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THE IMPORTANCE OF EDHF IN FUNCTIONAL SYMPATHOLYSIS IN HEALTHY YOUNG ADULTS

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ABSTRACT

Functional Sympatholysis allows for the matching of increased demands in skeletal muscle oxygenation by increasing exercise hyperemia despite elevated sympathetic outflow. Endothelial-Derived Hyperpolarizing Factor (EDHF) may be involved in functional sympatholysis although its role is not clear. **PURPOSE:** The purpose of the present study was to determine the importance of EDHF in functional sympatholysis in healthy young adults. **METHODS:** 14 participants (8 Females) participated in three study visits (2 experimental visits). Experimental visits were identical with the exception of ingestion of a placebo (PLA) (250 mg microcrystalline cellulose) or 150 mg of fluconazole (FLZ) 120 minutes before testing (randomized and counter-balanced). Forearm blood flow, (FBF, doppler and echo ultrasound), mean arterial pressure (MAP, finger photoplethysmography), vascular conductance (FVC, FBF/MAP × 100mmHg), and muscle oxygenation (near-infrared spectroscopy) were compared during rest, -20mmHg of lower body negative pressure (LBNP), rhythmic forearm exercise at 20% maximum voluntary contraction (MVC) for 5 minutes, and forearm exercise with LBNP for 2 minutes **RESULTS:** Data are percent change (Δ%) ± SD. FVC declined from rest to LBNP but did not differ between PLA and FLZ (Δ -32.02 ± 18.99% vs Δ -38.16 ± 15.97, p=0.35). During exercise, FVC was unaltered in PLA with the addition of LBNP (Δ 2.92 ± 12.69%, p=0.81), however, declined in FLZ (Δ –11.58 ± 15.06%, p=0.01). exercise within FLZ, exercise + LBNP between PLA vs FLZ, (p = 0.0003). **CONCLUSION**: Our results indicate that young, healthy adults were able to maintain FVC with the addition of LBNP induced sympathetic stress during exercise in the PLA, however, when EDHF was inhibited with FLZ, FVC declined. Therefore, EDHF may have an important role in functional sympatholysis.

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CHAPTER I: INTRODUCTION

Introduction to the problem

Exercise intolerance and poor cardiorespiratory fitness are well established to elevate risk of CVD and predict all-cause mortality (Kodama et al., 2009). Further, poor fitness and exercise tolerance are often linked to an inadequacy to match blood flow to the metabolic demand of active tissues (Joyner & Casey, 2015). During exercise, there is an increased demand for skeletal muscle oxygenation and removal of metabolites. Therefore, to sustain exercise, blood vessels in active muscle need to vasodilate to increase blood flow, such that, oxygen delivery is paired muscle metabolic demand and mean arterial pressure (MAP) is regulated (Joyner & Casey, 2015). To achieve the balance of increased arterial outflow directed to active muscle blood flow and control of MAP, increases in exercise intensity are coupled with increases in muscle sympathetic nervous system activity (MSNA) and norepinephrine (NE) release causing systemic α-adrenergic vasoconstriction limiting blood flow to inactive tissues (Tschakowsky & Pyke, 2008). The ability to locally vasodilate in exercising skeletal muscle despite increases in α-adrenergic vasoconstriction via MSNA is called functional sympatholysis (Hearon et al., 2016; Dinenno et al., 2003). Functional sympatholysis plays an important role in vasodilator and vasoconstrictor responses, and thus the matching of blood flow to the increased metabolic demands during exercise and stands in direct competition with blood pressure control (Mortensesn et al., 2014).

The mechanisms of functional sympatholysis are not well understood. Early studies investigating functional sympatholysis have established that muscle blood flow stays consistent apart from additional sympathetic stress added to an exercise modality in healthy humans. For example, local arterial infusion of tyramine, which causes NE release from nerve terminals,

mimicking increases in MSNA, did not change muscle blood flow during leg extensor exercise. Therefore, there is a consistent dilation despite high amounts of NE/MSNA, indicating the existence of mechanisms local to the active muscle that have the ability of blunt sympathetic vasoconstriction (Saltin & Mortensen, 2012). Evidence suggests that vasodilatory metabolites can overrule sympathetic vasoconstriction via blunting the effectiveness of norepinephrine and epinephrine. The importance of each potential metabolite involved in functional sympatholysis, and mechanisms of these specific vasodilators involved are not well understood (Hearon et al., 2016, Petterson et al., 2021). Most studies to date focused on the involvement of Nitric Oxide (NO) and Prostaglandins (PG) as local vasodilators involved in the mechanisms of functional sympatholysis (Thomas & Victor, 1998, Hearon et al., 2016). However, the evidence suggests little involvement in of these vasodilators in functional sympatholysis (Crecelius et al., 2011; Hearon et al., 2016). Specifically, Hearon et al (2016) tried to determine if NO or PGs contributes to the ability to override sympathetic vasoconstriction during exercise (functional sympatholysis). The data in the study showed that NO/PG did not change ability to blunt sympathetic vasoconstriction, indicating NO/PG independent sympatholysis. Further, the infusion of the NO donor sodium nitroprusside (acting endothelium independent) did not blunt sympathetic vasoconstriction at rest or in addition to forearm muscle exercise at 5% MVC. However, the vasodilatory stimulus via an acetylcholine infusion as well as low dose infusion of the endothelium dependent vasodilator adenosine triphosphate (ATP), did blunt sympathetic vasoconstriction during mild (non-sympatholytic) intensity exercise to a similar magnitude as moderate exercise (15%) (sympatholytic), thereby indicating endothelial factors that are independent of NO and PG must be responsible.

A possible contributor to the sympatholytic mechanism is a yet to be identified factor downstream of cytochrome P450 (CYP450), termed Endothelium derived hyperpolarizing factor (EDHF). EDHFs potentially cause vasodilation by opening Ca^{2+} activated K channels, leading to vascular smooth muscle relaxation (Trinity et al., 2021). The potential involvement of EDHF in functional sympatholysis has been vastly overlooked in recent literature. While recent evidence suggests that EDHF may be important in control of vascular tone at rest (Petterson et al., 2021), data on the importance of EDHF to vascular tone during exercise are limited. The investigation of the potential metabolites involved in functional sympatholysis, including EDHF, is essential to further understand cardiovascular function and etiology of exercise intolerance.

The aim of the present study was to investigate the importance of EDHF in functional sympatholysis. This will be accomplished by measuring muscle blood flow and oxygenation responses to the blocking of EDHF pathways during forearm exercise and additional sympathetic stress. We hypothesized EDHF blockade significantly impairs functional sympatholysis in healthy, young adults. We also hypothesis that EDHF blockade does decrease forearm muscle blood flow, at exercise with sympathetic stress in healthy, young adults and that forearm muscle oxygenation does decrease after EDHF blockade during rest, sympathetic stress, exercise, and exercise with sympathetic stress

Purpose

Therefore, the purpose of the present study was to determine if inhibition of EDHF attenuates functional sympatholysis in healthy young adults.

Research Questions

1. Does the blockade of EDHF significantly impair functional sympatholysis in healthy, young adults?

2. Does EDHF blockade alter forearm blood flow & vascular conductance during rest, sympathetic stress, exercise, and exercise with sympathetic stress in healthy, young adults.
3. Does forearm muscle oxygenation decrease after EDHF blockade during rest, sympathetic stress, exercise, and exercise with sympathetic stress in healthy, young adults?

Hypotheses

1. EDHF blockade significantly impairs functional sympatholysis in healthy, young adults.
2. EDHF blockade does decrease forearm muscle blood flow & decrease vascular conductance during rest, sympathetic stress, exercise, and exercise with sympathetic stress in healthy, young adults.
3. Forearm muscle oxygenation does decrease after EDHF blockade during rest, sympathetic stress, exercise, and exercise with sympathetic stress in healthy, young adults.

Significance of the research problem

In healthy adults, studies indicated that the ability to blunt sympathetic vasoconstriction is necessary to match the increased demand for perfusion in exercising muscle. Locally formed metabolites like NO and ATP have the ability blunt sympathetic vasoconstriction. Though, the role of other metabolites like EDHF remains unclear. It is of clinical interest to discover the underlying mechanisms behind functional sympatholysis in healthy adults to build the basis for potential therapeutic approaches in individuals with damaged neural control, leading to an inability to effectively match the blood flow demands required during exercise.

Delimitations

1. Only males and females between the ages of 18 and 30 years were included.
2. Individuals diagnosed with an autonomic dysfunction (e.g. orthostatic hypotension, multiple system atrophy, autonomic neuropathy causing dizziness, urinary issue. Sexual difficulties, exercise intolerance) or cardiovascular disease (CVD) were excluded.
3. Only normotensive individuals (systolic blood pressure below 130 mmHg and or diastolic blood pressure below 85 mmHg) were included.
4. Individuals with a BMI over 30 kg/m^2 were excluded.
5. Smokers and tobacco users were excluded.
6. Individuals who are taking any prescription medication (except birth control) were excluded.
7. Females were tested in the first five days of the early follicular phase.

Limitations

1. The results of the study are only representative of college aged males and females in the Norman area.
2. The study design entailed the determination of forearm oxygenation measures through a near-infrared spectroscopy (NIRS) device. A 1 second contraction to 2 second relaxation ratio was utilized. If results transfer to whole body exercise remains not fully discovered.
3. Near-infrared spectroscopy (NIRS) does not directly measure vessel diameter and thus is an indirect measure of changes in dilation and constriction.
4. Fluconazole was not locally infused.

Assumptions

1. Participants adhered to all pre-testing protocols and answered all questionaries truthfully
2. Participants performed maximal effort during initial testing session (isometric handgrip testing).
3. Fluconazole inhibition of CYP450 enzymes prevents EDHF dilation (Petterson et al., 2021)
4. CYP450 enzyme blocking through 150mg of fluconazole is not influenced by bodyweight (Petterson et al., 2021)
5. The systemic peak concentration of fluconazole is reached 120 minutes after ingestion of a 150mg pill (Petterson et al., 2021, Debruynev& Ryckelynck, 1993).
6. – 20 mmHg of Lower Body Negative Pressure (LBNP) significantly increases MSNA (Vongpatanasin at al., 2011)
7. 72 hours between visit 2 and visit 3 (counter balanced) is an adequate time to dispute the half-life of 26.8 ± 3.9 hours of fluconazole – (Hollier & Cox, 1996)
8. Female hormonal levels are similar between trial and all trials are performed within days 1-5 of the early follicular phase (Williams et al., 2020)
9. Subjects were blinded to experimental conditions (PLA vs. FLZ)

Operational definitions

1. **Functional sympatholysis:** The ability to blunt sympathetic vasoconstriction of in active skeletal muscle.
2. **Endothelium Derived Hyperpolarizing Factor (EDHF)**: Proposed endothelial-derived substance which is responsible for hyperpolarization and relaxation of the smooth muscle around a vessel and independent of nitric oxide and prostaglandins. For the purposes of this study, we will define EDHF as the downstream metabolites of cytochrome P450 epoxygenases, most likely 11,12 epoxyeicosatrienoic acid (EETs) (Fitzgerald et al, 2005).
3. **FinaPres NOVA:** Non-Invasive system to measure continuous arterial blood pressure displayed in waveform. Adjustments are made through infrared light and pressure signaling through the fleshy part of the digitorum (Silke & McAuley,1998).
4. **OxiplexTS NIRS:** Device which can determine levels of oxygenated and deoxygenated hemoglobin levels in active tissue through near-infrared spectroscopy within the skeletal muscle (Lipcsey et al, 2012).
5. **Ultrasonography**: Non-invasive device for the imaging of veins and arteries through soundwaves as well as determining blood velocity via the doppler effect.
6. **Sympathetic Nervous System (SNS)**: Division of the autonomic nervous system responsible for control of functions to prepare body for activity (fight or flight) (Hearon et al, 2016)
7. **Hand grip dynamometer**: Used on the nondominant arm in a slightly abducted position for the purpose of forearm exercise (Hamilton, 1992)
8. **HbO_2 :** Oxygenated hemoglobin.
9. **HHb:** Deoxygenated hemoglobin

10. **tHb:** Total hemoglobin
11. **Heart Rate (HR):** Number of heart beats per minute (BPM) based on ventricular contractions in.
12. **Mean Arterial Pressure (MAP):** Average arterial blood pressure during one cardiac cycle, which includes the systole and diastole (mmHg).
13. **Cardiac Output (CO);** Amount of blood ejected by each ventricle per minute (L/min).
14. **Stroke Volume (SV):** Amount of blood ejected by the left ventricle per systolic contraction (ml/beat).
15. **Total Peripheral Resistance (TPR):** Total resistance to blood flow within the systemic circulation.
16. **Forearm Muscle Blood Flow (FBF):** The volume of blood moving through the brachial artery to the forearm per minute. Calculated via (mean blood velocity x 60 x π x (brachial diameter/2)^2) (ml/min).
17. **Forearm Vascular Conductance (FVC):** The amount of FBF relative to 100 mmHg of arterial pressure. Use as an assessment of dilation vs constriction in the forearm circulation. Calculated via (FBF/MAP) $\times$ 100mmHg (ml/min/100mmHg).
18. **Placebo (PLA):** 250 mg microcrystalline cellulose ingested as a single dose.
19. **Fluconazole (FLZ):** 150 mg pill ingested as a single dose to block CYP450.

CHAPTER II: LITERATURE REVIEW

The purpose of this study was to investigate the importance of EDHF in functional sympatholysis in healthy young adults. The research question was whether the blocking of EDHF increases α-adrenergic vasoconstriction and thus decreases forearm muscle blood flow and oxygenation during handgrip exercise. To our knowledge, this was the first study to investigate the effects of blocking EDHF pathways in forearm muscle blood flow in healthy young adults during exercise. Therefore, this chapter does discuss the potential mechanisms behind functional sympatholysis and sympathetic restraint, the use of methods to block EDHF, and different methods to induce a sympathetic response.

Cardiorespiratory responses to exercise

The cardiovascular system responds to exercise in a unique way when compared to other stressors. A rapid increase in HR and CO can be expected to redistribute oxygenated blood to active tissues. Additionally, ventilation (VE), oxygen consumption (VO_2), ventilation of carbon dioxide (VCO_2) and the respiratory exchange ratio (RER) are increasing (Chambers & Wisley, 2019, Lewis et al., 1983). HR, systolic BP (SBP), A-VO_2 difference and VE all increase linearly with the increase in absolute work intensity. Total peripheral resistance (TPR) is typically characterized by an initial drop followed by an increase, typically above baseline values. Norepinephrine spillover increases with exercise intensity as it relates to BP regulation (Lewis et al., 1983).

Exercise Hyperemia

Exercise hyperemia is the increase in skeletal muscle blood flow during an increase in muscular activity. The demand of skeletal muscle blood flow is strongly dependent on exercise intensity and is closely related to metabolism. As exercise intensity gradually increases, so does

the demand of blood flow to match metabolic demands. The average oxygen consumption for a healthy male is a round 3.5 ml/kg/min at rest and around 50-60 ml/kg/min at maximal exercise in men, while it is around 40-50 ml*kg*min for women. Maximal oxygen consumption, or VO_2max, is highly dependent on CO and total hemoglobin levels. As skeletal muscle blood flow increases so should the amount of oxyhemoglobin to the active musculature (Joyner & Casey, 2015). The primary reason for the increased oxygen consumption is the increased demand in skeletal muscle oxygenation to maintain ATP production needed to match ATP demands. While brain blood flow remains mostly unchanged and the demands of flow to organs slightly increase, muscle blood flow demands increase by up to a 20-fold (Joyner & Casey, 2015).

The increase in oxygen uptake requires an increase in muscle perfusion, ultimately leading to a shift in arterio-venous oxygen content difference ($(A-V)O_2$). This difference occurs not only through in increased demand on oxygenation in skeletal muscle but also through vasoconstriction via baroreflexes towards peripheral organs to maintain blood flow and pressure to active muscle. This overall increase in oxygen demands is due to the increased overall oxygen extraction from 30-40% to 70-80% (Korthuis, 2011).

The function of almost all tissues in the human body depends on the adequate supply of oxygen through red blood cells. To match the demand of oxygenated blood, vessels need to be able to properly vasodilate and constrict. The largest vessels found in the body are arteries and veins. Within these vessels, blood supply is largely controlled by endothelial cells. Anatomically, the center of a blood vessel is the lumen of an artery, surrounded by the basal lumen and endothelial lining. The outer parts of vessels are smooth muscle, surrounded by loose connective tissue. The signaling from the endothelium as well as the surrounding smooth muscle is essential

for regulation of vascular tone and thus the oxygenation of surrounding tissue including the skeletal musculature **(**Alberts et al., 2002).

Saltin & Andersen (1985) found a strong correlation between limb blood flow and workload increases indicating that exercise intensity is closely related to muscle oxygen demands. Increases in exercise intensity cause an increased demand for muscle perfusion, leading to a linear increase in local muscle blood flow. The hypothesis has been tested using knee extensor exercise as well as handgrip exercise. Both exercise modalities, despite differences in overall muscle mass, showed comparable results when looking at percent changes. Additionally, the data indicated that maximal perfusion of skeletal muscle based on increased muscle blood flow and oxygenation is dependent on physical fitness. It is estimated that in untrained subjects' maximal perfusion is 2.5 L/kg/min, while trained aerobic subjects can reach 3.8 L/kg/min. The increase in flow and perfusion may be due to increased endothelial function and ability to properly control vascular tone (Saltin & Andersen, 1985).

The hemodynamic responses to exercise are unique when compared to other sympathetic stressors, specifically when looking at muscle blood flow. As discussed, the increase in blood flow occurs to match increased metabolic demands during exercise. This increase in flow is characterized by biphasic rapid vasodilation. The increase in dilation and exercise hyperemia at the onset of exercise is characterized by a rapid increase in flow over approximately 45-60 second (Phase I), followed by a steady state (phase II). In terms of cardiovascular responses, a rapid increase in HR occurs followed by a drop to a steady state, controlled by the central command (Tschakowksy & Pyke, 2008).

At the onset of exercise, a rapid drop in MAP, followed by an increase to a steady state in pressure can be observed. The rapid drop is characterized by a point of time at which arterial

outflow exceed inflow due to increased perfusion, leading to the increased metabolic demands and need to match blood flow. Simultaneously, heart rate shows a rapid increase followed by a slight decrease to a steady state above resting level. This occurs shortly after a drop in MAP due to signaling of the central command to the autonomic pathways involved in regulating arterial blood pressure. (Tschakovsky & Pyke, 2008). The potential mechanisms behind rapid dilation have not been fully explored although evidence suggests that the blocking of Potassium (1+) (K)-stimulated hyperpolarization and well as the blocking of NO and PG lead to a rapid reduction in hyperemia and vasodilation at the onset of exercise. These findings suggest that K, NO and PG are primary mediators in rapid vasodilation. The involvement of other mediators, including EDHF remains unclear (Crecelius et al,, 2013).

The role of the autonomic nervous system in arterial pressure regulation and vasoconstriction

The autonomic nervous system is responsible for regulating secretory glands, cardiac and smooth muscle and is responsible for the control of involuntary processes including heart rate, blood pressure and the control of circulation. Control of blood vessels is vastly dependent on functions of the sympathetic nervous system. More specifically, postganglionic nerves are responsible for the release of neurotransmitters epinephrine and norepinephrine, which bind to α1- and α2-adrenergic receptors. While α1 receptors (located at arterioles and veins) are primarily responsible for smooth muscle contraction and vasoconstriction to limit blood flow to inactive tissue, the function of α 2 receptors (located at parasympathetic nerve terminals) is inhibition of transmitter release. This inhibition leads to a decrease in sympathetic outflow. Evidence suggests that the hyperpolarization of smooth muscle results in vasodilation and a blunting of the Ca2+ influx. Simultaneously, K+ channel opening leads to a K+ flux and

hyperpolarization. Reversely, α1-recptor activation causes vasoconstriction and the control of pressure by K+ channel closing and a simultaneous Ca2+ flux into the sarcoplasm, leading to smooth muscle contraction (Thomas & Segal, 2004).

Another factor playing into the control of blood flow is the role of the arterial baroreflex and chemoreceptors. Generally, blood pressure is controlled and maintained through chemo and baroreceptors in a negative feedback loop and are located in the carotid sinus and aortic arch. Baroreflexes are activated by increases or decreases in pressure in the carotid sinus wall (Wehrwein & Joyner, 2013).

Muscle mechanoreflexes are reflexes activated by mechanoreceptor stimulation. They play in key role blood pressure regulation as evidence suggests that the stimulation leads to a reset of baroreflexes, and thus are important to arterial pressure regulation. Through a negative feedback loop the arterial baroreflex can detect pressure changes and send signals to the medulla oblongata, located in the brain stem, and central nervous system to adjust peripheral resistance (Thomas, 2011). If BP decreases, HR increases and if BP increases, HR decreases with the purpose of adjusting arterial pressure changes caused to an increased arterial outflow due to muscle perfusion. The metaboreflex is also through to be a key factor in increasing blood pressure through cardiac output increases. SNS activity is regulated up by muscle contraction, metabolite accumulation and metaboreflex activation (Nobrega et al., 2014). The neurofeedback involving the integration of the mechanoreflex and metaboreflex is also known as the exercise pressor reflex and is responsible for upregulating sympathetic tone and downregulating vagal tone to increase arterial pressure and HR. In summary, the Exercise pressor reflex modulates HR, contractability, preload and afterload to adapt blood pressure responses (Nobrega et al., 2014).

It is hypothesized that these responses are primarily driven by central commend, also known as the feedforward theory. The motor cortex and hypothalamus are potentially responsible for the neural signals to the feedforward controller leading to adjustments in the autonomic pathways to regulate BP through baroreceptors (Tschakowsky & Pyke, 2008). Central command is ultimately responsible for the somamotor activation leading to cardiovascular adjustments during specific stressors including exercise situated in the medulla (Nobrega et al., 2014).

In summary, the purpose of the sympathetic nervous system is to restrain blood flow to active muscle with the goal to regulate blood pressure during various stressors including exercise. Reasonable MAP is generally considered 100 mmHg and needs to be maintained to prevent syncope and to maintain adequate blood flow to active and inactive tissue (Casey & Joyner, 2015).

Local Vasodilation

Local vasodilation can be seen as competition to sympathetic vasoconstriction and is largely responsible for local control of blood flow. Smooth muscle contractions are highly dependent on calcium (Ca^{2+}) concentration. An increase in Ca^{2+} released by the sarcoplasmic reticulum leads to activation of myosin. The contraction of vascular smooth muscle is important to maintain a partially constricted state to support basal vascular tone due to its prevalence in vascular walls. The increase in vasoconstriction through a flux in Ca^{2+} leads to an increase in blood pressure.

In opposition, early evidence suggests that local vasodilation primarily occurs though the opening of potassium (K^{+}) channels, which induces vasodilation through the hyperpolarization and thus relaxation of smooth muscle. Through this opening, an increase in potassium

conductance can occur leading to a potassium flux, and thus, hyperpolarization. This leads to calcium channel closure and local vasodilation through the decrease in Ca^{2+} concentration (Korthuis et al., 2011 & Brozovich et al., 2016). More specifically, calcium activated potassium channels play a major role in the hyperpolarization of the cell membrane and cause a closure of voltage sensitive dependent calcium channels, leading to muscle relaxation and increased dilation. Evidence suggests that ATP-sensitive potassium channels are responsible for this closure of voltage sensitive dependent calcium channel (Korthuis et al., 2011). Jackson et al (1993), found that prostacyclin, a type of PG, is an important mediator in vascular tone through the opening of ATP-sensitive potassium channels in rabbit hearts. This hypothesis has been tested through inhibiting coronary vasodilation by blocking ATP-sensitive potassium channels. Whether we can make the same assumptions in humans remains mostly unclear.

Proper vascular control is regulated by an integrated redundant system that relies on the proper function of many localized cells. The endothelium, which is the inner membrane of a blood vessels, main function is the vascular control of relaxation and contraction. Additionally, erythrocytes, whose primary function is the transport of O_2, also have important vascular control responsibilities. Evidence suggests responsible sites for the release of vasodilatory metabolites predominantly include erythrocytes (which release ATP and NO) and endothelial cells (which release NO, ATP, PG, as well as EDHF) into the vessel towards the smooth musculature. Further, the surrounding smooth muscle responsible for the release to ATP and NO (Clifford & Hellsten, 2004).

Endothelial cells can detect mechanical signals, more specifically through shear stress, and thus can detect changes in blood and is able to convert changes in this mechanical stress into intracellular signals. Detection of increased mechanical stress on the endothelium activates the

enzyme nitric oxide synthase (NOS) producing NO (Bellien et al., 2006). Additionally, Ellsworth et al (2009) found that vasodilation may be due to the release of NO and ATP from erythrocytes during blood flow through capillaries. ATP is released from erythrocytes and bind to P2Y purinergic receptors on the endothelium cell Intravascular ATP has the ability to stimulate production and release of NO and prostacyclin in the endothelium towards the smooth muscle, causing potassium channel opening (Mortensen & Saltin, 2014).

ATP and Exercise Vasodilation

Local vasodilation is strongly signaled by ATP, especially during exercise. Evidence suggests that plasma ATP increases during exercise. Similarly, to NO, ATP is offloaded by O_2 from hemoglobin, and binds to P2Y receptors on the endothelial cell. Though, the main source of arterial ATP seems to be through shear stress, while O_2 may be the main source for venous ATP, as the half-life is very low. Additionally, evidence suggests that ATP can be released from the endothelium cell through mechanical stress including shear stress and hypoxia (Mortensen et al., 2011). In terms of O_2 offloading being a potential source of venous ATP, Ellsworth et al (1995) found that erythrocytes release ATP. Based on these findings, it was proposed that O_2 offloading of ATP plays a major role in the control of vascular tone as it binds to P2Y receptors, which increased NO and PG production (Ellsworth et al., 1995). ATP under PO_2 as well as low pH conditions. Regarding sympatholytic mechanisms, ATP has been suggested to play in important role in flow mediated dilation via the activation of P2Y receptors causing an increase in Ca^{2+}, causing a K^+ outflow toward the surrounding smooth muscle and hyperpolarization. (González-Alonso, 2012, Hearon et al., 2016).

As stated, arterial ATP is mainly released through shear stress, while venous ATP is primarily associated with offloading due to its small half-life of under 1 second (Mortensen &

Saltin, 2014). Despite its short half-life, plasma ATP levels can be measured through the use of intravascular microdialysis probes, specifically targeting the vasculature responsible for the supply and drainage of working muscles. Evidence suggests that plasma ATP sources are erythrocytes and endothelial cells. Results from a 2011 study indicated that exercise causes a release of ATP into local vasculature, while the levels are reduced during acetylcholine infusion despite increases in leg blood flow. Interestingly, resting leg blood flow and plasma ATP levels did not change, supporting the hypothesis that ATP plays a major role local vasodilation during exercise and may play a significant role in functional sympatholysis (Mortensen et al., 2011). In an early study, Jagger et al (2001) used CO with the intention of blocking venous plasma ATP in rats in an attempt to explain the importance of ATP derived through offloading from erythrocytes. The data suggests that the inhibition of ATP efflux through CO did not lower plasma ATP, but plasma ATP levels were increased during exercise and the simulations increase in skeletal muscle blood flow (exercise hyperemia).

Nitric Oxide, Prostaglandins and Exercise Vasodilation

Current theories suggest that NO is formed as a local vasodilator in multiple ways. Mainly, it is formed by converting O_2 and L-arginine into NO through nitric oxide synthase (NOS) enzymes and cofactors of tetrahydrobiopterin, flavins, iron protoporphyrin-IX and phosphate hydrogens (Tejero et al., 2019). Additionally, endothelial NOS (eNOS) and neuronal NOS (nNOS), which are two isoforms of NOS, can be found in the endothelium and skeletal muscle. eNOS is activated through the offloading of ATP and through shear stress from erythrocytes and the interaction of ATP with P2Y receptors, which are located on the endothelial cell. nNOS primarily occurs on the plasma membrane, the caveolae and sarcoplasmic reticulum, while eNOS primarily occurs in the plasma membrane, lipid rafts and caveolae. Due to the close

relationship between eNOS and nNOS, study design which aim at NO blockage are particularly challenging to design (Tejero et al., 2019, Hearon & Dinneno, 2016). Another NO source, less studied is nNOS. NNOS is primarily found in the cytosol as opposed to eNOS, which is primarily found in the membrane itself. Evidence suggests that nNOS id highly Ca2+ dependent and thus may play an important role in blunting sympathetic vasoconstriction (Hansen et al., 2000, Mortensen & Saltin, 2014, Mortensen et al., 2014).

Similar to NO, PG is formed through ATP offloading though shear stress and is converted into PG through the enzyme cyclo-oxygenase (COX). PG is strongly related to NO in local vasodilation as they are produced through COX-1 and COX-2 pathways along with terminal PG synthesis (Zarghi & Arfaei, 2011). Specifically, PGI2 is involved in local vasodilation along with bronchodilation and bronchoconstriction. (Zarghi & Arfaei, 2011). Dinenno & Joyner (2004) found that PG itself has little effect as a vasodilator, but rather the combination of NO and PG are responsible for significant contributions to exercise hyperemia. For future studies, it has been well established that non-steroidal anti-inflammatory drugs (NSAIDs) can block COX-2 pathways and thus the effects of PGI2s (Zarghi & Arfaei, 2011).

Endothelium Dependent hyperpolarization factor and Exercise Vasodilation

EDHF is a proposed metabolite which may play a larger role in local vasodilation then initially assumed in recent literature. Although the pathways of EDHF are not fully explored, the vasodilator is thought to be synthesized in the endothelial cell. As opposed to NO, PG as well as ATP, it has been vastly ignored in recent studies (Petterson et al., 2020).

It has been well established that the release of NO and PG through the endothelium plays a major role in vascular tone. EDHF has been proposed as the primary factor in smooth muscle relaxation around vessels. Results of a 2018 study, date collected by Goto et al. indicated that Endothelium-

dependent hyperpolarization is impaired in individuals with hypertension due to endothelial ion channel changes with prolonged development. The study found that in hypertensive patients, a reduction of endothelium dependent hyperpolarization seems to occur through reduced activation of small and intermediate conductance Ca2+-activated K+ channels as well as a downregulation of vanilloid type 4 channel (TRPV4). TRPV4 is known as a protein involved in the function of calcium channels. To determine the mechanisms of EDHF and vasodilatory responses are of clinical interest to determine underlying causes of CVD as well as reasons for exercise intolerance in diabetic individuals (Goto, 2018).

Early evidence suggests that EDHFs are primarily responsible for the acting on calcium-ion channels within the endothelium. The same pathways may release NO as a primary vasodilator as well. Other data suggests that EDHF may lead to muscle relaxation through potassium channels. EDHF activation may be a mechanism to back up NO in the case of rapid NO-level drops to maintain sufficient vasodilation. Thus, discovering the effect and underlying mechanisms of both, NO-KO and EDHF-KO is of clinical interest. The same study also found that EDHF is less reactive than NO, which creates specific difficulties in determining the impact of EDHF on sympatholysis. (Garland et al., 1995).

Later evidence suggests that EDHF may be activated by an increase in calcium, while calcium activated potassium channels are activated leading to the hyperpolarization of smooth muscle. This mechanism seems to be independent of NO and PG since it does not interfere with eNOS or COX pathways despite their potential close relationship in vasodilation (Goto et al., 2018). Crane et al (2003) found that small conductance and intermediate conductance calcium activated potassium channels are of importance in smooth muscle hyperpolarization. Additionally, the data suggests that the stimulation of the endothelial cell through acetylcholine

evoked endothelial hyperpolarization and is mediated by the opening of small conductance calcium activated channels.

Further evidence supports the idea that that vascular endothelium responses may be driven by EDHF to a higher extent then researchers initially thought. Data indicated that blocking the vasodilatory effects of EDHF using fluconazole significantly reduced popliteal and brachial artery blood flow. Healthy individuals showed a significant increase in flow mediated constriction after the blockade of EDHF in the brachial artery (Petterson et al., 2021). Additionally, flow mediated dilation seemed to not be significantly changed. These results as well as future discoveries in the pathophysiology of EDHF are of importance because of its possible role in individuals with low fitness levels linked to CVD. Evidence suggests that people with cardiovascular disease are blunted in flow mediated vasoconstrictor responses. Pre-exiting diseases cause an overall increase in sympathetic nervous system activity (Vongpatanasin, 2011). Results which suggest that EDHF-KO may reduce muscle blood flow indicates downstream actions of EDHF may be altered in these populations (Petterson et al., 2021). These findings add to the pool of knowledge surrounding flow mediated vasodilation as well as functional sympatholysis since data suggests that sedentary older individuals are less efficient in lactate buffering as well as impaired in functional sympatholysis compared to active older adults (Saltin et al., 2012).

Previous studies which attempted to explain the importance of EDHF during functional sympatholysis used EDHF and well as ACh knock out methods during local vasoconstrictor responses through phenylephrine (PE), while K+-mediated vasodilatation as well as endothelium independent vasodilators like PG and NO are playing a significantly smaller role in sympatholysis then originally assumed. PE is a drug used to increase blood pressure in

hypotensive individuals through α-adrenoreceptor stimulation and thus shows similar effects on vasoconstrictor responses as NE. Data revealed that forearm blood flow and vasodilation was significantly blunted during forearm exercise in addition to local vasoconstriction. To isolate endothelium-like hyperpolarization through ACh, NO and PG were blocked during exercise and baseline data. The results indicated no significant differences between the NO-PG blockage trial during exercise and ACh stimulation when compared to the control data during PE infusion. These data support the data surrounding the hypothesis that NO-PG is not involved in functional sympatholysis in humans. Although PE has been well established as a local sympathetic α-adrenergic vasoconstrictor, it remains unclear whether systemic sympathetic vasoconstriction through the use of LBNP would show similar results (Hearon et al., 2016).

Recent evidence focusing on hyperemia induced by passive leg movement, which is a widely accepted measurement to determine endothelial function, showed that although NO is the primary mediator in vasodilation, EDHF may play a different role then initially assumed. The data suggests that EDHF plays an important role in local vasodilation, and it seems to act independently from NOS since it does not work through COX pathways. This hypothesis was tested through infusion of N-monomethyl-L-arginine, a NOS inhibitor. To inhibit EDHF, Fluconazole was used to inhibit the cytochrome P-450 (CYP-450). CYP450 is known as an enzyme responsible to produce cholesterol and prostacyclin, which is an effective vasodilator (Trinity et al. 2021).

During exercise, Mortensen at al. (2007) found that EDHF does not seem to be a backup for NO and PG, as originally assumed. Rather, EDHF is an independent vasodilator from NO and PG. Additionally, the data found that the blocking of EDHF with tetraethylammonium chloride (TEA) did not change flow mediated dilation during exercise. These findings contradict

with data from Petterson et al (2021). In the study CYP450 receptors were blocked using fluconazole, suggesting that TEA may not be ideal to block all EDHFs during exercise. Hilig et al (2003) found that the blocking cytochrome P450 2C9, which is a EDHF synthase, in addition to blocking of NOS resulted in a significant reduction of blood flow and vascular conductance during exercise. Interestingly, CYP450 2C9 blockage alone did not significantly alter, which does supports to original idea of EDHF being a backup system for NOS. In the study, sulfaphenazole was used to block to effects of CYP450 2C9. In summary, the pathways of EDHF remain not fully understood and the literature discussing EDHF and its relationship to NO/PG as well as its involvement in functional sympatholysis is limited.

Functional Sympatholysis

As discussed, functional sympatholysis is a mechanism which causes reduced vasoconstriction despite increased sympathetic nervous system activity (SNA) during exercise. It is well established that whole body exercise causes an increase in SNA due to its necessity in controlling hemodynamics. The increase in efferent outflow leads to α -adrenergic receptor stimulation which ultimately leads to norepinephrine release and vasoconstriction in the absence of the ability to blunt sympathetic vasoconstriction (Remensnyder et al., 1962). As SNA increases, so does cardiac output (CO), heart rate (HR), and stroke volume (SV) to redistribute blood to guarantee proper muscle oxygenation of working muscle and is of importance to maintain mean arterial (MAP) during exercise as arterial outflow briefly exceeds arterial inflow, leading to a decrease in compartmental blood volume at the onset of exercise (Saltin at al., 2012, Tschakovsky & Pyke, 2008).

Strange (1999) was among the first who attempted to explain the phenomenon of functional sympatholysis in healthy young individuals itself. In the study, subjects performed

three bouts of resistance training. One bout of maximum isometric handgrip exercise was added in addition to forearm ischemia to induce a sympathetic stress. Results indicated that there was no significant reduction in muscle blood flow despite the increase in factors involved in sympathetic vasoconstriction. Though, MAP, CO, and forearm vascular conductance increased indicating that the stress was increased enough to induce blood flow responses. Muscle sympathetic nervous system activity (MSNA) was measured using microneurography, a method used to visualize nerve impulses, and indicated that SNA was significantly increased in the experimental trials. These findings were first indicators of the existence of functional sympatholysis and raised questions about the mechanisms behind the phenomenon.

Moore et al, (2010) found that the activation of α-adrenoreceptors causes a blunting in local vasodilation in mice. A series of experiments tested whether α-adrenoreceptor activation impacts rapid onset vasodilation, which occurs within 1-2 seconds within an initial contraction. Results indicated that the activation of α-adrenoreceptors caused a reduction in vasodilation, while the blocking of α-adrenoreceptors with phentolamine caused and enhancement of rapid onset vasodilation in the gluteal muscle of mice. Resting diameters showed no significant difference, indicating that the amount of diameter change in rapid onset vasodilation is highly dependent on SNS activity.

The primary mediators involved in functional sympatholysis still remain unclear. Though, Dinenno et al, (2004) discovered that nitric oxide (NO) and prostaglandins (PG) may be primary vasodilators during exercise. Results indicated that the inhibition of NO and PG increases α -adrenergic vasoconstriction in the forearm musculature during forearm exercise. The data collected showed a local response through inducing tyramine. Additional data collected within the parameters of the same study indicated that neither NO nor PG alone have the ability

of override sympathetic vasoconstriction by themselves. Rather, only the combination of both metabolites was effective as a mediator in sympathetic restraint. Whether systemic stressors including the cold pressure test (CPT) and or lower body negative pressure (LBNP) as opposed to local constriction induced by tyramine ingestion show different results remain to be determined.

On the other hand, Crecelius et al, (2015) found that sympathetic vasoconstriction was not able to be blunted when blocking inwardly rectifying potassium Na^+/K^+-ATPase. Additionally, data published in an additional study indicates that NO and PG are likely not the key pathways involved in modulating sympathetic tone (Crecelius et al et al., 2011). In the study, NO and PG combination did not significantly contribute to forearm blood flow and vascular conductance after ATP infusion, which was used at a P2Y agonist and *N*-monomethyl-l-arginine was used to block NO. Other locally formed vasodilators include carbon-dioxide, potassium, adenosine, ATP, acetylcholine, lactate as well as EDHFs. The balance of local these local vasodilators is the traditional way of attempting to explain the mechanisms behind local vascular conductance. The potential mechanisms behind functional sympatholysis are not fully explained either although we do know that they blunt the effects of norepinephrine rather than reducing norepinephrine. Other studies conducted on animals proposed that NO is the primary vasodilator in functional sympatholysis although in humans the combination of metabolites involved remain mostly unclear (Mortensen et al., 2014 & saltine et al., 2012, Just et al., 2016).

Functional Sympatholysis – effects of aging and CVD

It has been well established that aging as well as the development of cardiovascular disease are linked to reduced exercise hyperemia as well as a decreased ability to blunt α-adrenergic vasoconstriction. (Mortensen et al., 2012). Taddei et al (1995) found that the aging

process is correlated with a reduction in endothelial function, in both, normotensive and hypertensive individuals. This theory has been tested through inducing acetylcholine to test weather endothelial-dependent vasodilation may be impaired. Evidence suggests that the contribution of NO in exercise hyperemia is blunted in older adults, resulting in reduced ability to vasodilate (Casey & Joyner, 2015).

CVD is often characterized by abnormal sympathoexcitatory responses during exercise due to damages to neural control of muscle blood flow. Evidence suggests that exercise intolerance in hypertensive and pre-diabetic individuals is due to the inability to match the metabolic demands of muscle oxygenation (Vongpatanasin et al., 2011). In the study SNS activation was increased using LBNP in addition to handgrip exercise. The results shows that hypertensive subjects had significantly decreased forearm muscle oxygenation and decreased blood flow in the forearm musculature when compared to the control group. Additionally, SNS activity increased at a higher rate during exercise alone when compared to the control group. This is of no surprise as it is well established that individuals with CVD have increased SNS activity levels than, healthy individuals. It remains unclear what the exact cause for the decreased ability of hypertensive individuals to sufficiently blunt sympathetic vasoconstriction is (Vongpatanasin et al., 2011).

Price et al (2013) found that functional sympatholysis is impaired in both hypertensive as well as normotensive elderly adults. If sympathetic restraint is further inhibited, for example through older generation β-blockers, exercise intolerance may increase. In the study, SNS activity was evoked using LBNP and handgrip exercise while forearm muscle oxygenation was determined to determine the effects of different β-blockers on functional sympatholysis in hypertensive individuals.

Mortensen et al (2012) found that regular exercise improves the ability to blunt sympathetic vasoconstriction in healthy older adults. Due to an improvement in endothelium function, exercise may restore functional sympatholysis and thus O_2 delivery as well as exercise tolerance. Additionally, previous studies indicated that both, hypertension, and the aging process cause increased resting norepinephrine levels, which could lead to increased sympathetic vasoconstriction. (Mortensen et al., 2014, Mortensen et al., 2012). Understanding the mechanisms behind the ability to blunt sympathetic vasoconstriction during exercise helps us understand the underlying causes for exercise intolerance in individuals with CVD and in older adults.

Methodology considerations – Lower body negative pressure

Lower body negative pressure (LBNP) is a commonly used method to assess cardiovascular function. The technique causes a redistribution of blood from the upper body to lower body causing a shift in central blood volume. This mechanism causes a decrease in central flow pressure, left ventricle and end diastolic volume and an increase in stroke volume (SV) and CO. This causes a sympathetic stimulation leading to sympathetic vasoconstriction through vagal withdrawal (Goswami, 2019).

Sundlöf & Walling (1978) were among the first who discovered that the main mechanism behind SNA increases during LBNP correlate with decreases in central blood volume. He discovered that arterial baroreflexes seem to be the underlying mechanism which elevate SNA as well as CO and SV. Arterial baroreflexes make up receptors which are responsible for recognizing changes in arterial pressure and flow to prevent rapid drops in peripheral flow, located in the aortic arch. The findings of the study also suggest that the amount of SNA is dependent on the levels of LBNP. Higher levels of LBNP exposure showed increased SNA.

These findings are unique to the use of LBNP when compared to other stressors causing SNA changes including the use of the cold pressor test (CPT).

Methodological considerations - measuring vasodilation and endothelial function

Endothelial function can be assessed using high-frequency ultrasound and is often tested using cuff occlusion followed by reactive hyperemia. Endothelial dysfunction is indicated by reduced ability to vasodilate following the release of the occlusion cuff. Other assessments include the monitoring of flow mediated dilation using acetylcholine, an endothelium dependent agonist (Vogel, 2001).

Another method to assess flow mediated dilation is through measuring muscle oxygenation with Near-Infrared spectroscopy (NIRS). McLay et al (2016) found that muscle oxygenation is strongly correlated with flow mediated dilation, assessed through an ultrasound assessment. The use of NIRS is advantageous due to its ease of use and high reproducibility. G´omez et al.(2008) found that the device is highly reliable and valid. Thus, the technique is ideal for an accurate measure of micro vessel reactivity and is superior to other techniques with rely on estimates.

Two indicators of vascular tone are vascular conductance (VC), or vascular resistance (VR), which can indicate the ease (VC) or hindrance (VR) of flow at certain pressure differences. VC is the flow measured divided by pressure, while vascular resistance is pressure divided by flow (Joyner & Casey, 2015, Joyce at al., 2019). Thus, mathematically, VC has an inverse relationship to vascular resistance, which leads to the common misconception that the two variables can be used interchangeably. Joyce et al (2019) explained the difference between the two variable and made the case to VC being the more accurate measure of blood flow. VC is in a linear relationship with blood flow, while resistance presents a non-linear relationship. The

reason for the difference is due to variability of temperatures, directly impacting local blood flow. (Joyce et al., 2019). These data indicate that the use of VC may be superior to reporting vascular resistance for functional sympatholysis, although both variables should be considered.

Fluconazole/inhibition of EDHF

Fluconazole is a drug which has originally been developed to treat fungal infections. It is typically prescribed in either 150, or 200 mg doses depending on the condition. It is sold as a solid crystalline which is highly water soluble. Studies have shown that Fluconazole acts as an inhibitor of vascular K_{Ca} channels a well as CYP peroxygenase 2C, the same channels through which EDHF has been proposed to be released to induce smooth muscle relaxation (Bellien et al., 2006). Ding et al (2002) found that cytochrome P450 monooxygenase (CYP450) significantly contributes to the pathways involved in control of vascular tone in wild type mice. The blocking of these pathways through miconazole significantly reduced endothelium dependent vasodilation. The blocking occurs through acting on K-ATP channels, which are proposed pathways of EDHF. Gill et al (1996) found that fluconazole can block P450-mediated bioactivation better than other proposed substances which could contribute to P450 inhibition including ketoconazole. Yang et al (2017) found that the ingestion of fluconazole significantly blunts bradykinin-induced vasodilation in COPD patients. Bradykinin is a known vasodilator which works through the release of PG and NO, while EDHF functions independently from these local vasodilators. Fluconazole blunts Epoxyeicosatrienoic acid (EET) synthesis, which is a key component of vascular function as it supports vasodilation as it is a type of EDHF (Sudhahar et al., 2010).

The absorption time of fluconazole is between one and three hours with a bioavailability of over 90%. It has also been established that food consumption does not affect the

bioavailability. While the bioavailability is high, the rate of plasma protein binding is low at 11-12%, meaning the risk of toxic consequences of consequences is very low (Debruyne & Ryckelynck, 1993).

Sex differences in local vasodilation

Sex differences and genetic differences are crucial factors to consider when it comes to the ability to uptake oxygen. Males have a significantly higher VO_2 max on average when compared to Females with similar fitness levels (Sharma, 2016). These findings have been well established even though data indicates that females are able to blunt sympathetic vasoconstriction (sympatholysis) better than males (Just & DeLorey ,2017). In the study, different amounts of sympathetic stimulus were used in combination with NOS blockage in Sprague-Dawley rats. The data indicated that blunting of sympathetic vasoconstriction was reduced in females at high amounts of SNS stimulation. This indicates that female vascular control is more NOS dependent along with the finding that females are more efficient in the mechanisms behind functional sympatholysis. In humans, Bunsawat et al (2018) found that males may have higher basal sympathetic vasoconstriction them females and show a larger increase in leg blood flow after blocking α-adrenergic receptors.

Hogarth et al (2007) found that women have a lower muscle sympathetic nervous system activity then men largely due to arterial baroreflex inhibition. This is true for both, single units and multi-unit bursts. Simultaneously, resting blood flow was not significantly different in men and women and correlated with sympathetic nervous system activity. These results suggest that this may contribute to the lower occurrence of cardiovascular events in women then in men.

Conclusion

Functional sympatholysis is described as the ability to blunt sympathetic vasoconstriction during exercise to match the increased demands of muscle blood flow. The mechanisms behind functional sympatholysis remain not fully understood. Most studies to date focused on NO and PG as primary dilators and sympatholytic factors. EDHF may be an important contributor to the blunting of sympathetic vasoconstriction during exercise. However, to our knowledge has not been directly investigated. More recent studies used alterations of the sympathetic nervous system with LBNP to measure the changes in vascular conductance during exercise.

CHAPTER III: METHODOLOGY

The aim of this study was to investigate the importance of EDHF in functional sympatholysis. We tested the hypothesis that functional sympatholysis as determined by muscle forearm muscle blood flow/vascular conductance and oxygenation responses would be blunted if EDHF pathways are inhibited during forearm exercise. To our knowledge this was the first study testing the effects of EDHF blocking during exercise in addition to systemic sympathetic nervous systemic elevation. This chapter covers subject recruiting, a description of instrumentation utilized, description of the experimental protocol as well as data analysis/ data management procedures.

Participants

This study includes the results of 14 participants, 6 Males and 8 Females. Recruiting methods included the use of flyers as well as mass emails within the University of Oklahoma, Norman Campus and the greater area of Norman Oklahoma. Individuals interested in participating contacted the primary investigator (PI) and/or sub-investigators.

The study used a young, healthy, adult population to test all hypotheses. For enrollment into the study, all participants were required to fit the described inclusion criteria and none of the exclusion criteria.

Ethical approval

All procedures had ethical approval from the Institutional Review Board at the University of Oklahoma Health Sciences Center (IRB# 14056). Participants were given a verbal description of all procedures, purposes, and risks involved before providing their informed, written consent. The study conformed to the standards set by the Declaration of Helsinki and was registered with ClincalTrials.gov (NCT05176379).

Inclusion criteria:

1. Males and Females between the ages of 18-30 years
2. Normotensive (systolic blood pressure < 130 mmHg and/or diastolic blood pressure < 85 mmHg) individuals
3. Individuals free of cardiovascular disease and metabolic disease
4. Individuals free of any form of autonomic dysfunction
5. Individuals with a BMI under 30 kg/m^2

Exclusion criteria:

1. Individuals with a history of autonomic dysfunction
2. Individuals with CVD and or diabetes.
3. Smokers, tobacco users (or former tobacco users who have not quit within the last 6 months).
4. Individuals with a blood pressure ≥ 130/85
5. Unable to secure an adequate ultrasound signal/image
6. Subjects who use any prescription medication (except birth control)

Out of 14 participants, one subject was excluded in data analyses to determine changes in FVC and FBF due to a brachial artery bifurcation occurring above the antecubital fossa, making determination of FBF with ultrasound techniques via the brachial artery uninterpretable. Therefore, a total of 13 subjects for FVC and FBF were analyzed.

Study design

The study design was a single blind, randomized, placebo controlled, repeated measures, counterbalance design (true experiment). Participants completed three laboratory visits. Visit 1 was informed consent, determination of eligibility, anthropometric and demographic data collection, and experimental procedure familiarization. Visits 2 and 3 experimental visits, which were identical with the exception of ingestion of fluconazole or a placebo 120 min prior to experimentation. To investigate the contribution of EDHF pathways to functional sympatholysis, oral fluconazole was used as an inhibitor of cytochrome P450 as it is an effective way of blocking EDHF pathways (Dinneno, 2003, Petterson, 2021).

Experimental protocol

Visit 1

The purpose of the first study visit was to determine eligibility of a participant, provide informed consent and participants were familiarized with the study protocol.

An IRB approved informed consent document was provided along with a health history questionnaire to determine if the subject met inclusion and exclusion criteria, the international physical activity questionnaire (IPAQ) to determine the subject's physical activity level as well as a Health Insurance Portability and Accountability Act (HIPPA) authorization form were provided.

Anthropometric measurements were recorded including height, weight waist circumference, hip circumference along with arm dominance (right vs. left) as well as grip strength of non-dominate hand.

Once eligibly was established, body composition via a Dual-energy X-ray absorptiometry (DEXA) scan was measured. A pregnancy test (females only) was completed before the scan.

Subject were then familiarized with the experimental protocol, including fitting of the LBNP device, and a brief recording of the brief recording of the brachial artery diameter and velocity to screen for a potential brachial artery bifurcation.

Visits 2 & 3

During both experimental trials (counter balanced), participants ingested either 150 mg of fluconazole (FLZ) (Petterson at al, 2021) or a placebo (PLA, 250mg tablet of microcrystalline cellulose, Zeebo Effect, Burlington, VT). Before the PLA or FLZ ingestion and instrumentation, female participants were tested for pregnancy. All subjects gave verbal confirmation that they adhered to all pre-testing guidelines (abstaining from vigorous exercise and NSAIDS for 24 hours, caffeine for 12 hours and eating for 8 hours) and if they have been diagnosed with a new medical condition since the last visit. Once, confirmation of pre-testing guideline adherence and the pregnancy test result was negative, the participant ingested the pill. Before the instrumentation, subjects were instructed to stay in a relaxed supine position. Roughly 90 minutes after the ingestion, participants were instrumented and placed in a supine position on the examination table. After the instrumentation, a 5-minute baseline was initiated. Next, 5 minutes of LBNP exposure at -20mmHg was used as a baseline measure for SNA elevation. A 5-minute rest period as well as a 5-minute baseline period followed. Next, subjects began 7 minutes of continuous rhythmic handgrip exercise (20 contractions/min) at an intensity of 20% of their maximal voluntary contraction. During the last 2 minutes of exercise, -20mmHg of LBNP was added until exercise completion. A 10-minute rest period followed. Finally, 5 minutes of reactive hyperemia occlusion (RH OCC) followed to determine the nadir of near-infrared spectroscopy (NIRS) signals, such that the total liable signal (TLS) could be determined. After RH OCC, the

participant was given an additional 5 minutes of rest. The subject was de-instrumented at the end of the protocol.

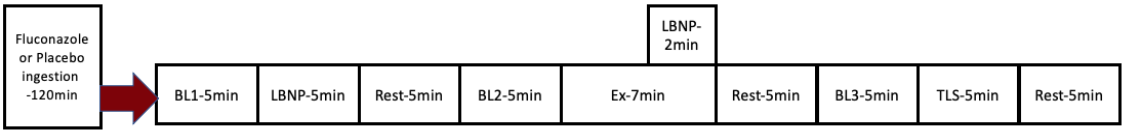

Figure 1. Protocol outline. BL = Baseline, LBNP = Lower Body Negative Pressure stimulus at -20mmHg, Ex = forearm muscle exercise at 20% MVC, TLS = total liable signal via occlusion.

Threats to validity

Threats to internal validity included equipment failure and practitioner error. In terms of practitioner error, the most common threat to internal validity was imprecise calibration of the NIRS. The NIRS used for all trials is calibrated through a calibration as well as a check block. All calibrations needed to be performed in a dark room after a 30-minute warm up period of the equipment. If any of the steps are taken improperly, measurement error and inconsistency can occur. Additionally, placement of the equipment of the appropriate muscle is necessary for inter/ intra-rater reliability and consistency. The second most common threat to internal validity was inconsistency in research protocols. It was of importance to keep track of exact timepoints during the experimental phase to have consistent measurements. A threat to external validity was population validity. The study population is specific to healthy young adults. Results may not be applicable to special and older populations. Evidence suggests that people with increased risk of cardiovascular disease are at increased risk for neurological damage (Firoz et al, 2015). Damage to the central nervous system may alter sympathetic outflow and generally increases sympathetic nervous system activity. Functional sympatholysis may be dysfunctional in these specific populations and our results may not apply beyond the study population.

To control for internal validity, it was of importance to follow all standard operating procedures for all the equipment to create consistent measurements. Each laboratory member was required to be trained on the standard operating procedures within the laboratory. Checklists were created for each lab member operating equipment.

Instrumentation

- Dual Energy X-Ray Absorptiometry (DEXA): Total body composition as well as forearm fat free mass (FFFM) was measured with a GE Lunar Prodigy bone densitometer (GE Lunar, Madison, WI). FFFM was measured with a custom region of interest (ROI) analysis. FFFM of the non-dominant forearm was determined with a custom region of interest analysis with the anatomical landmarks being the articular surface of the ulna and the medial epicondyle of the humerus.

- Near - Infrared Spectroscopy (NIRS) - Forearm muscle oxygenation was measured using a near infrared spectroscopy oximeter (Oxiplex TS, ISS Inc., Illinois USA) while being placed on the on the Flexor Digitorum Profundis muscle.

- Equivital Life Monitoring System - Heart rate and breathing rate were measured with a 2-lead electrocardiogram (ECG) from Equivital Life Monitoring Systems (ADinstraments, Denver, CO, USA).

- Doppler Ultrasound - Forearm muscle blood flow was measured using a Logiq S8 Doppler Ultrasound in B-mode to measure brachial artery diameter and blood velocity (GE Healthcare, Madison, WI, USA).

- Finapres : Mean Arterial Blood Pressure (MAP), Cardiac Output (CO), Stroke Volume (SV), Total Peripheral Resistance (TPR) were measured with a NOVA Finapres finger photoplethysmography device with a Finapres NANO CORE (Enschede, Netherlands).

- Hokanson cuff: forearm muscle oxygenation was measured during a forearm muscle occlusion with a Hokinson cuff (Hokanson, Bellevue, WA). Pressure was decreased to 230mmHg.
- Lower Body Negative Pressure (LBNP): Sympathetic stimulus at -20 mmHg was evoked with the subject being sealed with their lower body into an air sealed chamber connected to a vacuum (Techavance, Inc, Austin, TX, USA). Subjects were paces into the chamber up to the iliac crest. Pressure was decreased to -20mmHg at two different time points for 2 and 5 minutes.

Data processing

All data were stored and recorded in LabChart software (ADInstruments, Colorado, USA) at a 1 KHz sampling rate. All data collected was numerical and continuous data. Central cardiovascular variables, LBNP pressure, forearm muscle oxygenation data and pulse velocity were collected through Power Lab (ADInstruments, Colorado, USA). Blood velocity waveforms were collected continuously in Power Lab with a qDAT audio converter (Herr at al, 2010), Central Cardiovascular variables and forearm muscle oxygenation data reported were averaged over beat-to-beat averages and then processed in 3 second bins for simplicity of data analysis. Hemodynamic data was averaged in the last 30 seconds of each stage except for the Rest+LBNP, which was averaged during minute 1:30-2:00 (Rosenberry et al 2018).
Brachial Artery velocity data was averaged in LabChart directly to avoid the inclusion of data without proper doppler ultrasound signal and was extracted out of data pad. Only blood velocity envelopes that were clear and fully captured within the duty cycles (1s contraction and 2s relaxation) were reported.

Brachial Artery Diameter was collected via a capture card and processed with a Brachial Analyzer Software during the appropriate stages (MIA LLC, IA, USA).

The aim of the primary analysis was to determine whether FLZ ingestion will blunt FBF/FVC and muscle oxygenation responses. FBF was calculated as FBF calculated with the following equation: FBF = mean blood velocity x 60 x π x (brachial diameter/2)^2. FVC was calculated as FVC = FBF/MAP x 100mmHg.

ΔHbO_2 (%TLS) at rest was calculated as ((ΔHbO_2(rest)- ΔHbO_2(rest+LBNP))/(ΔHbO_2(max)- ΔHbO_2(nadir)) x 100.

ΔHbO_2 (%TLS) at exercise was calculated as ((ΔHbO_2(exercise)- ΔHbO_2(Exercise+LBNP))/(ΔHbO_2(max)- ΔHbO_2(nadir)) x100.

Functional sympatholysis (%FS) (exercise induced attenuation of oxyhemoglobin reduction) was calculated as ((ΔHbO_2 (rest) (%TLS)- ΔHbO_2 (exercise) (%TLS))/ (ΔHbO_2 (rest) (%TLS)) x 100.

Data analyses

Data were analyzed using SPSS Statistics for Windows, Version 28.0 (IBM SPSS Statistics for Windows, Version 28.0. Armonk, NY: IBM Corp). Level of significance was set at $p<0.05$.

Paired sample t-tests were used to compare the main differences in central cardiovascular variables between the conditions (FLZ vs. PLA) within each experimental event stage (rest, LBNP, exercise, exercise + LBNP). Paired sample t-tests were also used to compare the main differences in raw forearm vascular hemodynamic values and delta values between the conditions (FLZ vs. PLA) within each experimental event stages (rest, LBNP, exercise, exercise + LBNP). A repeated measures analysis of variance (ANOVA) was used to determine differences and observed power of Δ% FBF, Δ% FVC and %FS between the conditions (FLZ vs. PLA). A Spilt Plot ANOVA was used to determine sex differences in Δ% FBF (sex x condition). To determine sex differences within the Δ% FBF data, t-tests were used.

Normality was tested for all variables with the Shapiro Wilk test. The Wilcoxon and Friedman tests were used as non-parametric alternatives to t-tests and the repeated measures ANOVA if assumptions were violated. Cohen's *d* effect size was calculated as $d = M_1 - M_2 / \sigma_{pooled}$ with M being the mean of the compared values and σ being the pooled SD reported. Post Hoc Observed power was performed for Δ% FBF, Δ% FVC and %FS, based on the effect size, sample size, and α error probability. Data is reported as mean ± standard deviation (SD) unless otherwise stated.

CHAPTER IV: RESULTS

Subject characteristics

A total of 14 participants (6 Males, 8 Females) completed all three study visits (Table 1). A total of 4 Subjects either did not return to the lab after visit 1 or visit 2 or were diagnosed with a new medical condition between the visits, making them ineligible to participate. All subjects were healthy, young (<30 years), non-obese (BMI<30), nonsmokers who were not currently taking any prescription medication. All subjects declared to be right hand dominant, which resulted in all subjects performing rhythmic forearm muscle exercise with the left arm.

Table 1. *Participant Characteristics (14 right hand dominant and 0 left hand dominant subjects)*

	Whole Sample (n=14)	**Males (n=6)**	**Females (n=8)**
Age (years)	23.64 ± 3.31	25.33 ± 2.65	22.37 ± 3.35
Height (cm)	170.50 ± 10.82	178.66 ± 10.27*	164.37 ± 6.50*
Bodyweight (kg)	70.52 ± 12.84	79.28 ± 13.71*	63.95 ± 7.54*
BMI (kg/m^2)	24.14 ± 2.92	24.69 ± 2.44	23.72 ± 2.34
SBP (mmHg)	113.07 ± 10.21	122.83 ± 5.94*	105.75 ± 5.06*
DBP (mmHg)	70.29 ± 5.46	69.83 ± 6.70	70.62 ± 4.80
MVC (kg)	32.33 ± 13.95	48.73 ± 9.55*	25.12 ± 4.79*
20% MVC (kg)	6.54 ± 2.79	9.75 ± 1.91*	5.02 ± 0.95*
IPAQ (Met/min/wk)	4062.90 ± 3669.93	5524.5 ± 4530.59	2810.129 ± 2421.63
HC (cm)	93.05 ± 27.39	100.97 ± 6.99	57.54 ± 35.49
WC (cm)	80.12 ± 10.01	81.78 ± 6.89	77.92 ± 12.11
TB-FFM (g)	48840.60 ± 14421.50	61365.14 ± 10320.84*	37881.63 ± 5525.96*
FFFM (g)	791.43 ± 355.58	1046.83 ± 275.8*	599.87 ± 287.88*

Values are displayed as mean ± SD
**Indicates significant differences between Males and Females $p<0.05$*
BMI = Body Mass Index, SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure, MVC = Maximum Voluntary Contraction, IPAQ = International Physical Activity Questionnaire, HC = Hip Circumference, WC = Waist Circumference, TB- FFM = Total Body Fat Free Mass, FFFM = Forearm Fat Free Mass

Central cardiovascular variables

As shown in Table 2, stroke volume was significantly different between the PLA and the FLZ conditions during exercise only (p= 0.046) but not during rest (p= 0.42), LBNP at rest (p=0.17) and Exercise + LBNP (p = 0.052)

TPR was significantly different between the conditions at rest (p = 0.04), LBNP (p = 0.04), Exercise + LBNP (p: 0.007) but not Exercise alone (p = 0.056).

Heart rate was not significantly different between the conditions at rest (p: 0.44), LBNP (p: 0.34), Exercise (p: 0.49) or Exercise + LBNP (p: 0.99).

Table 2 : *Central cardiovascular variables (n=14, 6 Males, 8 Females)*

	Rest	**LBNP**	**Exercise**	**Exercise + LBNP**
SV, ml				
PLA	86.51 ± 1.12	75.66 ± 8.91	83.25 ± 16.56*	71.21 ± 15.47
FLZ	83.52 ± 19.58	71.46 ± 16.16	75.97 ± 16.15*	65.04 ± 16.818
TPR				
PLA	110.80 ± 26.07*	122.14 ± 8.91 *	117.56 ± 31.86	131.76 ± 30.99*
FLZ	126.96 ± 38.45*	141.89 ± 42.45*	133.35 ± 28.83	151.04 ± 31. 73*
HR, BPM				
PLA	63.25 ± 7.25	62.85 ± 8.91	74.20 ± 9.10	76.31 ± 20.11
FLZ	62.21 ± 5.42	64.84 ± 9.57	72.42 ± 8.75	76.37 ±11.22

Values are displayed as mean ± SD
FLZ= Fluconazole, PLA= placebo, LBNP = Lower Body Negative Pressure at -20mmHg during rest, RHG Exercise, rhythmic handgrip exercise, RHG Exercise + LBNP , rhythmic handgrip exercise during Lower Body Negative Pressure stimulus, BPM = beats per minute
**Represents significant differences between FLZ and PLA trial p<0.05*

Forearm vascular hemodynamics; comparison of raw and delta values

Responses to Sympathetic stress at rest (Table 3)

As shown in Table 3, at rest, LBNP at -20 mmHg evoked a significant reduction in brachial artery velocity, in both the PLA ($p = 0.01$) as well as the FLZ condition ($p=0.003$). There was no significant difference between the conditions in when comparing Δ Rest-LBNP velocity ($p= 0.24$).

LBNP evoked a significant change in brachial artery diameter, in both the PLA ($p=0.002$) as well as the FLZ condition ($p=0.04$). There was no significant difference between the conditions in when comparing Δ Rest-LBNP diameter ($p=0.21$).

LBNP evoked a significant reduction in FBF, in both the PLA ($p<0.001$) as well as the FLZ condition ($p<0.001$). There was no significant difference between the conditions in when comparing Δ Rest-LBNP FBF ($p=0.21$).

LBNP did not significantly change in MAP, in both the PLA ($p=0.98$) as well as the FLZ condition ($p= 0.90$). There was no significant difference between the conditions in when comparing Δ Rest-LBNP MAP ($p=0.17$).

LBNP evoked a significant reduction in FVC, in both the PLA ($p<0.001$) as well as the FLZ condition ($p<0.001$). There was no significant difference between the conditions in when comparing Δ Rest-LBNP FVC ($p=0.21$)

Table 3: *Raw forearm vascular hemodynamic values and Δ changes during rest (n=13, 6 Males, 7 Females)*

	Rest	LBNP	Δ Rest-LBNP
Velocity, cm/s			
PLA	3.95 ± 2.02	2.68 ± 1.38	-1.27 ± 1.16
FLZ	4.97 ± 1.62	3.17 ± 1.35	-1.80 ± 1.00
Diameter, mm			
PLA	3.49 ± 0.59	3.39 ± 0.59	-0.09 ± 0.08
FLZ	3.46 ± 0.57	3.41 ± 0.57	-0.05 ±0.08
FBF, ml/min			
PLA	23.78 ± 17.82	15.19 ± 10.76	-8.59 ± 9.03
FLZ	28.62 ± 15.26	17.25 ± 8.42	-11.37 ±8.79
MAP, mmHg			
PLA	99.91 ± 7.02	99.95 ± 8.67	0.03 ± 3.65
FLZ	100.30 ± 8.35	100.42 ± 7.70	0.12 ± 3.17
FVC, ml/min/100mmHg			
PLA	23.86 ± 18.19	15.23 ± 11.09	-8.62 ± 8.87
FLZ	28.91 ± 17.13	17.54 ±10.02	-11.36 ± 9.00

Values are displayed as mean ± SD
FLZ= Fluconazole, PLA= placebo, LBNP = Lower Body Negative Pressure at -20 mmHg during rest, RHG Exercise, rhythmic handgrip exercise, RHG Exercise + LBNP, rhythmic handgrip exercise during Lower Body Negative Pressure stimulus.
**Represents significant differences between FLZ and PLA trial $p<0.05$*

Exercise Responses (Table 4)

As shown in Table 4, when looking at the raw values, paired sample t-tests revealed that rhythmic forearm exercise at 20% MVC evoked a significant increase in brachial artery velocity, in both the PLA ($p<0.001$) as well as the FLZ ($p<0.001$) condition when compared to resting values. There was no significant difference in brachial artery velocities between the conditions ($p=0.15$) during rhythmic handgrip (RHG) exercise without sympathetic stimulus. There was also no significant difference between the conditions during RHG Exercise + LBNP ($p=0.59$). There was, however, a significant difference between the PLA and the FLZ condition when looking at Δ Ex-Ex/LBNP ($p=0.02$).

Rhythmic forearm exercise alone evoked a significant increase in brachial artery diameter, in both the PLA condition ($p=0.02$) but a not in the FLZ condition ($p= 0.25$) when compared to resting values. There was no significant difference is brachial artery diameters between the conditions ($p=0.26$) during RHG exercise without sympathetic stimulus. There was no significant difference between the conditions during RHG Exercise + LBNP ($p=0.15$). There was no significant difference between the conditions when looking at Δ Ex-Ex/LBNP ($p=0.15$). Rhythmic forearm exercise alone evoked a significant increase in FBF, in both the PLA condition ($p<0.001$) as well as the FLZ condition ($p<0.001$) when compared to resting values.

There was no significant difference in FBF between the conditions ($p= 0.54$) during RHG exercise without sympathetic stimulus. There was also no significant difference between the conditions during RHG Exercise + LBNP ($p=0.46$). There was, however, a significant difference between the PLA and the FLZ condition when looking at Δ Ex-Ex/LBNP ($p=0.009$).

When looking at MAP, rhythmic forearm exercise alone evoked a significant increase, in both the PLA condition ($p<0.001$) as well as the FLZ condition ($p<0.001$) when compared to

resting values. There were no significant differences in MAP between the conditions ($p=0.20$) during RHG exercise without sympathetic stimulus. There was also no significant difference between the conditions during RHG Exercise + LBNP ($p= 0.44$). There was no significant difference between the conditions when looking at Δ Ex-Ex/LBNP ($p= 0.21$).

Rhythmic forearm exercise alone also evoked a significant increase in FVC, in both the PLA condition ($p<0.001$) as well as the FLZ condition ($p<0.001$) when compared to resting values. There was no significant difference in FBF between the conditions ($p= 0.46$) during RHG exercise without sympathetic stimulus. There was also no significant difference between the conditions during RHG Exercise + LBNP ($p= 0.73$). There was, however, a significant difference between the conditions when looking at Δ Ex-Ex/LBNP ($p=0.007$).

Table 4: *Raw forearm vascular hemodynamic values and Δ changes during Exercise (n=6 Males, 7 Females)*

	RHG Exercise	**RHG Exercise + LBNP**	**Δ Change Ex-Ex/LBNP**
Velocity, cm/s			
PLA	31.58 ± 8.95	33.68.38 ± 9.06	2.10 ± 3.62*
FLZ	36.94 ± 10.57	35.10 ± 9.06	-1.83 ± 4.2*
Diameter, mm			
PLA	3.66 ± 0.59	3.61 ± 0.63	-0.04 ± 0.09
FLZ	3.56 ± 0.67	3.46 ± 0.69	-0.11 ± 0.15
FBF, ml/min			
PLA	206.14 ± 85.30	208.61 ± 76.16	2.47 ± 19.97*
FLZ	219.22 ± 92.90	195.03 ± 82.06	-24.19±30.74*
MAP, mmHg			
PLA	118.76 ± 12.59	119.90 ± 13.18	1.14 ± 3.06
FLZ	115.02 ± 8.79	117.36 ± 7.73	2.33 ± 2.98
FVC, ml/min/100mmHg			
PLA	176.96 ± 77.45	175.41 ± 61.61	3.55 ± 21.96*
FLZ	194.56 ± 82.37	169.15 ± 68.65	-25.42 ± 30.23 *

Values are displayed as mean ± SD
FLZ= Fluconazole, PLA= placebo, LBNP = Lower Body Negative Pressure at -20 mmHg during rest, RHG Exercise = rhythmic handgrip exercise, RHG Exercise + LBNP = steady state rhythmic handgrip exercise during Lower Body Negative Pressure stimulus.
**Represents significant differences between FLZ and PLA trial $p<0.05$*

Forearm muscle blood flow responses as Δ%changes

When looking at Δ%changes at rest, FBF decreased similarly in the FLZ and PLA condition (PLA: 31.65 ± 19.79% vs FLZ: 37.81 ± 16.29%) in response to LBNP stimulus at -20mmHg. A repeated measures ANOVA revealed a non-significant difference between the conditions (p= 0.335, observed power= 0.152, *d*= 0.34) (Figure 2).

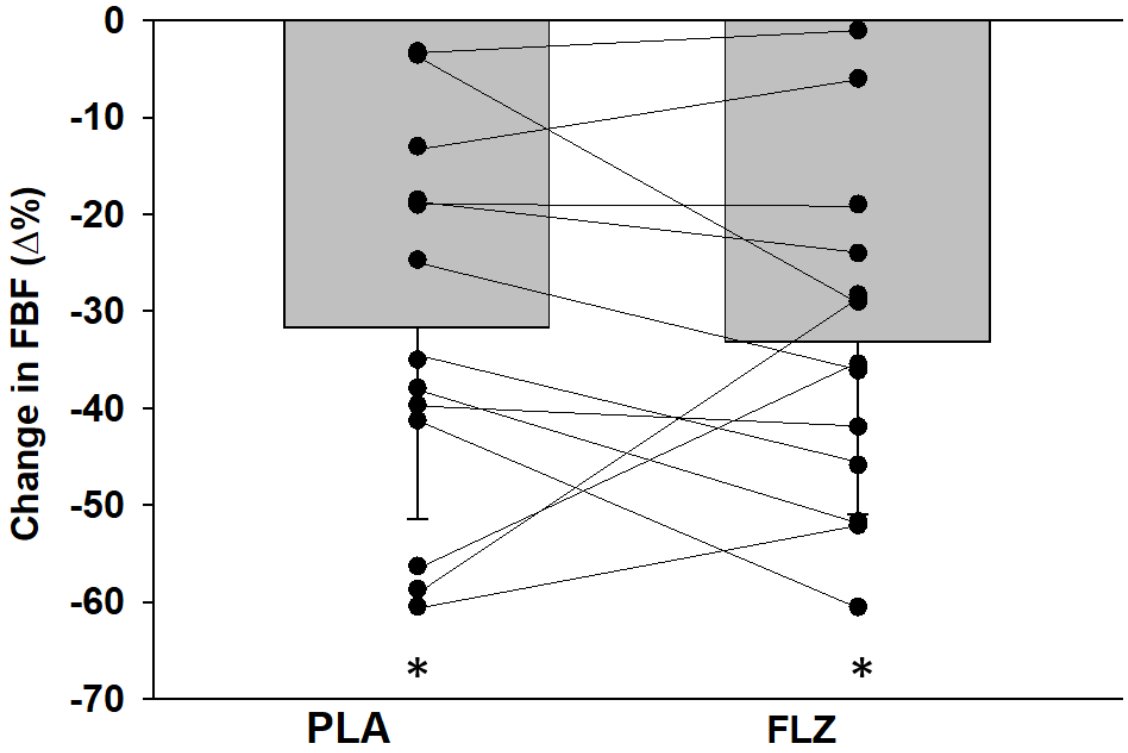

Figure 2. Individual data and group mean %changes in forearm muscle blood flow in the FLZ and PLA condition in response to LBNP at -20 mmHg induced reduction in forearm muscle blood flow at rest. P >0.05 between conditions.

**Indicates significant difference between rest and rest+LBNP*

When looking at Δ%changes during exercise without sympathetic stress, FBF increased similarly in the FLZ and PLA condition from Rest (PLA: 1064.13 ± 666.58% vs FLZ: 818.30 ± 589.86%). There was no significant difference between the conditions (p= 0.40, observed power= 0.156, *d* = 0.39) (Figure 3).

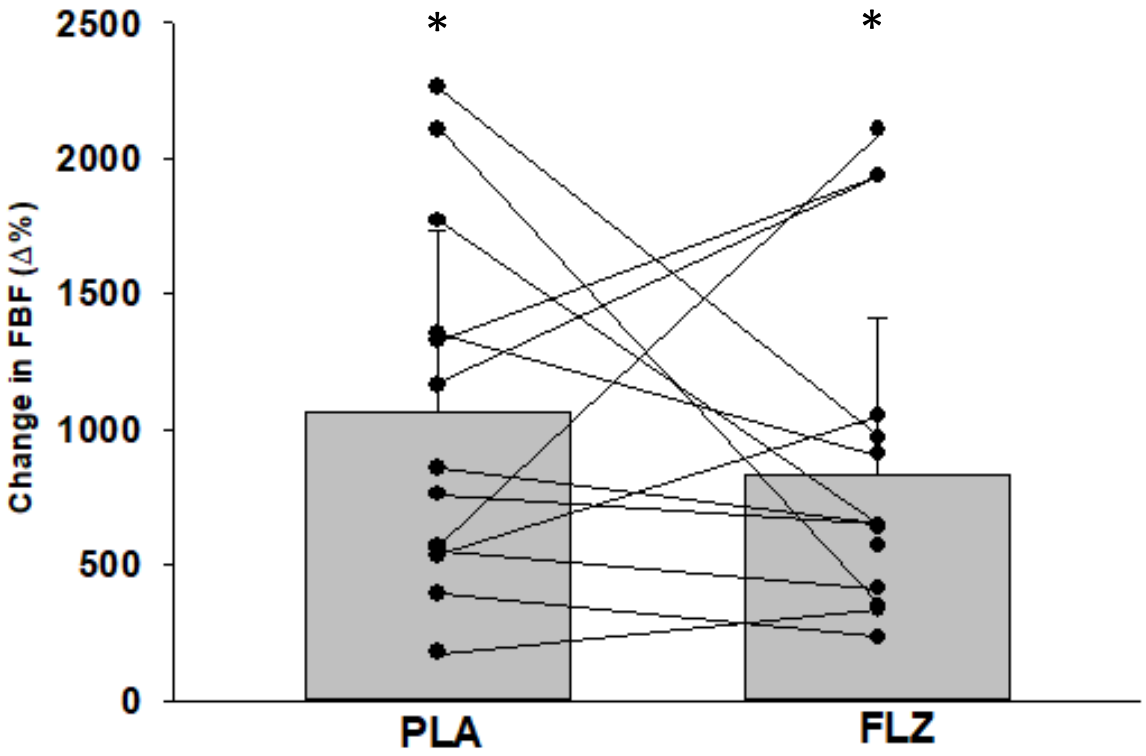

Figure 3. Individual data and group mean %changes in forearm muscle blood flow in the FLZ and PLA condition in response to exercise induced responses in forearm muscle blood flow. P >0.05 between conditions.

**Indicates significant difference from baseline.*

When looking at Δ%changes in response to LBNP induced sympathetic stimulus during forearm muscle exercise, FBF decreased significantly by -9.86. ± 15.90% (p= 0.02) in the FLZ condition but did not significantly change from exercise alone in the PLA condition (3.92 ± 11.03%, p= 0.66). There was a significant difference between the conditions (p=0.004, observed power= 0.906, *d*= 1.0) (Figure 4).

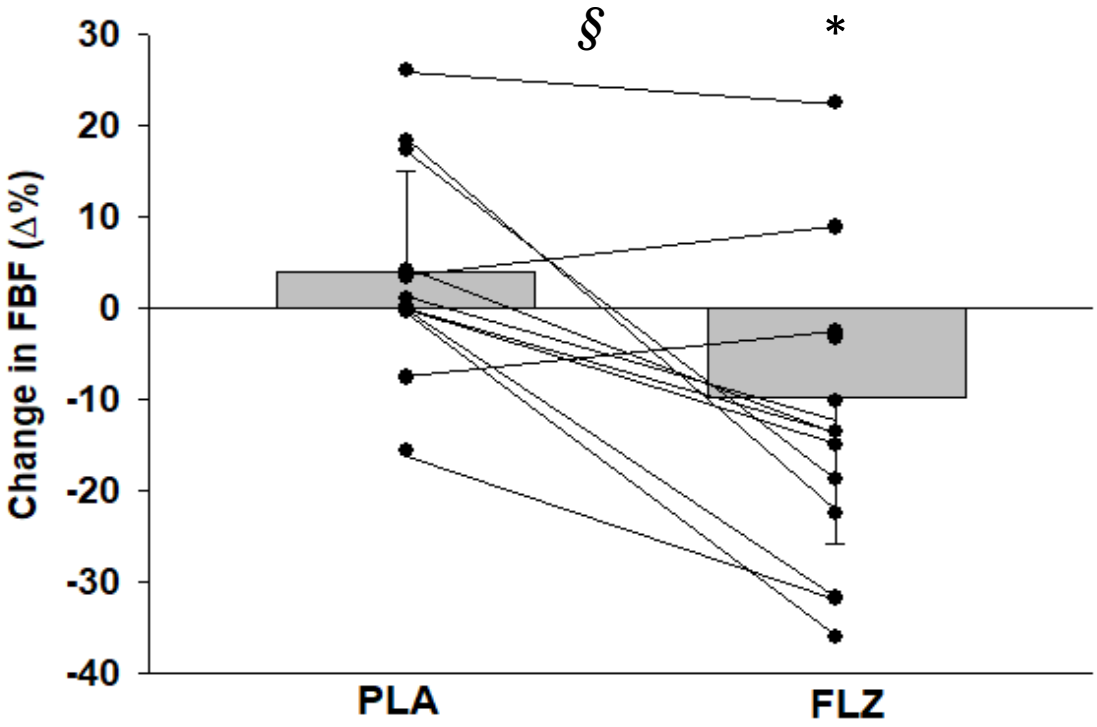

Figure 4. Individual data and group mean %changes in forearm muscle blood flow in the FLZ and PLA condition in response to LBNP induced responses in forearm muscle blood flow during rhythmic forearm muscle exercise at 20% MVC. P <0.05 between conditions.

**Indicates significant difference from baseline.*

§ indicates differences between the PLA and FLZ condition

Forearm vascular conductance responses as Δ%changes

When looking at Δ%changes at rest, FVC decreased similarly in the FLZ and PLA condition (PLA: -32.02 ± 18.99% vs FLZ: -38.16 ± 15.97%) in response to LBNP stimulus at -20mmHg. There was no significant difference between the conditions (p= 0.308, observed power= 0.165 *d*= 0.35) (Figure 5).

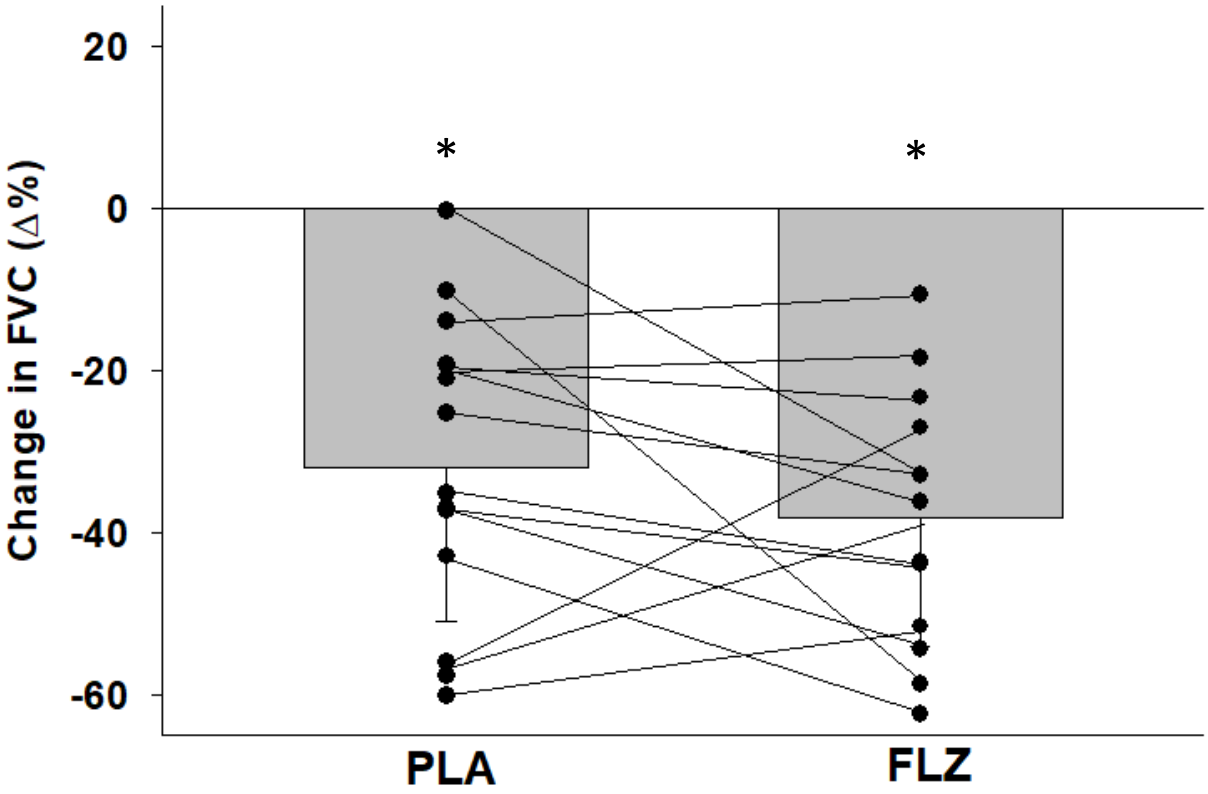

Figure 5. Individual data and group mean %changes in forearm vascular conductance in the FLZ and PLA condition in response to LBNP induced reduction in forearm muscle blood flow at rest. P <0.05 between conditions.

**Indicates significant difference from baseline.*

When looking at Δ%changes during exercise without LBNP induced sympathetic stimulus, FVC increased similarly in the FLZ and PLA condition (PLA: 873.96 ± 529.99% vs FLZ: 706.47 ± 484.20%), meaning there was no significant difference between the conditions (p= 0.166, observed power= 0.124, *d*= 0.3) (Figure 6).

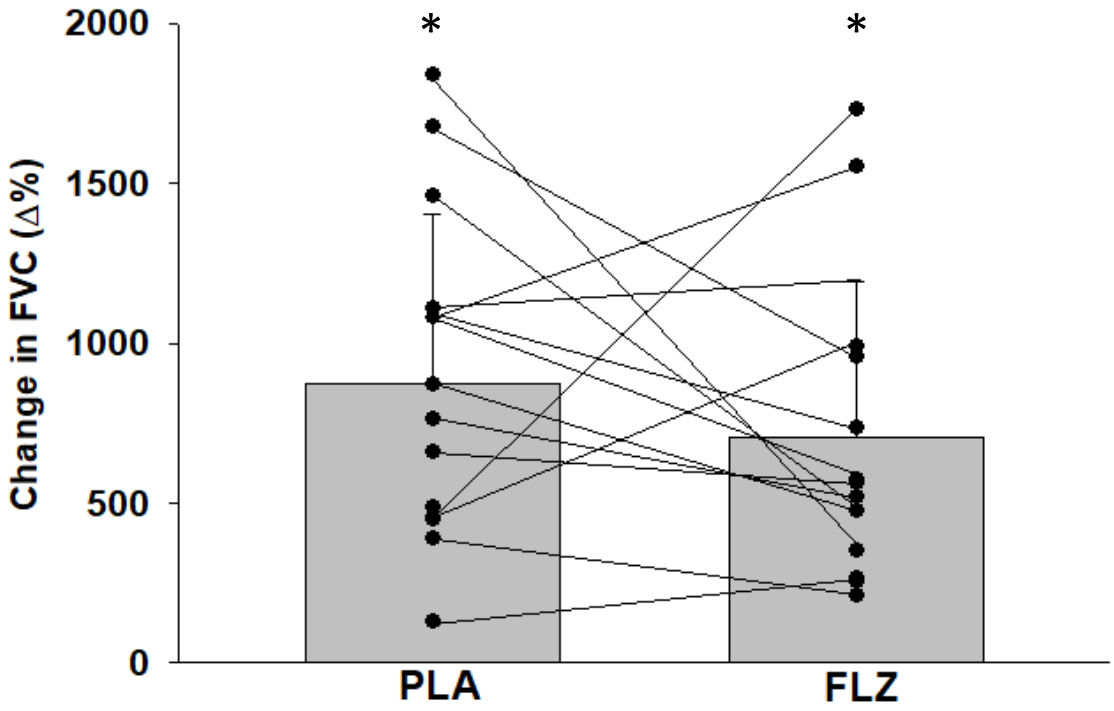

Figure 6. Individual data and group mean %changes in forearm vascular conductance in the FLZ and PLA condition in response to exercise induced responses. P >0.05 between conditions.

**Indicates significant difference from baseline.*

When looking at Δ%changes FVC in response to LBNP induced sympathetic stimulus during forearm muscle exercise, FVC decreased significantly by –11.58 ± 15.06% (p= 0.011) in the FLZ condition but did not significantly change in the PLA (2.92 ± 12.69%, p=0.81)). There was a significant difference between the conditions (p= 0.003, observed power= 0.933, *d*= 1.1) (Figure 7).

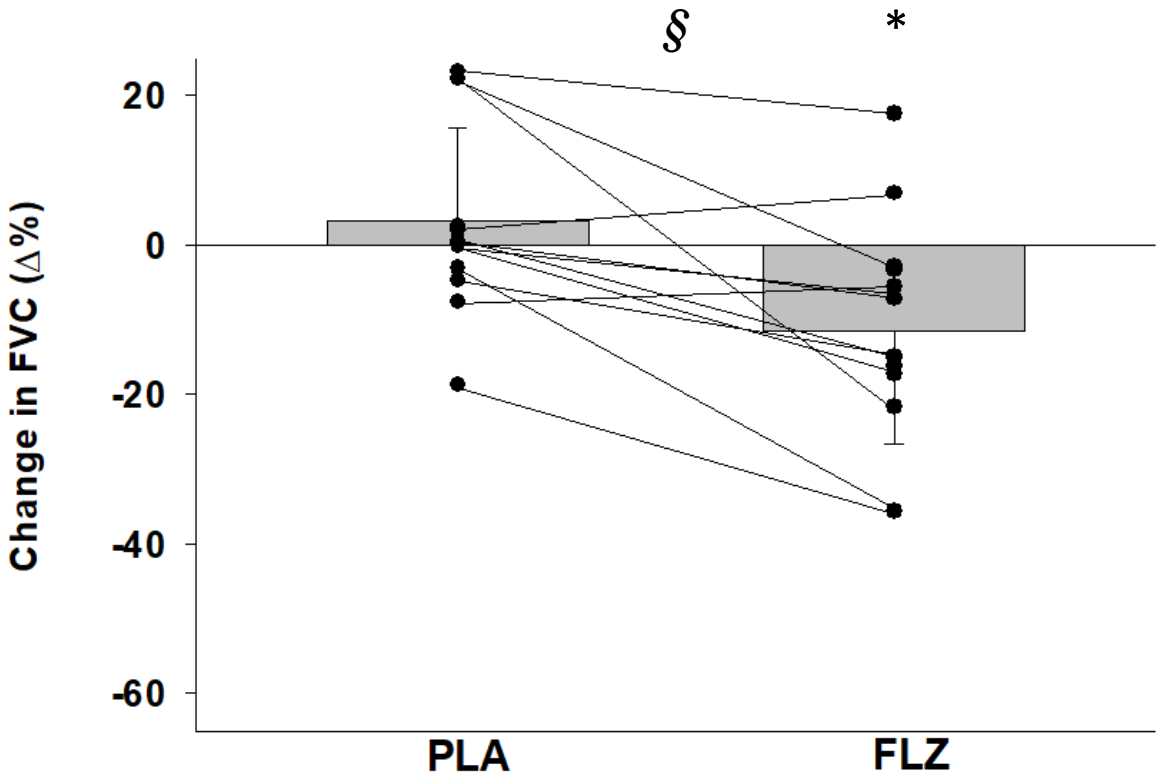

Figure 7. Individual data and group mean %changes in forearm vascular conductance in the FLZ and PLA condition in response to LBNP induced responses during rhythmic forearm muscle exercise at 20% MVC. P <0.05 between conditions.

**Indicates significant difference from resting measurements.*

§ indicates differences between the PLA and FLZ condition

Forearm muscle oxygenation responses

A repeated measures ANOVA revealed a no significant difference in exercise-induced attenuation of the oxyhemoglobin reduction (p=0.122, observed power=0.335, *d*= 0.36) between the PLA and the FLZ condition (Figure 8).

There was no significant difference in ΔHbO2 at rest when LBNP was added (normalized to the total liable signal) (PLA: 15.07 ± 8.28 vs FLZ: 17.06±12.51, p=1.0) (Figure 9 and 10).

There was also no significant difference in ΔhbO2 at rest when LBNP was added (normalized to the total liable signal) (PLA: 5.60 ± 5.87 vs FLZ: 8.11 ± 7.01 p= 0.14) (Figure 9 and 10).

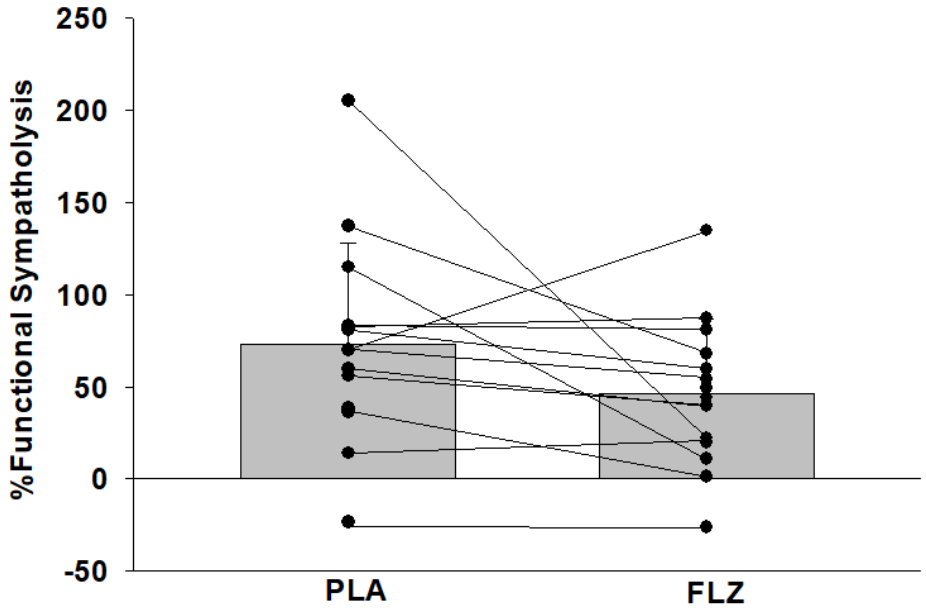

Figure 8. Individual and group differences in %functional sympatholysis as in exercise-induced attenuation of the oxyhemoglobin reduction. P > 0.05 between the conditions.

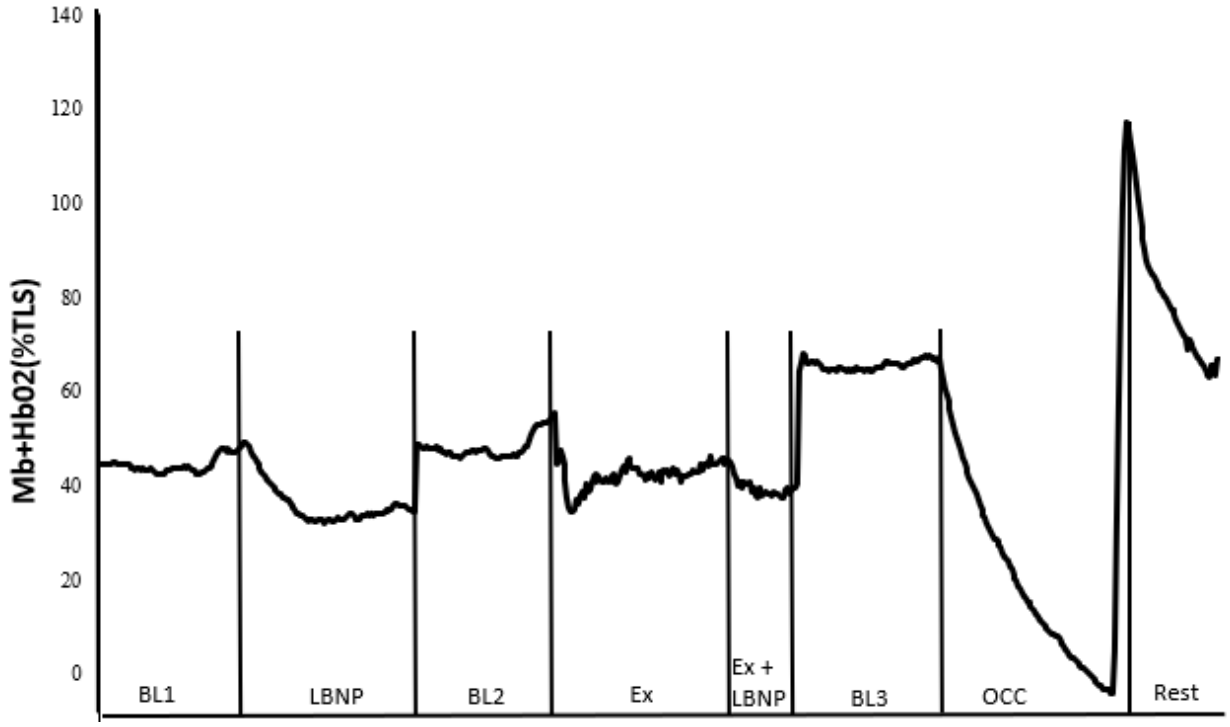

Figure 9. Functional sympatholysis protocol tracing HbO_2 in the forearm musculature during PLA. Data tracing of Myoglobin and Oxyhemoglobin responses during LBNP (-20mmHg) exposure, followed by forearm muscle exercise at 20%MVC and Exercise with LNBP exposure. Followed were 5 minutes of reactive hyperemia occlusion at 230mmHG to determine the nadir to determine % total labile signal (TLS).

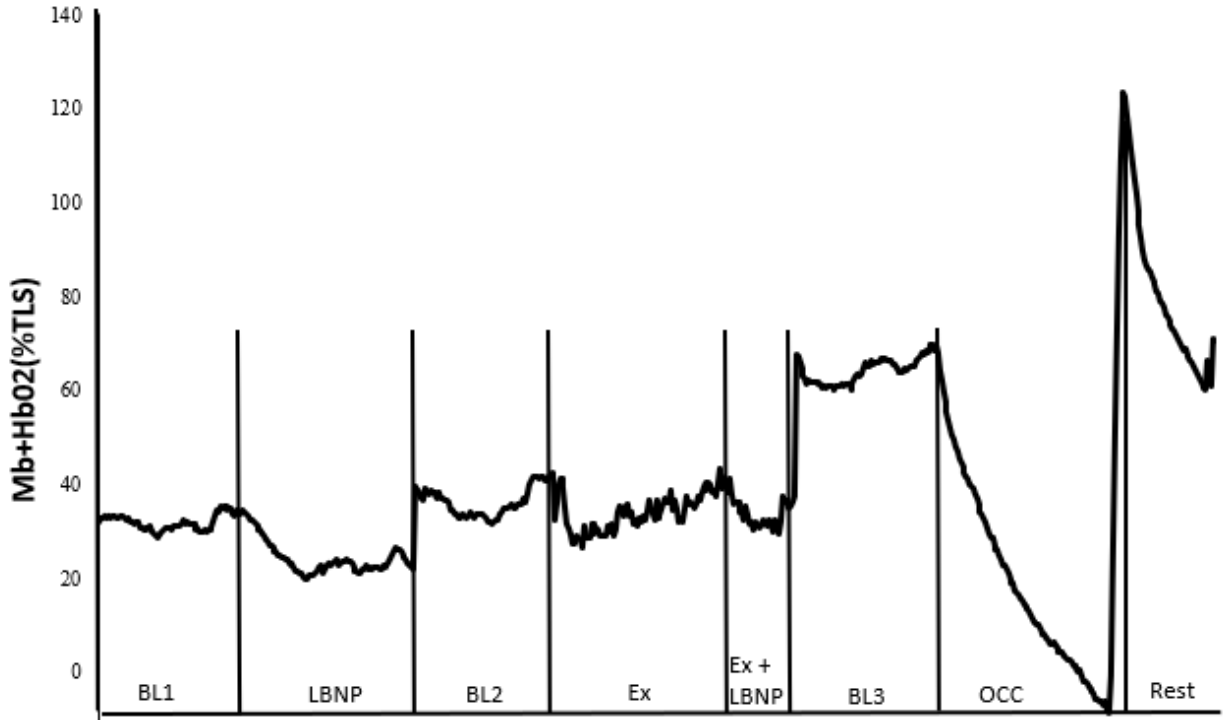

Figure 10. Functional sympatholysis protocol tracing HbO_2 in the forearm musculature during FLZ. Data tracing of Myoglobin and Oxyhemoglobin responses during LBNP (-20mmHG) exposure, followed by forearm muscle exercise at 20%MVC and Exercise with LNBP exposure. Followed were 5 minutes of reactive hyperemia occlusion at 230mmHG to determine the nadir to determine % total labile signal (TLS).

Sex differences

A Split Plot ANOVA revealed a significant main effect between the two conditions (p=0.041, Observed Power= 0.899) but a non-significant interaction effect (sex x condition) (p= 0.225, Observed Power: 0.217) (Table 5).

Paired Sample t-tests did reveal a significant sex difference in Δ% FBF Exercise – Exercise with LBNP at -20 mmHg during the placebo condition (p= 0.007) but no significant difference in the FLZ condition (p=0.84) (Figure 10).

Table 5: Sex differences in Δ%FBF Exercise – Exercise with LBNP

	Δ%FBF PLA	**Δ%FBF FLZ**
Males (n=6)	-3.05 ± 3.92*	-11.62 ± 13.94
Females (n=7)	9.90 ± 10.47*	-8.35 ± 18.39

Values are displayed as mean ± SD
*Indicates sex differences

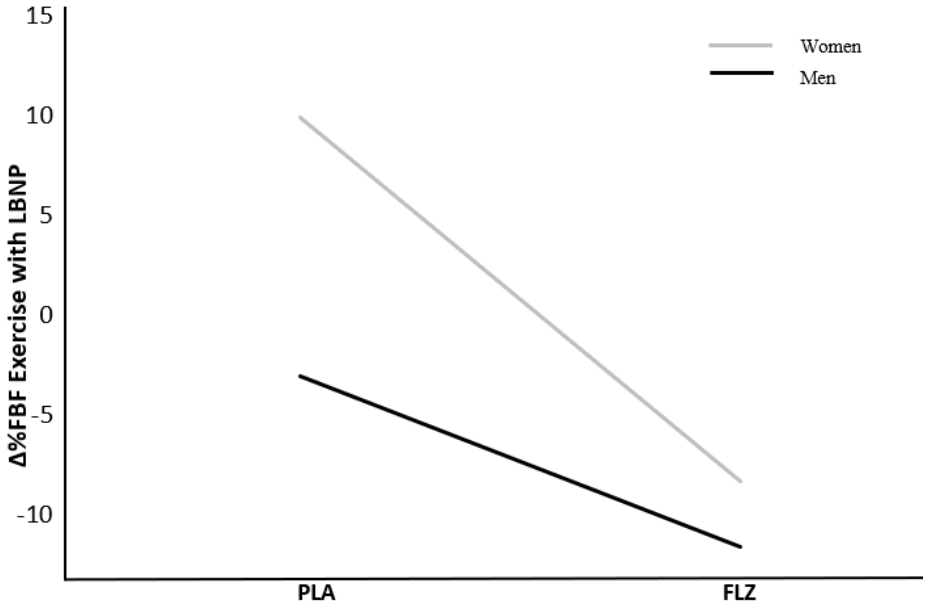

Figure 10: Sex differences in Δ%FBF Exercise – Exercise with LBNP at -20 mmHg responses

CHAPTER V: DISCUSSION

The purpose of the present study was to determine the importance of EDHF in functional sympatholysis in healthy young adults. To our knowledge, this was the first study to investigate the effects of an EDHF blockade via fluconazole during forearm muscle exercise in addition to sympathetic stress via LBNP. The main finding of this study is that the blockade of EDHF blunts the ability of skeletal muscle to override sympathetic vasoconstriction during exercise during sympathetic stress. In direct contrast, EDHF does not seem to influence the modulating of human skeletal muscle vasculature during sympathetic stress alone.

Resting hemodynamics

In resting conditions, we found no significant differences between the PLA and the FLZ conditions when comparing absolute brachial artery FVC, FBF, mean blood velocity and diameter (Table 3). These findings contradict findings by Petterson et al (2021), who found significant differences between control and Fluconazole ingestion in mean blood velocity, blood flow, shear stress, but not brachial artery diameter at rest.

In contrast, other studies investigating functional sympatholysis in clinical populations, as opposed to the investigation of local vasodilator including EDHF, reported in their baseline data that younger and older individuals did not significantly differ in baseline FBF and baseline FVC. Yet, functional sympatholysis, based on FVC and FBF, was impaired in the study groups (type 2 diabetes) (Bock et al, 2020). For example, Bock et al (2020) found no significant difference in resting FBF or FBF between healthy adults and subjects with type 2 diabetes despite impaired attenuated hyperemic and vasodilatory responses to exercise.

Contrary to our results, Ozkor et al (2011) found that the local infusion of FLZ significantly decreased resting FBF significantly in heathy adults. This indicates that that EDHF

significantly contributes to resting vasodilatory tone. A NO blockade via N-Methylarginine in addition to FLZ infusion reduced FBF even further, indicating that NO may also play an important role in resting vasodilatory tone. However, it is important to mention that, in this particular study, FBF was measured after intra-arterial infusion given at 0.4 μmol/min for 5 minutes, as opposed to a systematic EDHF inhibition via a FLZ pill, which may explain the discrepancies in results.

In contrast to Petterson at al (2021) and Ozkar et al (2011), our findings suggest that EDHF is not a modulator of skeletal muscle blood flow in resting conditions alone, keeping in mind the methodological differences.

% FBF/ FVC changes at Rest + LBNP Stimulus

In response to increased sympathetic nervous system stimulus via the use of LBNP reduced FBF in both FLZ and PLA with no difference between the conditions (Figure 2). LBNP stimulus causes sympathetic vasoconstriction via offloading of baroreceptors (Rickards et al. 2015), acting on the cardiovascular control center, ultimately leading to increased sympathetic vasomotor outflow. Baroreceptor offloading ultimately leads to increased sympathetic vasoconstriction and a decrease in FVC/FBF (Tschakowksy & Pyke, 2008). A LBNP stimulus of -20 mmHg causes an increase in sympathetic vasoconstriction, while MAP and HR are mostly maintained (Rickards et al., 2015). In both conditions, we found that LBNP created a robust sympathetic vasoconstriction without any difference between the PLA and the FLZ conditions, indicating that EDHF does not play a role in overriding sympathetic vasoconstriction at rest during sympathetic stimulation alone. This does match with our findings at rest without sympathetic stimulus. Similar to our findings in terms of FBF responses, we found a significant

decrease in Δ% in FVC in both, the PLA as well as the FLZ trial when comparing resting to rest+LBNP values without a significance between the conditions (Figure 5).

Our FBF findings match with previous studies using a similar study design but in different populations. Vongpatanasin, et al (2011) found that Hypertensive and Normotensive individuals showed similar FBF and FVC values during the increase of sympathetic stress via -20 mmHg LBNP stimulus. Our data indicates that EDHF may not be a key modulator in vascular tone during sympathetic stress in resting conditions.

% FBF/FVC changes - Exercise Without LBNP Stimulus

Unsurprisingly, we observed a significant increase in FBF during rhythmic forearm muscle exercise at 20% MVC from resting baseline in both conditions. However, similar to LBNP responses at rest, there was no significant difference in Δ% changes in FBF between conditions (Figure 3). Similar to our FBF data, we found a significant increase in Δ% FVC from resting values without significant difference between the conditions (Figure 6) .Our findings indicate that EDHF did not play a significant role in the ability to increase forearm muscle blood flow itself. However, exercise alone without added sympathetic stress is not sufficient to determine changes in functional sympatholysis (Bunsawat et al., 2019). Previous investigations in hypertensive individuals indicated that FBF and FVC was not statistically significant between normotensive and hypertensive subjects at steady state handgrip exercise. However, like our results, the same investigation indicated significant differences between the groups after LBNP was added to forearm muscle exercise (Vongpatanasin et al, 2011).

When taking our data and previous findings into consideration, we find that exercise responses itself may not be indicative of functional sympatholysis due to the lack to sympathetic stimulus. Due to the nature of functional sympatholysis, being the ability to blunt sympathetic

vasoconstriction, additional sympathetic stress needs to be added to exercise hyperemia to determine podetial mechanisms in the phenomenon.

% FBF/FVC changes Exercise + LBNP

In contrast to our exercising data without sympathetic stimulus, we found a significant difference between FLZ and PLA when comparing FBF and FVC responses to exercise during LBNP stimulus (Figure 4, Figure 7). We found a nonsignificant change in FBF and FVC when LBNP was added to forearm muscle exercise in the PLA condition, suggesting intact sympatholysis. On the other hand, in FLZ, FBF and FVC where compromised (Figure 4, Figure 7).

Our data adds to the knowledge gained from Hearon et al (2016). As stated before, the study found that the stimulation of the Endothelium Derived Hyperpolarization-like vasodilator acetylcholine as well as low dose infusion of the endothelium dependent vasodilator ATP blunts sympathetic vasoconstriction in exercising skeletal muscle during mild (non-sympatholytic) intensity RHG similar when compared to moderate intensity RHG at 15%MVC (sympatholytic) based on changes in ΔFVC. On the contrary, the NO donor sodium nitroprusside does not attenuate sympathetic vasoconstriction at rest or combined with mild exercise at 5% MVC. Overall, our and previous data indicates that EDHF may play an important role in blunting α1-adrenergic vasoconstriction (functional sympatholysis) when looking at changes on FBF and FVC.

Additionally, Crecelius et al. (2011) tried to determine the independent as well as the interactive roles of NO/PG in ATP-dependent vasodilation. Both, the individual and combined NO/PG inhibition via N-Methylarginine (blockade of NO) and Ketorolac (blockade of PG) brachial infusion indicated no effect on forearm vascular conductance.

Since EDHF acts independently of NO/PGs, endothelium derived hyperpolarization may play a more important role in exercise hyperemia then previously assumed. Our data, as indicated in Figure 4 and Figure 7, shows an important role of EDHF in our ability to blunt sympathetic constriction during exercise.

Forearm muscle oxygenation

We found no significant difference in %functional sympatholysis (Δ% HbO_2+MbO_2) when comparing the FLZ and well and the PLA condition (Figure 8). Our findings contradict with findings in terms of ΔFVC and ΔFBF changes. Our data also contradict our knowledge of the relationship between forearm muscle oxygenation and muscle blood flow during exercise. Previous studies have shown that the exercise induced redistribution of blood flow during exercise and the increase in sympathetic vasoconstriction based on baroreceptor offloading will lead to a reduction of oxygenated hemoglobin (Volianitis et al, 2003). Muscle tissue oxygenation and muscle blood flow are strongly correlated, which makes both valid and reliable tools to assess functional sympatholysis (Fadel et al, 2004).

The primary difference in our assessment of functional sympatholysis based on vascular conductance vs. oxygenation changes is the observed power we discovered. Our %Δ in FVC in response to Exercise with LBNP has indicated an observed power of 0.933 and d=1.1. On the other hand, our oxygenation data indicated an observed power was 0.335 with a d of 0.36. We can only speculate at the current progress of our data collection that a higher power due to an increased sample size may reveal potential differences between the conditions in terms of muscle oxygenation.

Sex differences.

Our data indicates no overall significant differences in functional sympatholysis responses (Figure 10) to an EDHF blockade but showed that the Δ%FBF Exercise – Exercise with LBNP was significantly lower in men in the PLA condition (Table 5). This may indicate that females are more dependent on EHDF more during functional sympatholysis then males. However, a major limitation of this analysis is the low observed power of the group differences. We can only speculate that an increased sample size may change our overall results.

Just & DeLorey (2017) were among the first who explore sex differences in functional sympatholysis and found that Female rodents are better at overriding sympathetic vasoconstriction the Males. Females were also more dependent on NO then males. Whether this applies to humans, and what role EDHF may play in not fully understood and needs to be explored further. Our findings can potentially add to our limited knowledge about sex differences in vasoconstrictor responses.

Limitations and experimental considerations

Our experimental design has a multitude of limitation in the experimental design. First, we asked all participants to abstain from alcohol, NSAIDs, and vigorous exercise for 24 hours, from caffeine for 12 hours and from eating for 8 hours leading up to the study visit. Additionally, we required all female participants to be tested in day 1-5 of the early follicular phase. We had to rely on the answers to be truthful as we were not able to control the pre-study requirements.

We tested sympatholytic responses in small muscle mass only. Whether our findings are applicable to large muscle mass in not fully clear. However, previous data indicated that

differences in muscle mass do not have an effect on the ability to inhibit sympathetic vasoconstriction (Wray et al., 2004).

A further limitation of our study design was that FLZ was given in a single dose as a 150mg pill. Previous studies investigating the effects of EDHF on skeletal muscle blood flow have utilized inter-arterial infusion of FLZ over a 5-minute time frame (Ozkor et al, 2011). This could be a potential cause for our resting data discrepancies when compared to the data to Ozkor et al (2011) and could have caused a blunted overall effect of a CYP450 blockade in our data, as our use of FLZ was systemically as opposed to local stimulation.

We were not able to measure sympathetic nervous system activity over the course of the LBNP stimulus. Previous data measuring functional sympatholysis also found differences in MSNA activity, giving further insight into vasoconstrictor responses (Vongpatanasin et al., 2011).

A potential critique point of our study analysis is the presentation of our FBF and FVC data as Δ% changes as opposed to absolute values. Data expression when investigating sympathetic vasomotion has been extensively debated in the previous literature by Tschakowsky et al (2001) Thomas et al (1997) & Buckwalter and Clifford (2001). In essence, percent changes in FBF and FVC are more appropriate to distinguish between vasoconstrictor responses as it will be more indicative of a percent reduction in vessel radius during differing flow conditions (e.g. high flow during exercise vs low flow during rest) within an individual (Tschakowsky et al, 2011). Therefore, interpreting percent changes allows us to quantify the relative vasoconstriction (e.g. change in vessel radius) caused by the sympathetic stimulus within each individual in each condition.

CHAPTER VI: CONCLUSION

The purpose of the present study was to determine the importance of EDHF in functional sympatholysis in healthy young adults.

Main findings

1. We accept the first hypothesis of the study indicating that EDHF blockade does significantly impair functional sympatholysis in healthy, young adults (Figures 4 and 7).
2. We also accept that the blockade of EDHF does decrease forearm muscle blood flow & decrease vascular conductance during exercise with sympathetic stress in healthy, young adults. However, we did not find significant differences between the conditions in terms of vascular conductance and muscle blood flow during rest and exercise alone (Figure 2 – Figure 7).
3. We reject the hypothesis that forearm muscle oxygenation does decrease after EDHF blockade during rest, sympathetic stress, exercise, and exercise with sympathetic stress in healthy, young adults, keeping in mind the low observed power compared to our vascular conductance data (Figure 8).

In conclusion, we found that EDHF potentially plays an important role blunting sympathetic vasoconstriction in healthy, young adults. Future direction should include the application of testing the blockade of EDHF in individuals with cardiovascular and cardiometabolic disease. Additionally, previous research indicates the importance of exercise training on functional sympatholysis. The importance of EDHF in these implications remains unknown. The importance of EDHF signaling in during exercise similarities and sex differences needs to be further explored as well.

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Appendix A

IRB approved documents

Consent Form to Participate in a Research Study
University of Oklahoma Health Sciences Center (OUHSC)
University of Oklahoma- Norman Campus

Study Title:
Endothelial Derived Hyperpolarization Factor and regulation of cerebral and muscle blood flow.

Sponsor: Department of Health and Exercise Science
Principal Investigator: J. Mikhail Kellawan
Phone Number: 405-325-9028

KEY INFORMATION ABOUT THE RESEARCH STUDY

You are being asked to participate in a research study. Research studies are voluntary and include only people who choose to take part. This consent form begins with a 'Key Information' section to provide important information to help you decide whether or not to participate in this study. More detailed information is provided after the key information. Please take your time, discuss this with family and friends, and ask the investigator and study team any questions you may have.

WHY HAVE I BEEN ASKED TO PARTICIPATE IN THIS STUDY?

You are being asked to participate in this research study because you are a healthy individual, young individual.

WHY IS THIS STUDY BEING DONE AND HOW LONG WILL IT LAST?

The purpose of this study is to determine the importance of a substance called endothelium-derived hyperpolarizing factor (EDHF) and how it may affect blood flow to your brain and muscles. You will be in the study for as little as 1 week or as long as three months. This time frame depends on your availability to complete the three study visits.

WHAT WILL I BE ASKED TO DO IN THIS STUDY?

If you decide to participate in this study, you will be asked to complete three study visits. The first visit is to ensure you qualify for the study and make you familiar with all equipment we will use in the experiment and what you are expected to do during your visits. The second and third visits are to test if the blocking EDHF will change blood flow to the muscles and brain. You will be given either fluconazole or a placebo pill and your blood flow will be measured using a lower body negative pressure device while you are performing handgrip exercise. During the second and third visits you will be randomly chosen to receive Fluconazole which is an anti-fungal medication and blocks EDHF or an inactive pill, also known as a placebo pill. You will not know which pill you are receiving during any visit. However, whatever pill you receive on visit two, you will receive the opposite pill on visit three.

WHY MIGHT I WANT TO PARTICIPATE IN THIS STUDY?

If you agree to take part in this study, there will not be direct medical benefit to you. It is hoped that the information learned from this study will benefit people with diseases that affect the cardiovascular system in the future.

WHY MIGHT I NOT WANT TO PARTICIPATE IN THIS STUDY?

You may not want to participate in the study because there is no direct benefit to you. Or the study may take up too much of your time. Further, the researchers do not know all the side effects/risks that could happen during the study. For a complete description of known risks, refer to the Detailed Information section of the consent form.

WHAT OTHER OPTIONS ARE THERE?

You may choose not to participate in this study. Please talk to your regular doctor about these and other options.

HOW WILL PARTICIPATING IN THE STUDY AFFECT ME FINANCIALLY?

There is no additional cost to you if you participate in this study. If you chose to participate and complete all three study visits you will receive a gift card for $50 for Amazon.com.

DETAILED INFORMATION ABOUT THE RESEARCH STUDY

The following pages of the consent form will provide you with more information about this study. Please take your time in reviewing this information and ask the investigator and study team any questions you may have.

HOW MANY PEOPLE WILL TAKE PART IN THE STUDY?

About 30 people will take part in this study at this location.

WHAT IS THE STATUS OF THE MEDICATION USED IN THIS STUDY?

Fluconazole also known as Diflucan, is a prescription medication that is typically used to treat fungal infections. It falls under the US Food and Drug Administration's Dietary health and Education Act as a **prescription drug.** Therefore, Fluconazole is approved & regulated by the US Food and Drug Administration. You will take one 150 mg dose in the form of a pill by mouth either during visit 2 or visit 3.

WHAT IS INVOLVED IN THE STUDY?

If you decide to participate in this study, you will be asked to complete three study visits. The first visit is to ensure you qualify for the study and make you familiar with all equipment and what you are expected to do during your visits. The second and third visits are to test if the blocking of EDHF, using the drug Fluconazole, will change the widening/narrowing of blood vessels and blood flow to your muscles and

brain while a lower body negative pressure device is turned on during handgrip exercise. Lower Body Negative Pressure causes a shifting of blood from your upper body to the lower body as well as an increase how fast your heart beats. During the second and third visits you will be randomly chosen to receive a Fluconazole pill, which is an anti-fungal medication and blocks EDHF, or a placebo pill. A placebo pill has no therapeutic value and thus will not change your brain and muscle blood flow. You will not know which treatment you are receiving during any visit. However, whatever treatment you receive on visit two, you will receive the opposite treatment on visit three.

Visit 1 (Screening Day): ~2 hours. This visit will include a variety of forms to be filled out including: informed consent, questionaries about your health history and physical activity as well as measurements of height, weight, waist and hip circumference, and blood pressure while you are lying down on your back. Women will also have to complete a pregnancy test. These are done to make sure you are eligible for the study and that it is safe for you to participate. Once you have been deemed eligible to participate, you will complete a Dual-energy X-ray absorptiometry (DEXA) scan to determine the amount of fat, muscle and bone is in your body. After the DEXA scan, your forearm grip strength in your non-dominate arm will be measured. Additionally, the proper positioning of the handgrip dynamometer will be determined for forearm exercise. The proper position of a transcranial doppler ultrasound device will also be determined.

Visit 2 & 3 (Study Day): ~3.5 hours. On these study days you will receive either a Fluconazole or a placebo in pill form. You will not be told on which visit you will receive Fluconazole and which day you will receive the placebo. After ~1.5 hours you will be asked to lie down. Next, we will setup and attach multiple pieces of equipment which measure several cardiovascular variables. All pieces of equipment used are described in the list below under "Instrumentation". You will rest for 30 minutes while all pieces of equipment are setup to start the experiment. After the rest period, a lower body negative pressure (LBNP) machine will be activated for 5 minutes. LBNP will cause a redistribution of your blood from your upper body to the lower body as well as an increase in heart beats per minute. You will then rest for 10 more minutes after which, you will start handgrip exercise at 20% of your maximal grip strength. You will squeeze a handgrip exercise device for 1 second and relax for 2 seconds in time with a metronome. You continue this exercise for 7 minutes. 5 minutes into the exercise an investigator will start the LBNP for 2 minutes. You will continue to exercise during the LBNP exposure for the remaining 2 minutes. After exercise is completed, you will rest for 10 minutes. Then a blood pressure cuff will inflate around the arm that you were exercising for 5 minutes. After 5 minutes, the cuff will deflate, and recovery measurements will be collected - after which, the visits end.

Roughly 24 hours after the 2nd and 3rd visit, but no later than 48 hours, you will be contacted by us with an email. We will ask you to fill out questions about any changes in your medical status and how you are feeling. We will also give you our contact information and instructions in case of an emergency.

Instrumentation: These are the devices what we will place on you. All of these devices are non-invasive.

- Body Composition: A Dual-energy X-ray absorptiometry (DEXA) scan of your whole body will use low dose X-rays to determine the amount of bone, fat, and muscle your body has.
- End-Tidal Carbon Dioxide ($EtCO_2$): The amount of carbon dioxide in your breath, while you breathe out will be measured using a mask and recorded using a metabolic cart.
- Forearm and Brain Oxygenation: The amount of oxygen in your forearm muscle and brain during the experiment will be measured via Near-Infrared Spectroscopy (NIRS). The NIRS device is able to

detect the amount of oxygen in your blood via near infrared light. We will place probes on your forehead and forearm secured with a tensor bandage.

- Heart Rate (HR): Heart beats per minute will be measured with a heart rate monitor that is you strap on your upper body directly on your skin.
- Blood Pressure: Is measured with a finger cuff. A larger blood pressure cuff is placed on both of your upper arm and small blood pressure cuff on your middle finger which is connected to monitor that is strapped on your wrist.
- Blood Oxygenation: The amount of oxygen in your blood is measured via infrared light with a clip on your ear.
- Brain and arm blood flow: will be measured using ultrasound on your temples and on the upper portion of your non-dominate arm.
- Maximal Voluntary Contraction (MVC): To determine your forearm muscle grip strength we will use a handgrip exercise device. Using your non-dominate hand you will squeeze the handgrip exercise device as hard as you can 3 separate times.
- Handgrip Exercise: Using your non-dominant hand at 20% of your maximal grip strength, A 1:2s cycle of squeezing the handgrip exercise device and relaxation will be used, for 7 minutes.
- Forearm Occlusion: using an inflation cuff on the nondominant hand, blood flow will be restricted to the forearm for 5 minutes.
- Lower Body Negative Pressure (LBNP): Lower Body (up to the upper part of your hips) is inserted in a sealed chamber connected to a vacuum. The device when activated provides suction at -20 mmHg for 2-5 minutes.
- Pregnancy Test: Female participants will take a commercially available pregnancy test prior to participation in any experimental visit.
- Placebo: 250 mg capsule of microcrystalline Cellulose (name: Zeebo relief). Microcrystalline Cellulose should not have an effect on blood flow measurements,
- Fluconazole: 150mg will be given in pill form.
-

CAN I WITHDRAW FROM THE STUDY?

You can stop participating in this study at any time. To withdraw from the study, simply inform one of the researchers of your decision.

There may be circumstances under which your participation may be terminated by the investigator without your consent.

- He/She feels that it is in your medical best interest.
- Your condition worsens.
- New information becomes available.
- You fail to follow study requirements.
- .

WHAT ARE THE RISKS OF THE STUDY?

In addition to the risks described in the Key Information section, you may also be at risk for these side effectsYou should discuss these with the researcher and/or your regular doctor. Many side effects go away shortly after the fluconazole/fasting/exercise/LBNP are stopped. The treatment or procedure may involve risks that are currently unforeseeable.

Risks and side effects related to Fluconazole and the procedures we are studying include:

This experiment is non-invasive. Therefore, we do not anticipate any risk or benefit to you personally. However, nothing is without risk, there is a small chance that you may have an adverse event. Some risks associated with the study may include:

a. Fluconazole: side effects to Fluconazole are rare but include headache, nausea, adnominal pain, skin rash, vomiting and diarrhea. Interactions with other medications you are taking could occur if other prescription drugs are being taken simultaneously. Other severe side effects could include elevated liver enzyme levels, liver damage, and the development of microbial resistance, meaning the effectiveness of the drug to fight a fungus might decrease. Most people recover from these side effects once they stop taking fluconazole. Considering you will only be taking Fluconazole one time, the chance you will experience these are small.
b. 8-hour fast: Side effects of an 8-hour fast include feeling hungry or weak. To reduce this risk, we will encourage you to eat a substantial meal prior to the fast, drink plenty of water while fasting. If you feel sign/symptoms such as tiredness, headache, stomachache, unsteadiness, anxiety, etc. that interfere with daily living, subjects should discontinue the fast. To avoid these sensations, we recommend fasting after a night-time meal and visiting the lab in the early morning.
c. Body size and composition Measurements: There are no risks to measuring height, weight, heart beats per minute, etc., which are measurements called anthropometrics. Measuring blood pressure with a forearm cuff and finger cuff may cause a temporary increase in pressure at the area of skin below the cuff, however, this discomfort is temporary and subsides when the cuff pressure is reduced.
d. Abstaining from exercise, caffeine, Non-steroidal anti-inflammatory drugs (NSAIDs): These poses minimal risk to you. Not using any caffeine may result in irritability and headache. Not exercising for 24 hours leading up to the experiment has no risk. NSAIDs include any drugs and that are designed to reduce pain and/or inflammation, for example Aspirin, Tylenol, Advil, Aleve etc. There is no risk from not using NSAIDs considering regular use of NSAIDS for a clinical condition would exclude you from taking part in the study.
e. DEXA Scan: "If you participate in the research, you will receive a DXA scan, a type of x ray procedure. The DXA scan will be performed for research purposes only, and is not required for your medical care. The amount of additional radiation to which you will be exposed is approximately the amount that you receive in one day from natural, background sources of radiation. The risk of radiation exposure is cumulative over your lifetime
f. NIRS: A NIRS will be used to measure the amount of oxygen in your brain and muscle. It is a non-invasive device that continuously monitors the amount of oxygen in your tissues safely. It is secured on your forehead and forearm with a tensor bandage. This may feel tight on your forehead and arm.
g. Blood pressure monitoring: We will attach a non-invasive, automatic blood pressure cuff around the upper arm and finger. Around the finger a near-infrared light is used to measure changes in pressure during each heartbeat. When both the cuffs are inflated, it may feel uncomfortable, but this is temporary. These blood pressure measures are considered very safe.
h. Total Liable Signal determination: Uses a non-invasive, automatic blood pressure cuff around the upper arm. When the cuff is inflated it may feel uncomfortable, but this is temporary. It will be inflated for 5 minutes then released. While inflated, there may be some feeling some numbness and tingling in your hands. Once released those sensations will subside quickly. These procedures are considered very safe.
i. Lower Body Negative Pressure (LBNP): LBNP will be used to increase the production of the stress hormone norepinephrine. More norepinephrine causes an increase in heart beats per minute. The device will be adjusted to -20mmHg for 5 minutes during resting measurements and for 2 minutes in while you are doing handgrip exercise. Minor risk at this level of LBNP could include an increase heart rate, tightness around the lower abdomen, dizziness or the sensation of getting close to passing out or actually pass out, and slight discomfort around the legs.

j. Breach of Confidentiality: Personal information such as name, gender, date of birth, and medications will be stored in a locked file cabinet in the HCRL laboratory. Study records will be coded with a number and only study personnel will have access to the link connecting your name to the collected data. After the study is complete, we will remove all identifying information so that study data is coded during analysis and publication. Your information will be coded to remove any personal identifiers during data analysis or research publications.

We do not anticipate that there will be any direct benefits to you for participating.

For more information about risks and side effects, ask the researcher.

TO WHAT EXTENT WILL MY INFORMATION BE KEPT CONFIDENTIAL?

Efforts will be made to keep your personal information confidential. You will not be identifiable by name or description in any reports or publications about this study. We cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. You will be asked to sign a separate authorization form for use or sharing of your protected health information.

There are organizations outside the OUHSC that may inspect and/or copy your research records for quality assurance and data analysis. These organizations may include the US Food & Drug Administration and other regulatory agencies. The OUHSC Human Research Participant Program office, the OUHSC Institutional Review Board, OUHSC Office of Compliance, and other University administrative offices may also inspect and/or copy your research records for these purposes.

Identifiable Private Information:

Your information may be used for future studies without your additional consent. We will remove direct identifiers from your information and assign a code. The key to this code will be kept separately and only the researcher for this study will have access to the code. If your information is shared with another investigator for research purposes, they will not have access to the key code and will not be able to re-identify you.

WHAT IF I AM INJURED OR BECOME ILL WHILE PARTICIPATING IN THIS STUDY?

In the case of injury or illness results from this study, emergency medical treatment is available.

Emergency medical treatment should be sought at the nearest medical center and the study P.I, J. Mikhail Kellawan should be contacted immediately (405-325-9028, kellawan@ou.edu).

You or your insurance may be charged for this treatment.

Complications arising as a result of the natural progression of an underlying or pre-existing condition will be billed to you or your insurance. Please check with the investigator or with your insurance company if you have questions.
No other funds have been set aside by the University of Oklahoma Health Sciences Center, University of Oklahoma – Norman Campus to compensate you in the event of injury, illness, or for other damages related to your event of injury or illness.

WHAT ARE MY RIGHTS AS A PARTICIPANT?
Taking part in this study is voluntary. You may choose not to participate. Refusal to participate will involve no penalty or loss of benefits to which you are otherwise entitled.

If you agree to participate and then decide against it, you can withdraw for any reason and leave the study at any time. You may discontinue your participation at any time without penalty or loss of benefits to which you are otherwise entitled.

You have the right to access the medical information that has been collected about you as a part of this research study. However, you may not have access to this medical information until the entire research study has completely finished. You consent to this temporary restriction.

DO I HAVE ANY OTHER RIGHTS OVER MY DATA?
Depending on where the sponsor for your study is located and other factors, you may have additional rights over your personal data collected in this study. For example, the European Union General Data Protection Regulation (GDPR) and some state privacy laws might apply. If the GDPR applies, generally you may have the following rights:

1. The right to request the information collected to be corrected.
2. The right to withdraw your consent for the use of your personal information at any time.
3. The right, in some circumstances, to receive your personal information in a structured, commonly used, and machine-readable format and the right to provide your information to a third party.
4. The right to strict confidentiality of your personal data when it is used/shared.
5. The right to limit the use/sharing of your personal information in certain circumstances.
6. The right under some circumstances to request the erasure of your personal data.
7. The right to file a complaint with a privacy protection regulator if you believe any of the rights above have been violated.

You can receive more information regarding these rights in the Privacy Notice for Research Participants, located on the OUHSC Office of Human Research Participant Protection (HRPP) website at *https://compliance.ouhsc.edu/HRPP/Participant/Privacy-Notice*.

If you have any questions and requests, please contact the HRPP Office at 405-271-2045.

WHOM DO I CALL IF I HAVE QUESTIONS, SUGGESTIONS, OR CONCERNS?

If you have questions, concerns, or complaints about the study or have a research-related injury, contact *J. Mikhail Kellawan* at *405-325-9028.*

If you cannot reach the Investigator or wish to speak to someone other than the investigator and for questions about your rights as a research participant, contact the OUHSC Director, Office of Human Research Participant Protection, at 405-271-2045.

SIGNATURE:

By signing this form, you are agreeing to participate in this research study under the conditions described. You have not given up any of your legal rights or released any individual or entity from liability for negligence. You have been given an opportunity to ask questions. You will be given a copy of this consent document.

I agree to participate in this study:

PARTICIPANT SIGNATURE (age ≥18)	**Printed Name**	**Date**
SIGNATURE OF PERSON OBTAINING CONSENT	**Printed Name**	**Date**

Subject ID: _____ Sex: M / W Date: / /

Medical History (General)

Body System	Have you ever had any conditions affecting these body systems?	*Only complete if 'Yes' for Diagnosed Condition*			
		Diagnosis/Condition/Surgery	Onset Date	Is it a current problem?	Are you currently taking a prescribed medication?*
Cardiovascular					
• Heart Attack	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
• Stroke	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
• Hypertension	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
• Coronary Artery Disease	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
• Other Cardiovascular	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
• Your 1st Degree Relatives (e.g. mother, brother, daughter)	☐ Yes ☐ No	List family members, their diagnosis, and approximately when they were diagnosed:			
Lungs					
• Asthma	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
• Exercise-Induced Bronchospasm	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
• Obstructive Lung Disease	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
• Other	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Musculoskeletal					
• Knee	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
• Hips	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
• Back					
• Other					
Head/Eyes/Ears/ Nose/Throat/Neck	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Endocrine/Metabolic					
Diabetes	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No

High blood sugar	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Liver	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Thyroid	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Kidney	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Pituitary Gland	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Neurological					
Nerve or Neurologic disorders	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Autonomic Neurological disorders	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Other	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Immunological					
Autoimmune disease	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Other	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Psychological					
Clinical depression	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Other	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No

Allergies	☐ Yes ☐ No			☐ Yes ☐ No	☐ Yes ☐ No
Tobacco Use					
Ever Smoked or used tobacco products (smoke, smokeless, vapor	☐ Yes ☐ No	For how long? (years): ___		*If no, when did you quit? _____/ _____/ _____	

ADDITIONAL NOTES: _____
_____
_____

Additional Questions (all subjects)

Have you recently experienced any of the following?	Yes	No	When?
Pain in the neck, jaw, or arms?			
Dizziness or fainting?			
Swelling in the ankles?			
Rapid heart rate while at rest?			
Leg pain or cramping while walking, relieved with rest?			
Has a doctor ever told you that you have a heart murmur?			
Unusual fatigue with usual activities?			

Additional COVID-19 Questions (all subjects)

Have you recently experienced any of the following in the 14 days?	Yes	No	When?
Fever or Chills?			
Cough?			
Shortness of Breath or Difficulty Breathing?			
Unusual fatigue with usual activities?			
Muscle of body aches?			
Headache?			
Sore Throat?			
Congestion or runny nose?			
Nausea or vomiting?			
Diarrhea?			
If you have answered YES to any of the above, **have you been Tested for COVID-19?**			

Have you experienced any of the following?	Yes	No	When?
Reason to believe you have had been infected with COVID-19?			
Received a positive test or confirmed diagnosis of COVID-19?			
Been hospitalized for COVID-19?			
Been Infected with COVID-19 and experienced symptoms?			
Been Infected with COVID-19 and experienced **NO** symptoms (Asymptomatic)?			
If you have had a confirmed case of COVID-19, did you experience any of the following symptoms:			
Fever or Chills?			
Cough?			
Shortness of Breath or Difficulty Breathing?			
Unusual fatigue with usual activities?			
Muscle of body aches?			
Sore Throat?			
Congestion or runny nose?			
Nausea or vomiting?			
Diarrhea?			
Have you recovered from your COVID-19 infection? (if applicable)			
Has a medical professional told you that you have you recovered from your COVID-19 infection? (if applicable)			

Please list all Medications or Supplements You Take

Medications/Supplements	
Are you currently taking Amiodarone?	☐ Yes ☐ No
Are you currently taking Sulphaphenazole?	☐ Yes ☐ No
Are you currently taking S-warfarin?	☐ Yes ☐ No
Are you currently taking Tolbutamine?	☐ Yes ☐ No
Are you currently taking Phenytoin?	☐ Yes ☐ No
Are you currently taking Lonafarnib?	☐ Yes ☐ No
Are you taking **ANY** Prescribed medications? (list below):	
Are you taking hormone replacement (e.g., estrogen) therapy?	
Do you take over the counter medications &/or supplements (aspirin, vitamins, etc.)?	

Do you have any reason you believe you should not participate in this research study? ☐ Yes ☐ No

Explain:

Are you currently enrolled in any other research studies or have you participated in any other research studies in the past 30 days? ☐ Yes ☐ No

If yes, when was your last study visit (MM/DD/YYYY)?

If yes, what is the date of your next visit (MM/DD/YYYY)?

Female Subjects Only ☐ N/A - subject is male

Subject currently pregnant? ☐ Yes ☐ No
Subject plans to become pregnant? ☐ Yes ☐ No

Currently using birth control? ☐ Yes ☐ No

If yes, method of birth control *[Select All That Apply]:*

☐[1] Oral Contraceptives	☐[5] NuvaRing	☐[10] Post-menopausal for ≥ 1 year
☐[2] Hormonal Injections	☐[6] Intrauterine device	☐[11] Tubal ligation, bilateral oophorectomy, or hysterectomy
☐[3] Hormonal Implants (i.e. Implanon)	☐[7] Hormonal Intrauterine device	☐[12] Abstinence
☐[4] Contraceptive Patches	☐[8] Non-hormonal Barrier method	☐[13] Other (specify in Reproductive field)
	☐[9] Spermicide	
Start Date of Birth Control (MM/DD/YY):	Brand Name: _____	

What is the date do you expect you next period? _____

Do you have a regular menstrual cycle (last 3 cycles consecutive)? ☐ Yes ☐ No

Past Menstrual History

Start Date of LAST menstrual cycle (MM/DD/YY):_____

End Date of LAST menstrual cycle (MM/DD/YY):_____

Have you ever consulted a doctor about menstrual problems (specifically, about irregular or missing periods)?

_____

Have you ever consulted a doctor about any problems relating to your hormonal system? If so, please explain.

_____

For HCRL Staff Only

Form Verified by:_____ **Date:** _____

COLLEGE OF ARTS AND SCIENCES
DEPARTMENT OF HEALTH AND EXERCISE SCIENCE
The UNIVERSITY of OKLAHOMA

Endothelial Derived Hyperpolarization Factor and regulation of cerebral and muscle blood flow.

Are you interested in helping us determine how humans control blood flow to their muscles and brains?

The purpose of this experiment is to determine if acute inhibition of Endothelial Derived Hyperpolarization Factor (EDHF) alters muscle and brain vascular responses to Lower Body Negative Pressure at rest and during dynamic handgrip exercise.

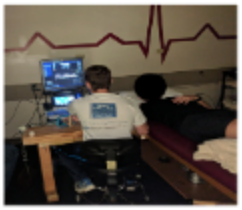
What is EDHF? EDHF is a proposed substance which contributes to the relaxation of smooth muscle, causing an increase in vessel diameter.

Time commitment: 3 visits (~12 hours total)

What will be measured: Brain and Muscle Oxygenation, Brain and Muscle blood flow, Blood Pressure, Heart Rate, Breathing Rate. ***All measures are noninvasive***.

Eligibility: Healthy women* and men between the ages of 18-30 years who do not take cardiometabolic medication, are not allergic to fluconazole, and do not use nicotine/tobacco products.

**Women need to have a regular menstrual cycle*



Compensation: A gift card valued at **$50** for Amazon.com will be given for completing **all** study requirements.

If interested, please contact:

Alexander Buelow: abuelow@ou.edu : Jacob Matney: matney@ou.edu

Principal Investigator: Dr. Mikhail Kellawan; kellawan@ou.edu; (405) 325-9028

The University of Oklahoma is an equal opportunity institution, IRB #14056

Scan QR Code to email!

IRB NUMBER: 14056

Post Visit Instructions and Emergency Contact Information

Non-Emergency Study Contact Information

If you have any questions or concerns, please contact:

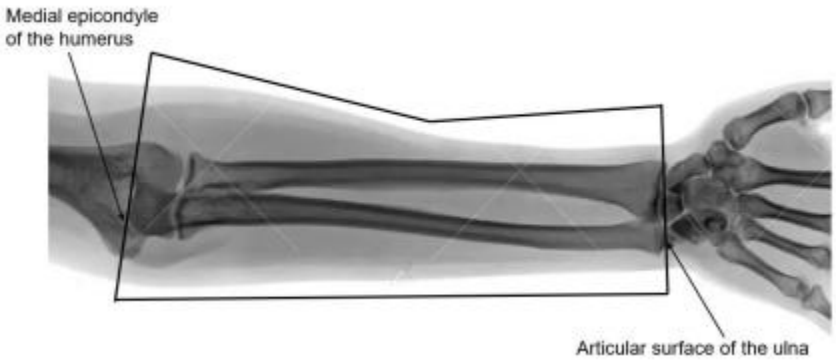
During Business Hours (8:00 to 5:00, M-F)
HCRL Staff
hcrl@groups.ou.edu

Dr. J. Mikhail Kellawan, PhD
Lab Director
kellawan@ou.edu
405-325-9028 (office)

24/7 Emergency Contact Information

In the event of an emergency, please **call 911 or go to the Emergency room**. Wait to contact study staff until after you have sought medical attention to report the emergency.

Anatomical Landmarks – Forearm ROI



Raw Data

PLA	Rest									
Sub_ID	Velocity	Diameter	FBF	MAP	FVC	CO	SV	TPR	HR	
101	1.85	4.12	14.79818007	101.5	14.5793523	4.46635667	79.803555	142.099	53.1895817	
103	4.37	2.92	17.55857582	95.02215833	18.4784014	6.2799725	90.7306808	82.9733333	62.5668025	
105	5.95	4.26	50.88365639	103.9677233	48.9417819	4.71850667	85.1583717	127.6535	53.6887317	
107	2.34	3.82	16.09106283	112.6621392	14.2825824	7.3388478	105.098788	84.2738409	64.5275098	
201	1.7	2.88	6.644710195	88.15942167	7.53715266	5.12408667	72.5752375	114.891417	68.104665	
202	3.42	3.44	19.07151257	110.2496792	17.2984744	4.47461833	66.9790167	171.175917	64.2600867	
205	7.66	3.05	33.57922	99.98774944	33.58333	6.23782084	96.0195384	89.7095286	63.2567389	
207	6.3	3.31	32.52660115	102.0190408	31.8828729	6.7700775	83.37504	95.9044167	77.6498767	
209	3.7	2.94	15.07087724	107.6034695	14.0059399	7.681321	101.180835	91.05525	75.4200075	
108	6.38	4.75	67.83440895	95.6953425	70.8857998	6.861395	103.86302	84.6133333	65.2851517	
206	2	3.17	9.470887272	91.7061	11.2559108	4.66547667	78.9304167	105.480667	56.185955	
106	1.26	4.05	9.739187766	101.51737	9.5936171	5.26249333	83.6725633	116.521	60.0689933	
210	4.54	2.73	15.94495	88.81676	17.95264	4.71620292	77.3334311	134.066698	58.0872629	
	3.959230769	3.495384615	23.78567925	99.91591953	23.8675273	5.7382443	86.5169611	110.801377	63.2531818	
n=13	2.022213036	0.596719621	17.82962249	7.020108485	18.1939303	1.12280516	11.7642183	26.0798219	7.28403947	

LBNP								
Velocity	Diameter	FBF	MAP	FVC	CO	SV	TPR	HR
1.9	4	14.325696	98.5999783	14.5291066	4.317125	71.6957033	145.7795	57.64172
3.07	2.67	10.3134281	97.7673683	10.5489472	5.30886	71.1931967	100.193667	70.6994558
2.31	4.09	22.2299186	103.125845	21.556108	3.94936	74.7706867	152.282	50.5791183
2.33	3.76	15.5229321	121.120292	12.8161284	5.44260667	100.952698	118.014	36.857435
1.07	2.82	4.0098132	84.455905	4.74781865	5.04831	68.687885	111.011083	70.9659917
1.46	3.31	7.53791074	102.834282	7.33015355	3.91971333	67.7241533	183.591	54.283955
5.98	3.1	27.08113	99.9125459	27.10483	5.04001984	79.7919546	108.830345	60.26529
2.91	3.13	13.4345713	105.662942	12.7145535	5.45940917	71.3771208	120.7195	72.94529
2.75	2.96	11.3542451	108.480085	10.4666631	108.480085	74.8841563	84.3600605	79.6221084
4.4	4.61	44.0652943	95.9405742	45.9297796	6.4035625	85.3235683	90.7906667	74.770495
1.82	3	7.7189112	91.1554593	8.88528288	4.82438333	79.23703	105.779333	59.0280667
0.79	4.03	6.04615576	100.483202	6.01708112	5.0539375	70.29769	122.013	69.2082867
4.07	2.69	13.87845	89.87386	15.44215	4.30529224	67.6645144	144.465795	60.2130127
2.68153846	3.39769231	15.1937274	99.9547952	15.2375848	12.8886665	75.661566	122.140765	62.852325
1.38930113	0.59034359	10.7625871	8.67841777	11.098689	28.6138665	8.9133099	26.8972842	11.6926406

Ex								
Velocity	Diameter	FBF	MAP	FVC	CO	SV	TPR	HR
37.14	4.47	349.7027866	135.1617795	258.72905	7.5202145	81.891189	106.42195	89.021708
53.92	3.17	255.3351209	117.1709127	217.91681	7.1666995	89.653168	92.649467	80.825091
37	4.4	337.5586368	125.3784602	269.23176	5.1718519	74.089765	139.9737	66.679358
21.99	4.43	203.3643529	146.94052	138.3991	10.038515	122.25475	78.47125	72.661035
21.07	3.09	94.80334079	103.970025	91.183339	5.1885017	65.579086	128.82208	76.795129
27.14	3.58	163.9147531	124.98421	131.14837	4.2292192	58.1172	200.68817	70.336834
23.39	2.91	93.33797	122.3312051	76.29939	7.4283019	91.321076	92.34687	77.437471
32.23	3.68	205.6828542	116.628196	176.35774	6.7944935	72.364601	104.84935	90.310086
29.29	3.23	144.00135	118.9334985	121.0772	7.7982105	88.473171	96.7308	85.491283
32.55	4.67	334.5237075	96.3954925	347.03252	7.0856975	104.83856	85.6755	65.775643
40.23	3.32	208.9624961	113.2758258	218.00037	5.0900567	80.59967	118.91533	61.919864
25.91	3.86	181.9215592	121.322995	149.94813	5.5078183	78.561393	132.28175	67.265593
28.7	2.81	106.7915	101.4534	105.2617	4.6922216	74.61811	150.48683	60.09373
31.58153846	3.663076923	206.1461868	118.7651169	176.96811	6.4393694	83.258595	117.56254	74.200987
8.951077992	0.593400628	85.30658969	12.56969184	77.452015	1.5413594	16.562167	31.869737	9.1054761

LBNP								
Velocity	Diameter	FBF	MAP	FVC	CO	SV	TPR	HR
39.26	4.42	361.440639	136.860157	264.0948592	7.5202145	81.8911885	106.42195	89.021708
56.56	3.11	257.793716	118.014725	218.4419925	5.822437167	70.28887367	115.73025	81.70687283
37.16	4.38	335.943369	130.896506	256.6480803	4.585805833	71.4660925	168.2313333	61.56491
21.07	4.55	205.555677	148.557148	138.3680823	6.924973333	106.7460567	113.4786667	25.806705
26.09	3.02	112.132141	100.517387	111.5549697	4.799230667	58.34264167	136.7505667	78.84412933
33.12	3.51	192.285576	119.893444	160.3803925	4.005698333	58.88497667	205.0580833	65.6600025
30.32	2.87	117.6888	125.221335	93.98462	6.351296702	68.71565308	108.9003017	89.57323711
38.88	3.42	214.299233	119.113504	179.9117858	5.608911333	55.41001483	131.4455833	98.55079667
32.08	3.14	149.051312	122.431287	121.7428287	5.581734545	51.99339636	136.8751212	104.8459497
26.77	4.73	282.236254	100.03247	282.1446421	8.2216645	92.992678	77.84985	89.264842
40.16	3.32	208.598902	112.659173	211.3162247	5.145703333	76.86007833	119.1136667	65.43657917
23.69	3.88	168.062438	125.577826	133.83	5.5164615	64.1803865	134.782	81.366759
32.8	2.63	106.9123	99.04606	107.942	4.291857973	68.06449065	158.2523319	60.39891157
33.68923077	3.613846154	208.615412	119.909309	175.4123444	5.721229979	71.21819442	131.7607465	76.31087714
9.192478205	0.636938751	76.1697447	13.1880894	61.61245184	1.178347695	15.47947575	30.99274276	20.11203682

Delta Rest to LBNP									Delta Ex to Ex wit LBNP				
Velocity	Diameter	MAP	FBF	FVC	CO	SV	TPR	HR	Velocity	Diameter	FBF	MAP	FVC
0.05	-0.12	2.900021667	-0.47248407	-0.05024562	-0.149231667	-8.107851667	3.6805	4.452138333	2.12	-0.05	11.7378523	-1.6983775	5.3658103
-1.3	-0.25	-2.74521	-7.24514771	-7.92945414	-0.9711125	-19.53748417	17.22033333	8.132653333	2.64	-0.06	2.4585948	-0.843811833	0.5251776
-3.64	-0.17	0.841878333	-28.65373783	-27.38567386	-0.769146667	-10.387685	24.6285	-3.109613333	0.16	-0.02	-1.6152675	-5.518045648	-12.5836811
-0.01	-0.06	-8.4581525	-0.56813072	-1.46645396	-1.896241136	-4.146089394	33.74015909	-27.67007477	-0.92	0.12	2.1913244	-1.616628333	-0.0310148
-0.63	-0.06	3.703516667	-2.634896991	-2.789334015	-0.075776667	-3.8873525	-3.880333333	2.861326667	5.02	-0.07	17.32879971	3.452638167	20.37163028
-1.96	-0.13	7.4153975	-11.53360183	-9.968320895	-0.554905	0.745136667	12.41508333	-9.976131667	5.98	-0.07	28.3708233	5.090765833	29.2320233
-1.68	0.05	0.075203494	-6.49809	-6.4785	-1.197801003	-16.22758383	19.1208167	-2.991448945	6.93	-0.04	24.35083	-2.890129679	17.68523
-3.39	-0.18	-3.643900833	-19.09202989	-19.16831941	-1.310668333	-11.99791917	24.81508333	-4.704586667	6.65	-0.26	8.6163783	-2.485308167	3.5540428
-0.95	0.02	-0.876615833	-3.71663218	-3.53927678	100.7987643	-26.29667869	-6.695189484	4.202100911	2.79	-0.09	5.049962	-3.49778847	0.6656285
-1.98	-0.14	-0.245231667	-23.76911469	-24.95602025	-0.4578325	-18.53945167	6.177333333	9.485343333	-5.78	0.06	-52.28745302	-3.6369775	-64.88787862
-0.18	-0.17	0.550640741	-1.751976072	-2.370627885	0.158906667	0.306613333	0.298666667	2.842111667	-0.07	0	-0.3635937	0.616653333	-6.6841433
-0.47	-0.02	1.034168333	-3.69303201	-3.576535979	-0.208555833	-13.37487333	5.492	9.139293333	-2.22	0.02	-13.8591212	-4.2548305	-16.1181275
-0.47	-0.04	-1.0571	-2.0665	-2.51049	-0.410910675	-9.668916709	10.39909746	2.125749845	4.1	-0.18	0.1208	2.40734	2.6803
-1.277692308	-0.097692308	-0.0388757	-8.591951846	-8.629942523	7.150422232	-10.85539509	11.33938849	-0.400856766	2.107692308	-0.049230769	2.469225338	-1.144192331	-1.555769426
1.162551265	0.083955469	3.653052965	9.033499722	8.872621217	27.03955221	7.702531189	11.62043153	9.614572989	3.623950572	0.093188109	19.20750775	3.060253923	21.96746027

FLZ	Rest									L
Sub_ID	Velocity	Diameter	FBF	MAP	FVC	CO	SV	TPR	HR	V
101	3.0631	3.93	22.29402	110.6899	20.14097	3.872792	66.03573	222.687	55.69627	
103	6.49	3.1	29.39072	96.68986	30.3969	6.012426	85.25363	102.2044	67.4558	
105	2.75	4.05	21.25616	94.18773	22.56787	4.111537	71.45594	151.8188	54.42575	
107	6.13	3.76	40.8393	115.5944	35.32982	6.712191	114.0807	90.37925	57.71842	
201	2.48	3.02	10.65879	96.23579	11.0757	4.784357	66.37975	112.2018	69.76758	
202	6.96	3.17	32.95869	113.7172	28.98303	4.574515	68.85667	167.8999	65.73199	
205	6.78	2.82	25.40798	97.88324	25.95743	5.609815	90.21833	114.2865	59.41029	
207	4.55	3.06	20.07689	108.5877	18.4891	5.938528	82.74968	106.8778	69.46759	
209	5.17	3.05	22.66378	98.18968	23.08163	5.521159	81.77522	110.5111	65.43405	
108	6.83	4.86	76.02132	89.51841	84.92255	9.226123	133.7163	64.36576	68.89384	
206	4.17	3.49	23.93476	91.7061	26.09942	3.743847	61.73335	154.0787	58.03056	
106	3.21	3.89	22.89003	96.79319	23.64839	5.207463	86.29383	119.2232	58.75643	
210	6.11	2.87	23.72579	94.16648	25.19558	4.716203	77.33343	134.0667	58.08726	
159.2308	4.976392308	3.466923	28.62448	100.3046	28.91449	5.386997	83.52942	126.9693	62.22122	
50.06662	1.625982222	0.574943	15.2668	8.356465	17.13449	1.397335	19.58374	38.45556	5.422226	

LBNP

Velocity	Diameter	FBF	MAP	FVC	CO	SV	TPR	HR
2.1702	3.87	15.3166517	107.5082	13.52849	3.53932	57.3871	246.2943	57.90138
2.81	2.96	11.60197404	101.453	11.43581	4.959674	65.0412	127.8703	72.70158
1.83	3.99	13.7290039	95.46575	14.38108	4.021831	60.30299	155.1641	64.91468
2.53	3.71	16.41007204	112.4042	14.59916	4.994478	105.0934	122.3583	47.64408
1.51	2.95	6.192458811	99.79104	6.205426	4.967107	57.73498	111.5511	82.95851
4.47	3.35	23.63955432	111.8275	21.1393	3.8288	61.41103	204.4603	57.86447
5.18	2.82	19.41199	97.55367	19.89878	5.009652	87.3171	124.9972	55.37243
2.43	2.91	9.696933089	108.3238	8.951806	5.042553	69.88138	126.6538	69.62604
3.46	2.98	14.47940695	93.49003	15.48765	4.418128	63.6446	135.5431	67.44923
3.79	4.8	41.14943078	85.825	47.94574	8.892127	105.3189	65.38962	76.42861
3.38	3.49	19.4003561	91.15546	21.28659	3.619472	60.2196	156.1157	56.76544
1.62	3.79	10.96568034	101.7524	10.77683	5.15111	68.0833	123.8259	73.12031
6.073631	2.79	22.27917	99.00113	22.50395	4.305292	67.66451	144.4658	60.21301
3.173372	3.416154	17.25174477	100.427	17.54928	4.826888	71.46924	141.8992	64.84306
1.350605	0.575494	8.420797337	7.702879	10.02254	1.300169	16.16712	42.4585	9.573235

Ex

Velocity	Diameter	FBF	MAP	FVC	CO	SV	TPR	HR
25.21	4.48	238.4356	108.504	219.7481	5.292932	69.91484	156.434	73.47262
52.21	3.47	296.2476	116.6525	253.9574	5.995827	78.05537	117.8371	74.83952
50.91	4.42	468.6944	113.3099	413.6396	4.718953	66.04713	150.6808	68.65613
38.08	3.9	272.9407	134.3822	203.1078	4.800275	87.77235	148.544	53.80613
42.76	3.28	216.7842	118.4105	183.0785	4.702668	54.40495	139.7986	82.2889
42.02	3.51	195.4	124.8029	195.4734	4.679596	58.58829	180.8472	75.24017
28.3	2.92	113.7089	119.5638	95.10311	6.195428	83.98173	121.9171	72.70717
52.96	3.04	230.6414	117.8156	195.7647	6.877348	73.23717	99.8376	90.82139
34.7	3.21	168.4929	110.213	152.8793	6.120326	80.97593	112.9956	72.72005
22.32	4.91	253.5708	95.92963	264.3301	9.050596	121.4744	68.32402	74.37317
25.89	2.92	104.0255	113.2758	91.83382	4.311545	62.59898	163.3695	67.58438
24.9	3.79	168.5466	115.0216	146.5347	5.933282	76.06989	122.4817	74.94528
39.97	2.55	122.4776	107.5012	113.9313	4.692222	74.61811	150.4868	60.09373
36.94077	3.569231	219.2282	115.0294	194.5678	5.643923	75.97993	133.3503	72.42682
10.57283	0.673515	92.90695	8.795659	82.37925	1.241984	16.15771	28.83872	8.758507

LBNP								
Velocity	Diameter	FBF	MAP	FVC	CO	SV	TPR	HR
30.07	4.28	259.5751	110.4416	235.0339	4.874179	66.38961	171.4926	68.88795
46.19	3.4	251.6216	119.7166	210.1811	5.282203	63.29366	136.5837	81.11436
47.15	4.35	420.4384	119.3216	352.3572	3.933963	55.48819	182.4896	72.08359
33.62	3.74	221.6068	130.2156	170.1845	4.736553	86.39117	151.4235	53.71074
35.69	3.16	167.9434	117.0925	143.428	4.482613	47.11626	146.4184	88.93858
42.16	3.51	190.3	128.8054	190.0304	4.197837	47.42909	208.0455	83.96338
34.43	2.93	139.2882	124.601	111.7874	5.411283	73.48512	144.8499	71.82086
47.94	2.97	199.2751	119.8005	166.3392	5.328159	56.29773	131.379	90.81602
34.62	3.16	162.9084	114.8822	141.8047	4.364034	54.43613	161.0882	76.66775
15.8	4.82	172.979	101.6352	170.196	9.67947	108.4382	66.5906	87.8732
26.49	2.31	66.61132	112.6592	59.12641	4.317663	60.55419	162.3709	68.42465
24.89	3.74	164.0628	118.5435	138.3989	5.268118	58.15184	142.5453	87.8781
37.3	2.6	118.8222	107.9725	110.0486	4.291858	68.06449	158.2523	60.39891
35.10385	3.459231	195.0333	117.3606	169.1474	5.089841	65.04121	151.0407	76.35216
9.06016	0.692825	82.06057	7.731176	68.6568	1.40672	16.18093	31.37565	11.22622

Delta Rest to LBNP					Delta Ex to Ex wit LBNP				
Velocity	Diameter	FBF	MAP	FVC	Velocity	Diameter	FBF	MAP	FVC
-0.8929	-0.06	-6.97737	3.181743	-6.61248	4.86	-0.2	21.13959	-1.93755	15.28578
-3.68	-0.14	-17.7887	-4.76317	-18.9611	-6.02	-0.07	-44.626	-3.06413	-43.7763
-0.92	-0.06	-7.52716	-1.27802	-8.18679	-3.76	-0.07	-48.256	-6.01177	-61.2823
-3.6	-0.05	-24.4292	3.190202	-20.7307	-4.46	-0.16	-51.3339	4.166553	-32.9233
-0.97	-0.07	-4.46633	-3.55525	-4.87027	-7.07	-0.12	-48.8408	1.318034	-39.6505
-2.49	0.18	-9.31913	1.889649	-7.84373	0.14	0	-5.1	-4.00246	-5.44305
-1.6	0	-5.99599	0.329577	-6.05865	6.13	0.01	25.5793	-5.03729	16.68429
-2.12	-0.15	-10.38	0.263928	-9.5373	-5.02	-0.07	-31.3663	-1.98483	-29.4255
-1.71	-0.07	-8.18437	4.699649	-7.59398	-0.08	-0.05	-5.58457	-4.66914	-11.0746
-3.04	-0.06	-34.8719	3.693412	-36.9768	-6.52	-0.09	-80.5919	-5.70554	-94.1341
-0.79	0	-4.5344	0.550641	-4.81283	0.6	-0.61	-37.4142	0.616653	-32.7074
-1.59	-0.1	-11.9244	-4.95921	-12.8716	-0.01	-0.05	-4.48372	-3.52186	-8.1358
-0.03637	-0.08	-1.44662	-4.83465	-2.69163	-2.67	0.05	-3.6554	-0.4713	-3.8827
-1.80302	-0.05077	-11.3727	-0.12242	-11.3652	-1.836923077	-0.11	-24.1949	-2.33112	-25.4204
1.000752	0.081428	8.79767	3.172266	9.005995	4.203760469	0.157444876	30.74645	2.981667	30.23149

%change	FBF_Rest_LBNP_PLA	FBF_Rest_LBNP_FLZ	FBF_rest_Ex_PLA	FBF_rest_Ex_FLZ	FBF_Ex_Ex_L	FBF_Ex_Ex_LBN
	PLA	FLZ	PLA	FLZ	PLA	FLZ
101	-3.192852552	-31.2970456	2263.147258	969.504454	3.35652238	8.86595647
103	-41.26272987	-60.52504373	1354.190383	907.962925	0.96288939	-15.0637361
105	-56.31226186	-35.41165733	563.3930436	2104.98122	-0.47851464	-10.2958418
107	-3.530722153	-59.81794203	1163.834186	568.328512	1.07753614	-18.8077158
201	-39.65405433	-41.90277182	1326.749068	1933.8542	18.2786804	-22.5296837
202	-60.47554846	-28.27519552	759.4743207	492.86341	17.3082793	-2.61003071
205	-19.35152157	-23.59884572	177.9634846	347.532232	26.0888789	22.4954247
207	-58.69666431	-51.70101524	532.3527418	1048.79065	4.1891573	-13.5995937
209	-24.66102086	-36.11213059	855.4941475	643.445809	3.50688518	-3.3144239
108	-35.0399083	-45.87119461	393.147523	233.552294	-15.6304178	-31.7827844
206	-18.49854213	-18.9448441	2106.366628	334.621138	-0.1739995	-35.9663638
106	-37.91930188	-52.09407841	1767.933585	636.331758	-7.61818515	-2.66022592
210	-12.96021624	-6.09724692	569.7512379	416.221378	0.11311762	-2.98454575
Mean	-31.65810343	-37.81915474	1064.138277	818.306923	3.92160228	-9.8656588
SD	19.79224591	16.29050356	666.5877834	589.864652	11.0310557	15.906069

	FVC_Rest_LBNP_PLA	FVC_Rest_LBNP_FLZ	FVC_rest_Ex_PLA	FVC_rest_Ex_FLZ	FVC_Ex_Ex_L	FVC_Ex_Ex_LI
	PLA	FLZ	PLA	FLZ	PLA	FLZ
	-0.344635476	-32.83101183	1674.626501	991.050368	2.07391104	6.95604524
	-42.91201374	-62.37837195	1079.30556	735.471395	0.24099912	-17.2376709
	-55.95561257	-36.27631276	450.106169	1732.86935	-4.67392147	-14.8153968
	-10.26742869	-58.67751449	869.0061189	474.890505	-0.02240968	-16.2097792
	-37.00779513	-43.97259823	1109.784962	1552.97447	22.3413953	-21.6576503
	-57.62543356	-27.06319784	658.1499146	574.440945	22.289277	-2.78454914
	-19.29082077	-23.34071593	127.1942359	266.381071	23.1787305	17.5433695
	-60.1210545	-51.58333589	453.1425709	958.811194	2.01524625	-15.0310585
	-25.26982704	-32.90055196	764.4703699	562.341838	0.54975544	-7.24399813
	-35.20595141	-43.54180398	389.5656414	211.260152	-18.6979245	-35.6123249
	-21.0611823	-18.44036715	1836.763472	251.861567	-3.0661156	-35.615868
	-37.2803703	-54.42891643	1462.998877	519.639081	-10.7491356	-5.55213171
	-13.98396002	-10.68294518	486.3299214	352.187646	2.54632027	-3.40793092
Mean	-32.0250835	-38.16289566	873.9572549	706.475352	2.92508678	-11.5899188
SD	18.99353155	15.97216949	529.9909081	484.200709	12.6937761	15.0656603