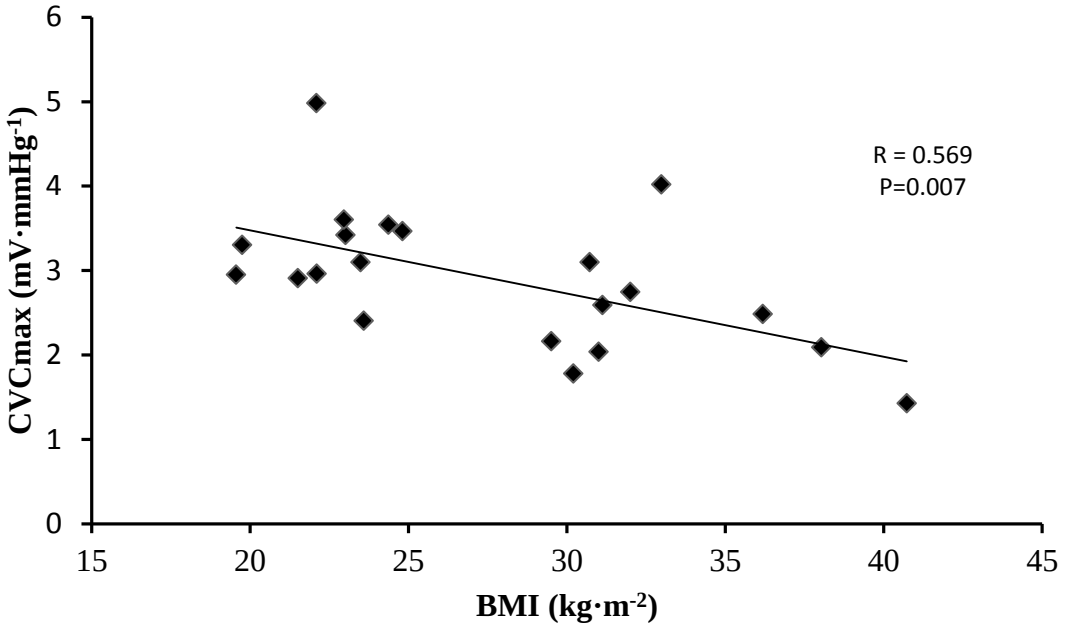

Figure 10: Relationship of maximal SNP CVC values to BMI. There is a significant negative correlation between SNP induced maximal CVC and BMI (P=0.007)

Chapter 5: Discussion

To the best of our knowledge, this is the first study investigating microvascular function in young, otherwise healthy obese adults using the microdialysis technique. Our results suggest that young obese individuals exhibit an attenuated cutaneous microvascular vasodilation in response to pharmacological stimuli. Decreased endothelium-independent vasodilation has been correlated with increasing BMI in the macrocirculation (Christou et al., 2012) as well as the microvasculature (Khan et al., 2003). Additionally, SNP-induced increases in forearm blood flow has been shown to be attenuated in obese subjects (Sivitz et al., 2007)

Impaired microvascular function is thought to precede the onset of hypertension (Levy et al., 2001; Noon et al., 1997), atherosclerosis (Al Suwaidi et al., 2000), and insulin resistance (Jonk et al., 2007; Serné et al., 2007). Additionally, the presence of microvascular dysfunction can worsen the prognosis for cardiovascular disease (Nakamura et al., 2001; Quyyumi, 2003). Since obesity is associated with these conditions, microvascular dysfunction is likely a contributing link between obesity and their development. This hypothesis is supported by the results of the current study suggesting that microvascular dysfunction occurs in young, at-risk individuals prior to the onset of diagnosable disease (Irving et al., 2002). Impairment at the level of the microvasculature can cause decreased perfusion and increased pressure, resulting in less surface area for glucose uptake and elevated blood pressure, as well as rarefaction, which only exacerbates these issues (Jonk et al., 2007).

Previous studies using iontophoresis to deliver pharmacological stimuli to the cutaneous microvascular have shown attenuations in vasodilatory response to endothelium-dependent stimuli such as ACh while showing no difference in endothelium-independent

vasodilation via SNP in obese versus their age-matched lean counterparts (de Jongh et al., 2004a; de Jongh et al., 2004b). However, caution must be taken when trying to interpret our results in light of these previous studies. These studies have mostly been performed on populations older than that of the current study and age itself has been known to effect microvascular function. The technique of iontophoresis involves the use an electrical charge for the infusion of substances across the skin and into the intradermal space. Non-specific effects of the electrical charge and individual difference in skin electrical resistance have been suggested and it is not possible to quantify the dose the dose that is actually being delivered intradermally (Droog & Sjöberg, 2003; Grossmann et al., 1995; Ramsay et al., 2002). Supporting the potential limitation to iontophoresis, severely obese adolescents have been shown to exhibit impaired brachial and cutaneous dilation to exogenous nitrate, yet no difference after SNP iontophoresis (Montero et al., 2014).

The results of the current study show attenuated endothelium-independent vasodilation across to multiple doses of SNP and an attenuated maximal endothelium dependent vasodilation in response to MCh. The impaired maximal response to MCh is difficult to interpret in the presence of attenuated response to SNP. SNP donates NO directly the VSMC to cause vasodilation; therefore our results indicate either an impaired response to NO at the VSMC or the degradation of NO prior to the relaxation of the VSMC. Either of these possible mechanisms would have an effect on the endothelium dependent response to vasodilation, since VSMC response to endothelial derived vasodilators is part of the process. Therefore, we cannot conclusively state that the endothelium is impaired in young, obese individuals. Any impairment in response to MCh could be attributed to impaired VSMC. Muscarinic stimulation of endothelium signals vasodilation of the VSMC with redundant mechanisms including NO, prostaglandins, and endothelial derived hyperpolarizing factor (EDHF) (Holowatz et al., 2005; Kellogg et al., 2005). Therefore, it is

possible that obesity could result in a greater impairment that is partially compensated and is thus not detectable without more mechanistic studies.

Oxidative stress, namely superoxide, limits endothelial-mediated dilation by reacting with NO before it can signal SG in the VSMC. Oxidative stress has been implicated in the microvascular impairment present in many at-risk population including: hypertension (Holowatz & Kenney, 2007), chronic kidney disease (DuPont et al., 2011), healthy aging (Holowatz, Thompson, & Kenney, 2006), and cigarette smoking (Fujii, Brunt, & Minson, 2014). These studies demonstrated augmented endothelial-dependent response after local infusion of anti-oxidants such as ascorbic acid or Tempol. In rats, obesity relates to elevated levels of superoxide and impaired microvascular response to both ACh and SNP (Frisbee & Stepp, 2001). When treated with anti-oxidants, obese, but not lean, rats show augmentation of both endothelium-dependent and independent vasodilation. Russo et al. found that the VSMC of an obese rat artery has a higher level of cGMP in the basal state, but cGMP levels do not rise to the same degree as seen in lean rats when exposed to NO. Anti-oxidant treatment decreased cGMP levels and partially restored the vascular relaxation occurring after a NO-donor in-vitro (Russo et al., 2008). In light of these studies, it is likely that oxidative stress is a factor in the microvascular responses seen in the current study.

However, other mechanisms may also contribute to the impaired response. Obesity is associated with increased plasma levels of the vasoconstrictor endothelin-1 (ET-1) (Ferri et al., 1995). ET receptor blockade produces vasodilation in obese that is not present in lean (Mather, Mirzamohammadi, Lteif, Steinberg, & Baron, 2002; Weil et al., 2011). Mather et al. then showed that ET receptor blockade increased NO availability suggesting that ET-1 acts through both elevated constriction and impaired NO-mediated dilation (Mather, Lteif,

Steinberg, & Baron, 2004). Future research should examine the role of ET-1 in young, apparently healthy obese individuals.

Commonly, microdialysis studies present results in terms of %CVCmax, where the CVC during any treatment is indexed to that specific site's maximal CVC. This relative method allows for comparison of multiple sites within the same person while accounting for any variablity in cutaneous blood flow across the forearm. Therefore, within one person, the effect of various enzmatic inhibitors or substrates can be accurately compared. In the present study, we chose to compare groups in terms of absolute CVC for a given treatment since we were comparing across groups and not treatments within subjects. As seen in Table 2, the baseline and maximal CVC did not differ between MCh and SNP sites within either the obese or lean group. Lastly, with similar EC_{50} values between groups, similar relative CVC dose response curves would be expected, as our data shows, suggesting similar sensitivity, but not necessarily response, to SNP and MCh between Obese and Lean. Taken together these data support the use of absolute, rather than relative, CVC for comparison across groups.

Chapter 6: Limitations

The current study has multiple limitations. A major limitiation of this study was the small sample size. The analysis at the MCh site included 12 subjects in each group while the SNP site only consisted of 11 lean and 10 obese subjects. This small sample size limits our ability to generalize our findings to a larger population and thus warrants further study. Additionally, the cutaneous microvasculature is considered a surrogate for whole body microvascular function, but may not reflect the functionality of other vascular beds. Furthermore, some may argue that the obese group could have received a lower effective dose of both MCh and SNP because they have a greater amount of tissue. However, we believe this not to be the case because the LDF probes were positioned immediately over the microdialysis membranes and microdialysis drug delivery is a local, not systemic, technique. The membranes were all inserted by the same investigator just below the surface of the skin, effectively standardizing the diffusion distance between obese and lean groups.

The use of only two microdialysis sites, each of which infusing separate pharmacolic stimuli, limits the conclusions that can be drawn from our data. Using multiple sites with the same stimulus, such as heat or MCh, while also administering blockers (eNOS, ET-1, or arginase inhibitors) or supplements (ascorbate, L-arginine, BH_4) and comparing responses both within individuals and across groups allows for a more thorough assessment of what pathways are being effected by obesity. Future studies should take a more mechanistic approach.

While subjects were instructed to avoid medications for 24 hours prior to testing, it has been shown that aspirin can impair endothelium-dependent vasodilation for at least three days via a decreased prostaglandin production (Durand et al., 2004). Though it is unlikely our obese group happened to consume COX-inhibitor drugs in the days prior to

testing at a higher rate than the lean group, future studies should prohibit medications for a week prior to testing.

Another limitation to our study is that we did not measure insulin sensitivity, waist circumference or total body fat, which have all been shown to correlate to microvascular dysfunction. Without these measurements, it is possible that this study did not include enough subjects of the central-obesity phenotype. Additionally, length of obesity should be assessed in regards to the development of microvascular dysfunction. Finally, our study only used a rough index of self-reported physical activity, so we are unable to determine if any group differences may just be related to differences in activity or fitness level between groups. Physical fitness has been shown to augment endothelium dependent vasodilation (Black, Green, & Cable, 2008; Pugh et al., 2013; Wang, 2005). Other studies have shown improved response to incremental heating, but no change in maximal response to SNP, after an exercise training program. Therefore it may not be likely that different activity levels in the current study would change the results, but future studies need to include accurate physical activity measures in order to avoid this limitation.

Chapter 7: Conclusions

To the best of our knowledge our study is the first to examine the cutaneous microvasculature in healthy young obese individuals using a microdialysis technique. Our major finding implicated the endothelium-independent impairments in this apparently disease-free population. Though the precise mechanism of the attenuated response to SNP warrants further study, previous research in animals suggests a role for elevated oxidative stress. Future research needs to elucidate the mechanisms contributing to the observed dysfunction in young, disease-free, obese humans.

Appendix A

Consent for Participation in Research Title: Effect of Obesity

on Peripheral Microvascular Function

Principal Investigator(s) (include faculty sponsor), UT affiliation, and Telephone Number(s):

R.Matthew Brothers, Ph.D. Assistant Professor: Department of Kinesiology and Health Education; College of Education; University of Texas at Austin. Phone: (512) 516-1961; Email: r.m.brothers@mail.utexas.edu.

Funding source: Currently this work is funded by the University of Texas at Austin.

Introduction
The purpose of this form is to provide you information that may affect your decision as to whether or not to participate in this research study. The person performing the research will answer any of your questions. Read the information below and ask any questions you might have before deciding whether or not to take part. If you decide to be involved in this study, this form will be used to record your consent.

Purpose of the Study
You have been asked to participate in a research study about the effect of body weight on substances that cause constriction (narrowing) of blood vessels. The purpose of this study is to determine if the role of these substances if effected by body weight.

What will you to be asked to do?
Before you can be admitted to the study, you will be given a brief examination during your visit to the laboratory. This examination will include filling out a brief health history questionnaire, which will include questions about your age, sex, and ethnic origin, as well as questions about lifestyle choices (i.e. tobacco use, alcohol consumption etc.). Additional measurements will include standard measures of bodyweight, height, blood pressure, and heart rate.

If you are eligible for the study you will be enrolled into the study which will be completed on the same day. The expected time for you to complete the study day is approximately 4 hours. Please refer to the following section for a description of the visit to the laboratory.

Laboratory visit – step-by-step protocol

1. Arrival at laboratory, health history questionnaire
2. Measures of body height and weight (using a standard scale similar to what is used in a doctor's office).
3. Measures of blood pressure, heart rate, and skin blood flow, and skin temperature during ~3 hrs of resting quietly.
    - ***Total time in the laboratory: ~ 4 hours***

A detailed list of procedures is described below. The potential risks and duration of each procedure are provided. If at any time you wish to discuss the information above or any

other risks you may experience, you may ask questions now or call the Principal Investigators listed on the front page of this form.

Skin blood flow:

- **Description of Procedure:** Skin blood flow will be measured at 4 different places on your forearm. This measurement will be done using laser-Doppler devices. These devices use very low power laser light to measure how fast blood is flowing in blood vessels in your skin. Four small probes will be placed on one of your forearms.
- **Potential Risks:** There are no risks associated with using these devices to measure skin blood flow; they are painless and harmless in all respects.
- **Duration of Procedure:** Skin blood flow will be measured during the entire experiment.

Manipulation of skin temperature:

- **Description of Procedure:** Control of local skin temperature is accomplished by locally heating the site where skin blood flow is measured. The highest temperature we will use is 108° F for 30 minutes.
- **Potential Risks:** There are no risks associated with these temperature changes, although there is a small risk of minor discomfort at the heated sites.
- **Duration of Procedure:** The total duration of local heating will be about 30 minutes.

Electrocardiogram:

- **Description of Procedure:** Sticky patches will be applied to your skin to measure the heart's electrical signals.
- **Potential Risks:** There is no risk or discomfort associated with this procedure.
- **Duration of Procedure:** The electrocardiogram will be measured during the entire experiment.

Blood pressure:

- **Description of Procedure:** Your blood pressure will also be monitored using a cuff placed on your upper arm that is inflated and deflated periodically.
- **Potential Risks:** Other than some potential discomfort associated with cuff inflation there is no risk to this procedure.
- **Duration of Procedure:** The cuff will be on your upper arm during the entire experiment. We will take blood pressure measurements at different time points during the experiment. Each measurement will last approximately 30 seconds.

Placement of Small Microdialysis Tubes:

- **Description of Procedure:** These tubes will be used to deliver small amounts of commonly used substances into your skin. To do this we will place 4 small microdialysis tubes into the skin of one of your forearms. The microdialysis tube is placed almost exactly like an "IV" in the hospital, except that veins are not entered. First the skin is cleaned with alcohol. Then four very thin (25 gauge) needles will be placed just under the surface of your skin on one of your forearms. Four special, sterile, thin tubes (about 1/100th of an inch in diameter; about the

size of fishing line) with microscopic holes in it will be placed through the needle. Then the needle will be removed, leaving the special tubing just under the surface of your skin on your forearm. Additional tubing (without holes) will be connected to the special tube in your skin and a sterile salt solution that is used in hospitals (Ringer's solution) will be pumped slowly through the tubing assembly. Only a tiny amount of the salt solution and drug will go into your skin. After the study ends, all tubes are removed.

- **Potential Risks:** You may experience some discomfort with the small needles used during the placement of the microdialysis tubes. However, after the needles have been removed, the microdialysis tubes should not hurt and will be physically unnoticeable.
- **Duration of Procedure:** These tubes will be in your arm during the entire experiment.

Drug Solutions:

- **Description of Procedure:** Throughout the experiment small amounts of commonly used drug solutions (see below for description of drug solutions) will be delivered through the tubes. These drugs will be used to measure the effect of various substances on the constriction (narrowing) of your blood vessels during the cold exposure. These drugs will be mixed in a solution called Ringer's solution (similar to a lightly salted solution that is used in hospitals). The drugs to be used include L-N^G-Nitroarginine methyl ester (L-NAME), sodium nitroprusside (SNP), Acetylcholine (Ach) and ascorbic acid.
- **Potential Risks:** It is possible that there will be some redness of the skin in the area where the drug is given. Due to the small concentration of the drugs delivered, there is no known risk associated with receiving these drugs. There are no known negative experiences in humans with the concentrations of each of the drugs.
- **Duration of Procedure:** These tubes will be in your forearm during the entire experiment.

All tests that are to be performed have safely been used in both healthy and diseased individuals. Throughout the tests you will be closely monitored by highly skilled and trained personnel.

Because of your participation in this study, you may experience any of the risks cited above. You should discuss these with the researchers and your regular health care provider. All the tests in this study are designed for research only, not for medical purposes. Even though the researchers are not looking at your tests to find or treat a medical problem, you will be told if they notice something unusual. You and your regular doctor can decide together whether to follow up with more tests or treatment. Because all research tests done in this study are not for medical purposes, the research results will not be sent to you or to your regular doctor.

This study will last approximately 4 hours, and will take place on a single day. It will include approximately 50 study participants.
This is a research study and, therefore, is not intended to provide a medical or therapeutic diagnosis or treatment. The measures and steps taken during the course of this study are not necessarily equivalent to the standard method of prevention, diagnosis, or treatment of a health condition.

What are the risks involved in this study?
All possible risks associated with this study are explained above. The principal investigator and laboratory team are experienced with all procedures outlined in this study in both healthy and diseased populations. Nevertheless, as with all studies involving human subjects, there are risks associated with experimentation that are currently unknown. To reduce these risks, you will only be able to join the study if you are healthy and you will have to complete a health history questionnaire as a precautionary measure to ensure the study poses no additional risks. Furthermore, potential risks will also be minimized by: (a) using only safe, well-established procedures; (b) constant, personal monitoring of each experimental session by the investigators and staff; and (c) knowledge that you can request to stop at any point during the procedure. You should immediately tell the research personnel if you are injured or harmed in any way during the study. Also, you should know that the principal investigator may also terminate the testing procedure at any time. All experimental procedures will conform to the University of Texas at Austin Institutional Review Boards for Human Subjects.

In addition, the privacy of the subject will be ensured as a result of the record keeping process to be employed. All data collected on individual subjects will only be viewed by the investigative team involved in the project (i.e., principal investigator, research assistant, pre- and postdoctoral research fellows), although these data will be provided to the subject. The individual data for each subject will be analyzed and stored by code, and at no time will any individual data point be identified with a particular individual subject. The data will almost exclusively be presented as average group differences. All data and information regarding the study will be kept in the personal, well-secured laboratory of the principal investigator.

What are the possible benefits of this study?
Direct benefits of the study are not guaranteed. The possible benefits of the study are: 1) information such as blood pressure readings, heart rate etc. free of charge and 2) rewarding contribution to a basis of scientific and clinical knowledge that is likely to improve our understanding of blood flow regulation during cold exposure.

Do you have to participate?
No, your participation is voluntary. You may decide not to participate at all or, if you start the study, you may withdraw at any time. Withdrawal or refusing to participate will not affect your relationship with The University of Texas at Austin (University) in anyway.
If you would like to participate, you will be asked to fully read and then sign this form. The researcher will also sign this form and will provide you with a copy of the signed form.

Will there be any compensation?
You will receive $50.00 upon completion of the entire protocol. If you only participate in part of the study day you will receive compensation at a rate of $12.50 per hour spent in the lab. Note that in order to process compensation you will be asked to provide your social security number. At all times, documents containing social security numbers will be stored securely in locked cabinets and in separate files from other study records.

Payments will be processed after the study has been completed and this process typically takes 3 to 4 weeks. You will be responsible for any taxes assessed on the compensation.

What if you are injured because of the study?
The University has no program or plan to provide treatment for research related injury or payment in the event of a medical problem. In the event of a research related injury, please contact the principal investigator.

What are my confidentiality or privacy protections when participating in this research study?
This study is **confidential** and your privacy will be ensured as a result of the record keeping process to be employed. All data collected on individual subjects will only be viewed by the investigative team involved in the project. The individual data for each subject will be analyzed and stored by code, and at no time will any individual data point be identified with a particular individual subject. All data and information regarding the study will be kept in the personal, well-secured laboratory of the principal investigator.

What should you expect if the study is collecting genetic information?
Genetic information will not be collected in this study.

Whom to contact with questions about the study?
Prior, during or after your participation you can contact the researcher (R. Matthew Brothers,
PhD) at (512) 232-6016 or send an email to r.m.brothers@austin.utexas.edu**.**

This study has been reviewed and approved by The University Institutional Review Board and the study number is **2013-05-0106.**

Whom to contact with questions concerning your rights as a research participant?
For questions about your rights or any dissatisfaction with any part of this study, you can
contact, anonymously if you wish, the Institutional Review Board by phone at (512) 471- 8871 or email at orsc@uts.cc.utexas.edu.

Participation
If you agree to participate in this study, the researcher will accept this form, and provide you with a copy.

Signature

You have been informed about this study's purpose, procedures, possible benefits and risks, and you have received a copy of this form. You have been given the opportunity to ask questions before you sign, and you have been told that you can ask other questions at any time. You voluntarily agree to participate in this study. By signing this form, you are not waiving any of your legal rights.

_____

Printed Name

_____ _____

Signature Date

As a representative of this study, I have explained the purpose, procedures, benefits, and the risks involved in this research study.

_____

Print Name of Person obtaining consent

_____ _____

Signature of Person obtaining consent Date

Appendix B

Research Health Questionnaire

The Environmental and Autonomic Physiology Laboratory

Personal Information
Name: _____

Today's Date: _____

Age _____ Sex □ Male
□ Female: Date of Last Menstrual Period: _____

Contact Information:
_____

Physician Name and Phone
Number:_____

Emergency Contact Info:
_____

Symptoms or Signs Suggestive of Disease
Check appropriate box:

Yes	No		
□	□	1.	Have you experienced unusual pain or discomfort in your check, neck, jaw, arms or other areas that may be due to heart problems?
□	□	2.	Have you experienced unusual fatigue or shortness of breath at rest, during usual activities, or during mild-to-moderate exercise (e.g., climbing stairs, carrying groceries, brisk walking, cycling)?
□	□	3.	When you stand up, or sometimes during the night while you are sleeping, do you have difficulty breathing?
□	□	4.	Do you lose your balance because of dizziness or do you ever lose consciousness?
□	□	5.	Do you suffer from swelling of the ankles (ankle edema)?
□	□	6.	Have you experienced an unusual and rapid throbbing or fluttering of the heart?
□	□	7.	Have you experienced severe pain in your leg muscles during walking?
□	□	8.	Has a doctor told you that you have a heart murmur?

Chronic Disease Risk Factors
Check appropriate box:

Yes	No		
□	□	9a.	Are you a male over age 45 years or a female over age 55 years?
□	□		b. Are you a female who has experienced premature menopause?

☐ ☐ c. If you answered "yes" to 9b, are you on estrogen replacement therapy?

☐ ☐ 10. Has your father or brother had a heart attack or died suddenly of heart disease before the age of 55; has your mother or sister experienced these heart problems before the age of 65?

☐ ☐ 11. Are you a current cigarette smoker?
If quit smoking, when? Date:

Yes	No		
☐	☐	12.	Has a doctor told you that you have high blood pressure (more than 140/90 mm Hg) or a heart condition?
☐	☐	13.	Is your total serum cholesterol greater than 200 mg/dl, or has a doctor told you that your cholesterol is at a high risk-level?
☐	☐	14.	Do you have diabetes mellitus?
☐	☐	15.	Are you physically inactive and sedentary (little physical activity on the job or during leisure time)?
☐	☐	16.	Do you have a bone or joint problem that could be made worse by a change in your physical activity?
☐	☐	17.	During the past year, would you say that you have experienced enough stress, strain, and pressure to have a significant effect on your health?
☐	☐	18.	Do you eat foods nearly every day that are high in fat and cholesterol such as fatty meats, cheese, fried foods, butter, whole milk, or eggs?
☐	☐	19.	Do you weigh 30 or more pounds than you should?
☐	☐	20.	Do you know of any other reason you should not do physical activity?

Medical History

21. *Please check which of the following conditions you have had or now have. Also check medical conditions in your family (father, mother, brother(s), or sister(s)). Check as many as apply.*

Self	Family	Medical Condition	Self	Family	Medical Condition
☐	☐	Coronary heart disease, heart attack; by-pass surgery	☐	☐	Major injury/fracture to foot, leg, knee
☐	☐	Arrhythmias	☐	☐	Major injury to back or
☐	☐	Autonomic Neuropathy	☐	☐	neck
☐	☐	Diabetic Nephropathy			Diabetic Retinopathy
☐	☐	Angina	☐	☐	Major injury/fracture to
☐	☐	Marfan's syndrome			hip or shoulder
☐	☐	High blood pressure	☐	☐	Recent leg trauma/injury
☐	☐	Peripheral vascular disease	☐	☐	Rheumatoid arthritis
☐	☐	Phlebitis or emboli	☐	☐	Osteoarthritis
☐	☐	Other heart problems	☐	☐	Osteoporosis
☐	☐	Stroke	☐	☐	Fibromyalgia

☐	☐	Asthma	☐	☐	Chronic fatigue syndrome
☐	☐	Bronchitis	☐	☐	Systemic lupus erythematosus
☐	☐	C.O.P.D. (emphysema)	☐	☐	Anemia (low iron)
☐	☐	Pulmonary embolism (blood clots in lungs)	☐	☐	Thyroid problems
			☐	☐	Gout
☐	☐	Deep vein thrombosis (blood clots in legs)	☐	☐	Kidney disease
			☐	☐	Nephrotic (kidney) syndrome
☐	☐	Antithrombin III deficiency	☐	☐	Gallstones/gallbladder disease
☐	☐	Inherited hypercoaguability	☐	☐	Liver disease (cirrhosis)
☐	☐	Acquired hypercoaguability	☐	☐	Hepatitis
☐	☐	Factor V leiden mutations	☐	☐	Diabetes mellitus
☐	☐	Protein C deficiency	☐	☐	Raynaud's disease
☐	☐	Protein S deficiency	☐	☐	Crohn's disease
☐	☐	Stomach/duodenal ulcer	☐	☐	Hysterectomy
☐	☐	Rectal growth or bleeding	☐	☐	Problems with menstruation

Self	**Family**	**Medical Condition**	**Self**	**Family**	**Medical Condition**
☐	☐	Irritable bowel syndrome	☐	☐	Cold-Induced Urticaria
			☐	☐	Post-menopausal
☐	☐	Lung cancer			Date:
☐	☐	Breast cancer	☐	☐	Allergies
☐	☐	Prostate cancer	☐	☐	Depression
☐	☐	Skin cancer	☐	☐	Anxiety, phobias
☐	☐	Colorectal cancer	☐	☐	Eating disorders
☐	☐	Other cancer Specify:	☐	☐	Substance abuse problems (alcohol, other drugs, etc.)
☐	☐	Hearing loss	☐	☐	Sleeping problems
☐	☐	Cataracts	☐	☐	Other
☐	☐	Glaucoma			Specify:
☐	☐	Raynaud's Disease			

Please specify and include information on any recent illnesses, hospitalizations, surgical procedures, or other health problems.

_____

_____

_____

22a. Are you currently pregnant, think you may be pregnant, or are currently trying to get pregnant?

☐ Yes ☐ No ☐ Not sure ☐ Not applicable (male or post-menopausal)

b. If you answered "yes" or "not sure" to 22a, do you need a pregnancy test? ☐ Yes ☐ No

23**.** In the past two weeks, have you had a barium test, a nuclear medicine scan, or x-rays with a dye injection?

☐ **Yes** ☐ **No**

24. Do you have any adverse reactions to cold water / temperatures?

☐ **Yes** ☐ **No**

25. *Please check any of the following medications you take regularly and give the name and dose of the medication.*

Medication	**Name of Medication**
☐ Heart medicine	_____
☐ Blood pressure medicine	_____
☐ Blood cholesterol medicine	_____
☐ Thromboembolic disease medicine	_____
☐ Hypercoaguability medicine	_____
☐ Steroids	_____
☐ Hormones/HRT	_____
☐ Birth control medicine	_____
☐ Medicine for breathing/lungs	_____
☐ Insulin	_____
☐ Other medicine for diabetes	_____
☐ Arthritis medicine	_____
☐ Medicine for depression	_____
☐ Medicine for anxiety	_____
☐ Thyroid medicine	_____
☐ Medicine for ulcers	_____
☐ Painkiller medicine	_____
☐ Allergy medicine	_____
☐ Dietary supplements (herbs, vitamins, etc)	_____
☐ Other (please specify)	_____

26. Do you have any known drug allergies? _____

Body Weight

27. What is the most you have ever weighed? _____

28. Are you now trying to:

☐ Lose weight ☐ Gain weight ☐ Stay about the same ☐ Not trying to do anything

Stress

29. During the past month, how would you rate your overall level of stress?
☐ Very high ☐ High ☐ Moderate ☐ Low

30. In the past year, how much effect has stress had on your health?
☐ A lot ☐ Some ☐ Hardly any or none

31. On average, how many hours of sleep do you get in a 24-hour period?
☐ Less than 5 ☐ 5-6 ☐ 7-9 ☐ More than 9

Substance Use

32. How would you describe your cigarette smoking habits?
☐ Never smoked
☐ Used to smoke. How many years has it been since you smoked? _____ years
☐ Still smoke. How many cigarettes a day do you smoke on average? _____ cigarettes/day

33. How many alcoholic drinks do you consume? (A "drink" is a glass of wine, a wine cooler, a 16oz bottle/12oz can of beer, a shot glass of liquor, or a mixed drink).
☐ Never use alcohol ☐ Less than 1 per week ☐ 1-6 per week
☐ 1 per day ☐ 2-3 per day ☐ More than 3 per day

34. In one sitting, how many drinks do you typically consume? _____
35. How many cups (8 ounces) of coffee do you drink per day? _____
36. How many ounces of sodas containing caffeine do you drink per day? _____

Physical Fitness, Physical Activity/Exercise

37. Considering a **7-Day period** (a week), how many times on the average do you do the following kinds of exercise for **more than 15 minutes** during your **free time** (write on each line the appropriate number).

a) **STRENUOUS EXERCISE (HEART BEATS RAPIDLY)** **Times Per Week**
(i.e. running, jogging, hockey, football, soccer, squash, basketball, cross country skiing, judo, roller skating, vigorous swimming, vigorous long distance bicycling) _____

b) **MODERATE EXERCISE (NOT EXHAUSTING)**
(i.e. fast walking, baseball, tennis, easy bicycling, volleyball, badminton, easy swimming, alpine skiing, popular and folk dancing) _____

c) **MILD EXERCISE (MINIMAL EFFORT)**
(i.e. yoga, archery, fishing from river bank, bowling, horseshoes, golf, snow-mobiling, easy walking) _____

38. Considering a 7-Day period (a week), during your leisure-time, how often do you engage in any regular activity long enough to work up a sweat (heart beats rapidly)?

□ OFTEN □ SOMETIMES □ NEVER/RARELY

39. How long have you exercised or played sports regularly?

□ I do not exercise regularly □ Less than 1 year □ 1-2 years
□ 2-5 years □ 5-10 years □ More than 10 years

Appendix C

Research Subject Information

The environmental and Autonomic Physiology Laboratory

Personal Information

Name: _____

Subject ID/SSN: _____

Date of Birth: _____ Age: _____ Sex □ Male
□ Female

Ethnic Background: □Hispanic or Latino □Not Hispanic or Latino

Race:
□White □American Indian/Alaskan Native □Pacific Islander
□Black or African American □Asian □Other: _____

Address: _____

Contact Information: _____

Emergency Contact Info: _____

Would you like to be contacted for future studies? □ Yes □ No

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