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DOES EDHF-BLOCKADE BY FLUCONAZOLE ALTER CEREBRAL AUTOREGULATION AND HEMODYNAMICS DURING LOWER-BODY NEGATIVE PRESSURE?

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DOES EDHF-BLOCKADE BY FLUCONAZOLE ALTER CEREBRAL AUTOREGULATION AND HEMODYNAMICS DURING LOWER-BODY NEGATIVE PRESSURE?

A THESIS APPROVED FOR THE

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BY THE COMMITTEE CONSISTING OF

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Table of Contents

List of Tables

Lists of Figures

Abstract

Animal models suggest that cytochrome P450 2C9 (CYP450), an enzyme within the EDHF pathway, plays a critical role in the control of cerebral hemodynamics and autoregulation. However, this observation has not been directly examined in humans. **PURPOSE:** To determine the contribution of EDHF to cerebrovascular hemodynamics in healthy young individuals at rest and during mild simulated hypovolemia via blockade of cytochrome P450. **METHODS:** 16 subjects (9 females, tested only during early follicular phase) participated in 1 familiarization and 2 experimental visits. In experimental visits, participants ingested either a CYP450 inhibitor, fluconazole (FLZ 150mg) or a microcrystalline cellulose placebo (PLA 250mg) in randomized, single-blind, crossover design. Following 120 minutes of supine rest after ingestion middle cerebral artery velocity (MCAv, cm/s, Transcranial Doppler), mean arterial pressure (MAP, mmHg, finger photoplethysmography), prefrontal cortex oxygenation (TSI, %, Near-Infrared Spectroscopy) were continuously measured during 5 minutes of supine rest and 5 minutes of lower-body negative pressure (LBNP, -20mmHg). Cerebrovascular conductance index was calculated (CVCi = MCAv/MAP, cm/s/mmHg). Further, gain, coherence, and phase were determined using transfer function analysis of MCAv and MAP data. **RESULTS:** Resting values for all variables were not different between treatments ($p > 0.05$). Therefore, all data are presented as a change (Δ) from rest to LBNP ± SD. MCAv decreased from rest to LBNP with FLZ ($p = 0.001$) but did not differ between treatments (PLA -3.11 ± 7.04 vs. FLZ -6.61 ± 6.00 cm/s, $p = 0.17$, $d = 0.36$). Similarly, CVCi decreased between rest and LBNP for FLZ ($p = 0.02$), but did not differ between treatments (PLA -0.04 ± 0.09 vs. FLZ -0.06 ± 0.06 cm/s/mmHg, $p = 0.19$, $d = 0.35$). FLZ responses to LBNP were significantly different than zero for both CVCi and MCAv measures (ΔMCAv -6.61 ± 6.00, $p = < 0.001$, $r = 0.622$; ΔCVCi -0.06 ± 0.06, p =

0.001, r = 0.549). TSI was unaltered from rest to LBNP, nor by treatment (PLA 0.03±1.45 vs. FLZ -0.02±1.25 %, p = 0.41, d = 0.213). Transfer function analysis was performed but not interpreted, as coherence values were not consistently above 0.5, which is not an unanticipated result when analysis is applied to healthy young adults. **CONCLUSION:** This study indicates that cytochrome P450 inhibition does not affect middle cerebral artery velocity, CVCi, or prefrontal cortex oxygenation at steady-state rest or during mild-hypovolemic stress. However, EDHF-blockade does appear to alter hemodynamic responses to sympathetic stress. Therefore, these data suggest that CYP450 is not compulsory for regulation of cerebrovascular hemodynamics in healthy young adults, however, may be critical for dynamic cerebral responses to drops in blood pressure.

Acknowledgements

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Chapter I

Introduction

The brain is one of the body's most metabolically demanding organs, requiring a great deal of nutrients in order to survive. However, the brain's inability to retain adequate essential nutrients within itself requires the body to provide a constant stream of blood to working neuronal tissues. Due to this high demand of nutrients, the body distributes nearly 15 percent of its resting cardiac output to the brain in order to maintain homeostatic conditions and must always maintain the same relative amount of flow regardless of changing conditions (Xing et al., 2017, Williams and Leggett, 1989). If the body is unable to properly deliver blood to the brain, stroke, neurological conditions, and death can quickly ensue. In the United States alone, incidences of stroke have increased nearly 8 percent in the last 10 years, accounting for nearly 1 in every 19 deaths within the country (Virani et al., 2020). Additionally, as of 2017, stroke has been identified as the fifth leading cause of mortality within Oklahoma, ranking the state ninth in the U.S.A for stroke-related deaths (CDC, 2022).

By nature, humans consistently experience many physiological and environmental stimuli (changes in posture, physical activity, ambient temperature, altitude, etc.) that necessitates the body to redistribute cardiac output to different regions of itself, thus altering systemic blood pressure in the process. These unavoidable changes often force the brain to modify its cerebral vasculature in order to regulate the flow of blood and the magnitude of perfusion pressure it receives. Cerebral autoregulation (CA), the cerebrovasculature's intrinsic mechanism which balances blood flow to the brain in response to changes in blood pressure, is essential for the health and wellbeing of the brain and body. Impaired CA has been linked to fatigue, dampened

cognitive ability, presyncope, possible neurodegenerative disease, and even death (Shekhar et al., 2017; Rickards, 2015).

The exact mechanisms that form cerebral autoregulation are unclear, and many conditions such as hemorrhage, alterations in altitude, stoke, disease and other obstacles are believed to cause impairment to this process (Iwasaki et al., 2011; Rickards, 2015; Shekhar et al., 2017). Because of this, it is crucial that we continue to identify the underlying components that contribute to cerebral autoregulation in order to better diagnose and treat those with impaired cerebrovascular control, as well as the psychological and physiological effects it has on one's body and wellbeing.

CA has been linked to neurogenic, myogenic, metabolic, and endothelium-dependent factors – but to what degree each mechanism contributes to overall vascular response within the brain is not well known (Rickards, 2015; Peterson et al., 2011). Of these contributions, endothelial-derived control of blood flow is considered a major component in blood flow dynamics within the brain, where metabolites such as nitric oxide (NO) and eicosanoids are linked to numerous vascular responses (Peterson et al., 2011). Evidence has shown that NO plays a role within CA's control of blood flow, where autoregulation is attenuated following blockage of NO synthesis (White et al., 2000). The cyclooxygenase pathway (COX), which produces many types of eicosanoids, has also been extensively studied and is known to create the vasodilators prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) within normal cerebral endothelium (Peterson et al., 2011). Subsequently, research has shown that blocking both NO and COX pathways within a vessel during purinoceptor stimulation causes an attenuated vasodilatory response but does not completely remove the relaxing effect of purinoceptor

stimulation – suggesting a third dilatory pathway at work (You et al., 1999; Petersson et al., 1995).

Endothelium-derived hyperpolarization factor (EDHF), which is the primary suspect for this sustained relaxation, is not well understood in any condition. Studies suggest that EDHF may be present within the brain's vasculature, however it is still unknown if it plays a role within cerebral autoregulation alone (Gervasini et al., 2004). There is little-to-no research into EDHF's role in autoregulation during a variety of different stressors, including hypovolemia. Due to the prevalence and numerous causes of hypovolemia (e.g. dehydration, hemorrhaging, illness, etc.), in addition to the lack of study into EDHF's effect within the cerebral circulation, the current study shows significant importance for clinical and physiological knowledge regarding autoregulation's control of blood within the brain during sympathetic activation via simulated hypovolemia.

The aim of this study is to define EDHF's role within the cerebral vasculature and its contribution within cerebral autoregulation using simulated hypovolemia in humans. Unlike previous studies, which focus on well-established endothelium-dependent pathways within the brain's vasculature, this study will focus on establishing EDHF's function within the autoregulation of healthy, young populations during increased sympathetic outflow by means of lower-body negative pressure. By inhibiting EHDF via fluconazole, an established EHDF antagonist, we hope to identify alterations in cerebral autoregulation by ways of transfer function analysis and alterations in cerebrovascular conductance index (CVCi) via the collection of several cardiovascular variables (Trinity et al., 2021; Petterson et al., 2021; Bellien et al., 2008). We hypothesize that EDHF blockade will result in a decreased autoregulatory capacity when compared to the placebo following sympathetic activation by lower-body negative pressure.

Purpose of Study

The purpose of this study is to determine the role of endothelium-derived hyperpolarization factor within the regulation of cerebral blood flow in healthy, young adults.

Research Questions

1) Does the antagonization of EDHF within the body affect cerebral hemodynamics during simulated hypovolemia?
2) Does the antagonization of EDHF within the body affect the cerebral vasculature's autoregulatory response to simulated hypovolemia?
3) Does the antagonization of EDHF within the body affect prefrontal-cortex oxygenation during simulated hypovolemia?

Research Hypotheses

1. Inhibition of EDHF will result in a significant decrease in CVCi, increase in MAP, and a decrease in MCAv during rest and during simulated hypovolemia in young, healthy adults.
2. Inhibition of EDHF will cause an impaired cerebral autoregulation (increase gain and decreased phase) during rest and simulated hypovolemia in young, healthy adults.

3. Inhibition of EDHF will result in a maintained oxygenation at rest, however, will result in a decrease in oxygenation during simulated hypovolemia in healthy, young adults.

Significance of Study

Autoregulation of cerebral blood flow and perfusion pressure is essential for the continued delivery of nutrients to the brain. Although the homeostatic effects of cerebral autoregulation can be observed during numerous different stressors, the mechanisms that cause these changes are not well established or understood. The factors that account for the maintenance of continued blood flow and perfusion pressure to the brain despite physiological challenges to blood volume and pressure remain elusive. EDHF has shown promise as an endothelium-dependent vasodilator within the periphery, however its effect within the brain's vasculature is not known. To this end, the determination of EDHF's effect within the cerebral vasculature during changes in central blood volume can add valuable insight into the protective mechanisms the body uses to maintain blood flow and pressure to the brain during physiological stress and disease.

Delimitations

The delimitations of this study include:

1. All subjects are 18-30 years of age.
2. All subjects are considered healthy with no chronic metabolic, pulmonary, or cardiovascular diseases.

3. All subjects are normotensive (systolic blood pressure <130 mmHg and diastolic blood pressure <85 mmHg).
4. All subjects have a BMI of less than 30kg/m^2.
5. All female subjects will be testing during days 1-5 of their menstrual cycle (early follicular phase).
6. All subjects are non-smokers or have ceased smoking for at least 6 months.
7. All subjects are not pregnant.
8. All subjects were not taking cardiometabolic prescription medications.
9. All subjects are not taking medications that also inhibit P450 cytochrome-C.

Limitations

The limitations of this study include:

1. Middle cerebral artery velocity (MCA_v) will be measured rather than directly measuring vessel diameter in order determine Δ cerebral blood flow.
2. Fluconazole will not be locally administered.
3. Whole brain oxygenation will not be measured, only prefrontal cortex oxygenation.
4. Oxygen extraction will not be measured directly globally within the brain.
5. Data may not represent adequate responses for those who are older or experiencing chronic disease.

6. End-tidal carbon dioxide ($EtCO_2$) will not be experimentally controlled.

Assumptions

The assumptions of this study include:

1. All subjects adhered to pre-testing protocols.
2. ΔMCAv accurately represents changes Δcerebral blood flow.
3. Δcerebral vascular conductance index (CVCi) accurately represents changes in Δcerebral vascular conductance.
4. 150mg of fluconazole antagonizes EDHF equally between subjects (Petterson et al., 2021).
5. 120 minutes is adequate time for peak EDHF concentrations within the blood (Grant and Clissold, 1990).
6. 48 hours is adequate time for fluconazole to dissipate due to its half-life (Grant and Clissold, 1990).
7. Subjects respond to -20mmHg LBNP equally.
8. Female estrogen levels will be similar between experimental trials (earlier follicular phase, days 1-5).
9. Subjects were properly blinded by placebo.
10. All subjects answered questionnaires truthfully.

Operational Definitions

The operational definitions of this study include:

1. **Cerebral Autoregulation (CA) -** The cerebral vasculature's intrinsic ability to maintain a constant perfusion pressure and blood flow regardless of fluctuating blood pressure (Aaslid et al., 1989).
2. **Cardiac Output (Q)** – The amount of blood that leaves the heart within one minute, calculated using number of heart beats per minute and volume of blood per beat.
3. **Cerebral Blood Flow (CBF)** – The volume of blood passing through the brain within a given time frame (ml/min).
4. **Cerebrovascular Conductance Index (CVCi) -** An index used to portray conductance, or the ease of flow, within an artery. A high CVCi score represents less resistance in the vessel therefore is a surrogate index of vasodilation/vasoconstriction.
5. **Coherence –** Represents the relationship between blood pressure oscillations and cerebral blood flow oscillations
6. **Deoxyhemoglobin (DeoxyHb)** – Hemoglobin without oxygen bound to itself.
7. **End-tidal CO_2 ($EtCO_2$)** – The volume of CO_2 exhaled at the end of a breath.
8. **Endothelium-derived Hyperpolarization Factor (EDHF) -** A system or molecule that causes a vasodilatory response within the vasculature that is derived from within the endothelium. It is not known exactly how this pathway or molecule works within the body other than its effect on the body's vessels.

9. **Fluconazole -** A pharmaceutical inhibitor that is used to block EDHF within the body by inhibiting CYP epoxygenase 2C9. It is often found within antifungal and antimycotic medication (Petterson et al., 2021).

10. **Gain –** Represents the diminishing effects of autoregulation on waveforms between blood pressure and MCAv (van Beek et al., 2008).

11. **Lower-body Negative Pressure (LBNP)** - A sealed air chamber that utilizes negative pressure to remove pressure surrounding the lower extremities, thus causing blood volume to shift from the center of the body to the lower half of the body (Hinojosa-Laborde et al., 2013).

12. **Mean Arterial Pressure (MAP)** – The mean arterial pressure throughout one cardiac cycle (DeMers and Wachs, 2021).

13. **Middle Cerebral Artery Velocity (MCAv)** - The speed at which erythrocytes travel within the middle cerebral artery.

14. **Near-infrared Spectroscopy (NIRS)** - A device that calculates hemodynamics by emitting 700-1000 nm wavelengths of light into a selected tissue. The amount of light absorbed by the cells determines the levels of oxyhemoglobin, deoxyhemoglobin, and total saturation within the tissue (Villringer et al., 1993).

15. **Oxyhemoglobin (O_2Hb)** – Hemoglobin with oxygen bound to itself.

16. **Phase –** Represents the displacement of one waveform to another and is commonly expressed in degrees of change (0° - 360°) or radians (0 - 2π) (van Beek et al., 2008).

17. **Stroke Volume (SV)** – The amount of blood that leaves the left ventricle per contraction of the heart (ml/beat).

18. **Total Peripheral Resistance (TPR)** – The total amount of force exerted on the body's circulating blood by surrounding vessels (Trammel and Sapra, 2021).

19. **Total Saturation Index (TSI)** – The ratio of oxyhemoglobin to total hemoglobin in each tissue (total oxy- and deoxyhemoglobin within a tissue) (Sanni and McCully, 2019).

20. **Transcranial Doppler Ultrasonography (TCD)** - A form of ultrasound that uses the Doppler effect, where ultrasonic waves are emitted and reflect off the moving erythrocytes within the cerebral vasculature to determine various cerebral blood flow velocities (Purkayastha and Sorond, 2013).

21. **Transfer Function Analysis** – An analysis, which transforms time-domain fluctuations of arterial pressure and cerebral blood flow into numerous oscillatory frequencies, that is used to evaluate the relationships between pressure and flow in terms of changes in gain, phase, and coherence (Rickards, 2015; Tzeng et al., 2012; van Beek et al., 2008).

Chapter 2

Literature Review

Due to its extremely high metabolic demand, the brain requires a constant flow of nutrient-rich blood in order to maintain autoregulatory and homeostatic conditions within the body. Without this continuous influx of oxygen and glucose, an individual can show signs of fatigue, lightheadedness, loss of consciousness, neurological disease, and death (Shekhar et al., 2017; Rickards, 2015). Because of this essential need for constant nutrient delivery, the autoregulation of cerebral blood flow serves as an essential function for the health and growth of all human processes. Although vital, cerebral autoregulation is not a perfect system, and many physiological stressors can alter and impair this intrinsic mechanism (changes in altitude, hypovolemia, and disease that affects homeostatic regulation); any of which can cause substantial psychological and physical damage to the brain (Rickards, 2015). By continuing to study the hemodynamics of blood within the brain, we as researchers can develop a better understanding of the protective systems that the cerebrovasculature has in place to defend the body from life threatening injuries, disease, and more. This study aims to identify the role of EDHF within the cerebrovasculature, in hopes of better understanding the hemodynamics of blood flow that occurs during increasing sympathetic stress caused by simulated hypovolemia.

This chapter will discuss the basic anatomy of the cerebral circulation, the theoretical components of cerebral hemodynamics, and EDHF's believed mechanism of action within the vasculature. Fluconazole's bioavailability, pharmacokinetics, and antagonizing effects to EDHF will also be presented. Lastly, the classification of hemorrhagic shock as they relate to the effect hypovolemia on the systemic cardiovascular and cerebrovascular systems, lower-body negative

pressure's role in simulated hypovolemia, and the use of transfer function analysis in quantifying autoregulatory responses to hypovolemia will be given.

Anatomy of the Cerebral Vasculature

The brain's vasculature is complex and often varies between individuals. Of the numerous arteries found within the cerebral vasculature, there are four feeding arteries which supply nutrient-rich blood to the brain: the left and right internal carotid arteries (ICAs) supply blood to the anterior half of the brain, while the left and right vertebral arteries (VAs) supply blood to the posterior half (Payne, 2016; Vavilala et al., 2002). As these vessels enter the cranium, the basilar artery is formed from the combining of the two vertebral arteries (Payne, 2016). In the anterior circulation, the left and right ICAs branch off into the left and right middle cerebral arteries (MCAs), then the anterior cerebral arteries (ACAs). From there, anterior communicating arteries (ACoA) and the posterior communicating arteries (PCoA) form with the posterior cerebral arteries (PCA) to fashion the Circle of Willis (Payne, 2016; Ashwini et al., 2008). The Circle of Willis offers redundancy within cerebral blood flow, ensuring adequate dispersal of blood throughout the brain.

After leaving the large arteries of the brain, blood continues to flow into arterioles, where vessel tone is regulated, thus aiding in the determination of blood flow (Payne, 2016). Following the arterioles, the blood is then passed through capillary beds, where nutrients are off-loaded, and metabolites are onloaded from the surrounding tissue. The blood then drains into the venules and veins, sending blood back to the heart.

Abnormalities Within the Cerebral Vasculature

It is widely accepted that there is great anatomical variability within humans' Circle of Willis. Research by Iqbal (2013) found that 52% of subjects had alterations in their Circle of Willis – 24% of which was caused by hypoplasia of the vessels. Similarly, another study in 2013 had found that nearly 58% of 500 patients had anatomical abnormalities within their Circle of Willis, where 35.6% of subjects experienced hypo- or aplasia of their left PCoA (Papantchev et al., 2013; Payne, 2016). This agrees with Alpers and colleagues' (1959) findings, where nearly 48% of the 350 subjects examined had abnormalities within their Circle of Willis. These subjects were believed to be free of vascular pathologies at the time of study, suggesting that a large mass of the healthy adult population have alterations within their cerebral vasculature (Alpers et al., 1959; Payne, 2016). This inconsistency in cerebrovascular anatomy may cause variation in blood flow between individuals, as an incomplete or abnormal Circle of Willis' can cause lack of flow to areas of the brain, especially if a blockage were to occur. The MCA – a vessel commonly used within cerebrovascular research – is also highly subject to individual variation. Examination of cadaver specimens revealed bifurcations, trifurcations, and quadfurcations within the vessel, where ~64% of cadavers were found to have a bifurcating artery and nearly 15% of bodies expressing either tri- or quadfurcation (Gunnal et al., 2019). Of the remaining ~20% of individuals, single main-stem arteries were seen (Gunnal et al., 2019). Subsequently, 60% of brains showed asymmetrical middle cerebral artery branching between each hemisphere, resulting in variating vasculature to each side of the brain (Gunnal et al., 2019).

Principles of Cerebrovascular Control of Blood Flow

Although the cerebral vasculature is equipped with unique mechanisms that allow it to manipulate the flow of blood throughout the brain's tissues, like any other part of the circulatory

system, it must still follow the foundational law of flow described by Poiseuille. Poiseuille's law states that a vessel's flow rate is influenced by changes in vessel's diameter, pressure, blood viscosity, and vessel length. Due to the vessel's radius being to the fourth power within Poiseuille's formulae, small changes in diameter result in large alterations in blood flow, as seen in the given equation below:

$$Q = \frac{\Delta P \pi r^4}{8 \eta l}$$

Poiseuille's Law, where ΔP = change in pressure, r = vessel radius, η = viscosity, l = vessel length

Poiseuille's equation gives additional, fundamental insight into the relationship between blood pressure and blood flow within the vasculature, where - assuming that the viscosity of blood, the length of the vessel, and the radius of the vessel are constant - a decrease in blood pressure will result in a linear decrease in blood flow. It is because of this decrease in pressure that a passive vessel shows a vasoconstrictive response, thus increasing pressure back to homeostatic levels (Payne, 2016). However, due to various myogenic, neural, endothelial-dependent, and metabolic components, the cerebral circulation reacts counterintuitively to a drop in blood pressure – where a decrease in pressure results in an increase in diameter and vice versa. This paradoxical, active response to changes in blood pressure, known as autoregulation, is found in numerous vascular beds such as the kidneys, brain, and heart and allows for a steady supply of blood to these organs regardless of changes in pressure (Johnson, 1986, Rickards, 2015).

Myogenic Contributions to Flow and Vessel Tone

The reflexive ability of vascular smooth muscle to regulate basal tone in response to changes in lumen pressures, without the intervention of neural or hormonal stimuli, is known as the myogenic response of vascular smooth muscle (Baek and Kim, 2011). This response, activated by the excessive stretching of the vessel's lumen, results in a constriction of the cerebrovasculature by means of altering membrane potential and the level of depolarization of cells (Bayliss, 1902; Peterson, 2011; Mchedlishvili, 1980; Tan et al., 2013; Harder et al., 1998). This decrease in membrane potential allows for an influx of Ca^{2+} into the smooth muscle, causing vasoconstriction (Tan et al., 2013; Harder et al., 1998).

Metabolic Contributions to Flow and Vessel Tone

Often considered the most potent regulator of blood flow, modifications in metabolism within the periphery and brain are known to greatly effect cerebral vascular tone and flow (DeFiley and Chilian, 1995). Neurovascular coupling (NVC), characterized by the alteration in vessel diameter to match the metabolic need of working neuronal tissues in the brain, as well as vessel CO_2 reactivity are believed to largely contribute to the determination of flow through given arteries (Murkin, 2007; Peterson et al., 2011; Faraci and Heistad, 1998; Rickards, 2015, Claassen et al., 2021). Described as having both feedback and feedforward mechanisms, NVC is known to increase regional flow within working neuronal tissues by means of endothelial-dependent signaling within the cerebral arteries (Iadecola, 2017; Aaslid, 1987; Claassen et al., 2021). Similarly, global decreases in metabolism within the brain via hypothermic anesthesia has resulted in a reduction of middle cerebral artery velocity as well, supporting previous investigations (Endoh and Shimoji, 1994).

Within hypercapnic conditions, cerebral blood vessels dilate and allow for greater flow to the surrounding tissues (Claassen et al., 2021; Rickards, 2015; Willie et al., 2011). These

transient changes seen during excessive $PaCO_2$ are often attributed to the concurrent changes in blood p.H (H^+ ions) that accompany CO_2 production, not the carbon dioxide itself (Kontos et al., 1977; Brian, 1998). Dagal and Lam (2009) observed a profound role of carbon dioxide within the cerebral vasculature - suggesting that every 1 mmHg increase of CO_2 causes a 3-4% increase in blood flow. In this way, blood acidosis is linked to vasodilation within the vasculature, while alkalosis is linked to vasoconstriction (Rickards, 2015). Studies have shown that the posterior cerebral artery and middle cerebral artery in subjects respond similarly to hypercapnic stress – where increases in $PaCO_2$ resulted in direct increases in blood velocity - providing evidence of a common, global response within the anterior and posterior conduit arteries of the brain to CO_2 (Willie et al., 2011). In fact, all arteries within the brain (large arteries, pial arteries, and arterioles) appear to be sensitive to changes in carbon dioxide (Claassen et al., 2021).

Neurogenic Contributions to Flow and Vessel Tone

The nervous system's contribution to the control of cerebral blood flow has been a critical topic in the field of physiology for decades. Within the cerebral vasculature, evidence of sympathetic innervation exists by the presence of adrenergic receptors within the vascular smooth muscle of cerebral arteries, as well as adrenergic nerve endings innervating them (Ainslie and Brassard, 2014). Studies suggests that sympathetic control protects the brain from excessive increases in flow at rest, whereas removing sympathetic outflow via ganglion blockade or gangliectomy results in higher resting blood flow within the brain (ter Laan et al., 2013; Jeng et al., 1999; Claassen et al., 2021). Observed predominantly within larger arteries of the brain, this protective mechanism helps prevent drastic increases in cerebral perfusion pressure (Willie et al., 2014). Like the sympathetic nervous system, the parasympathetic system also has numerous

nerve terminals spread throughout the cranial vessels, however most researchers believe that they play only a minor role on vasodilation in the human brain (Claassen et al., 2021).

Recent studies have found autonomic control of the brain's vasculature may be frequency dependent. Zhang and colleagues (2002) found that, in low frequencies (0.02-0.07 Hz), mean blood pressure variability decreased by 82% while maintaining MCAv following ganglion blockade using trimethaphan. In return, the gain between blood pressure and middle cerebral artery velocity significantly increased by 81% (Zhang et al., 2002). Another study concluded that blockade of α-adrenergic receptors resulted in a significant increase in gain between pressure and flow at frequencies greater than .05 Hz – indicating a frequency-dependent role of sympathetic regulation of blood flow (Hamner et al., 2010). Research by the same research team found a similar result in the parasympathetic system, where blockade of cholinergic receptors via glycopyrrolate administration resulted in significant increase in gain above .05 Hz, as well (Hamner et al., 2012).

Endothelial-Dependent Contributions to Flow and Vessel Tone

Numerous vasomodulatory molecules that are released from the endothelium have been shown to cause vasodilation and vasoconstriction in response to increases in shear stress within a vessel (Green and Lee, 2012). Depending on the chemical released, the mode that each endothelial-dependent ligand takes to cause alterations in a vessel's diameter differs, however, the removal of endothelial tissues attenuates each response regardless of the pathway (Wilkerson et al., 2005). This gives evidence that the vessel's endothelium plays a critical role within the regulation of vascular tone, reactivity, and flow, where endothelial dysfuncton is linked to numerous cerebral vascular diseases such as stroke and dementia (Peterson et al., 2011; Wang et al., 2018; Sheinberg et al., 2019; Cosentino et al., 2001).

Of the many endothelial-dependent factors that are released following incidences of shear stress, nitric oxide (NO), prostaglandins, and endothelial-derived hyperpolarization factor (EDHF) are the most cited in research. Unfortunately, the complex, interwoven nature of these pathways makes distinguishing the role of each molecule particularly difficult. Nitric oxide, a common vasodilator which utilizes cyclic GMP to relax smooth muscle via phosphorylation of myosin and actin, plays a significant role within cerebral hemodynamics and autoregulation (Preckel et al., 1996; Lehners et al., 2018). Studies have demonstrated that NO inhibition lowers cerebral blood flow throughout the brain within humans and limits the lower interval of autoregulation in rat models, causing decreases in blood pressure to be detrimental on cerebral blood flow (Carter et al., 2021, Preckel et al., 1996). Similarly, the cyclooxygenase pathway (COX), which causes vasodilation via the production of prostaglandin I_2, also seems to play a role within control of cerebral blood flow - where the use of the COX inhibitor indomethacin significantly decreased cerebral vascular conductance and autoregulation responses in humans (Kellawan et al., 2020, Ricciotti & FitzGerald, 2011; Smirl et al., 2014; Tanaka et al., 2004; Peltonen et al., 2015; Shoemaker et al., 2021).

Additionally, emerging evidence of a vasodilatory pathway(s) that act independently of NO or COX are beginning to be explored (You et al., 1999; Petersson et al., 1995). In a groundbreaking study, Peterson et al. (1995) reported a vasodilatory response following the inhibition of NO synthase and COX within in vitro human pial vessels when using substance P, a potent vasodilator. This observation demonstrates a vasodilatory pathway independent of nitric oxide or prostaglandin production within the pail arteries of the brain (Petersson et al., 1995). **Figure 1**, which depicts human pial arteries responses following use of substance P, a vasorelaxant, with NO and COX-blockade (Petersson et al., 1995).

EDHF, which has been known to work independently of NO and COX pathways within the periphery, is believed to be cause of this relaxation within the pial arteries – however the exact mechanism of this pathway is not known (You et al., 1999; Petersson et al., 1995, Golding et al., 2002). **Figure 2** demonstrates the theorized relative contribution of EDHF when agonistically induced vasodilation occurs with and without COX/NOS inhibition – as shown, the concentration of the antagonist drug alters the contribution of EDHF within a vasodilatory response.

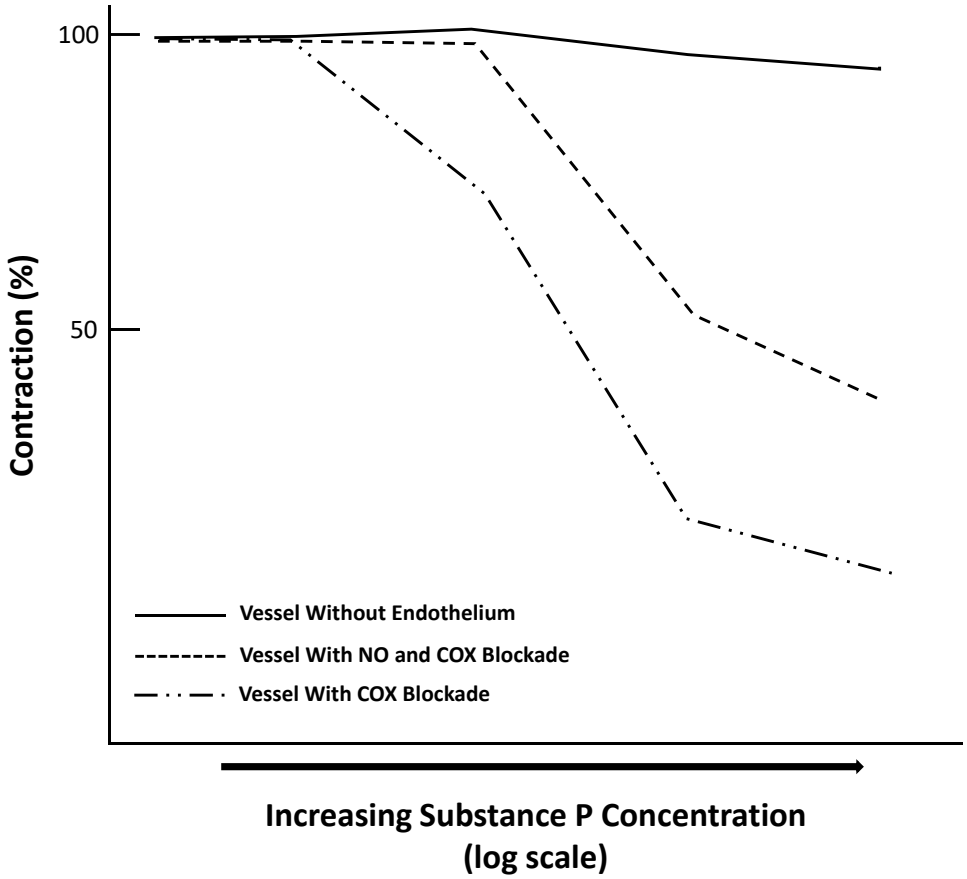

Figure 1. Graph demonstrating a vasorelaxation response within the cerebral vasculature following the blockade of NO via L-NOARG with COX blockade using indomethacin. Redrawn from Petersson et al., 1995 .

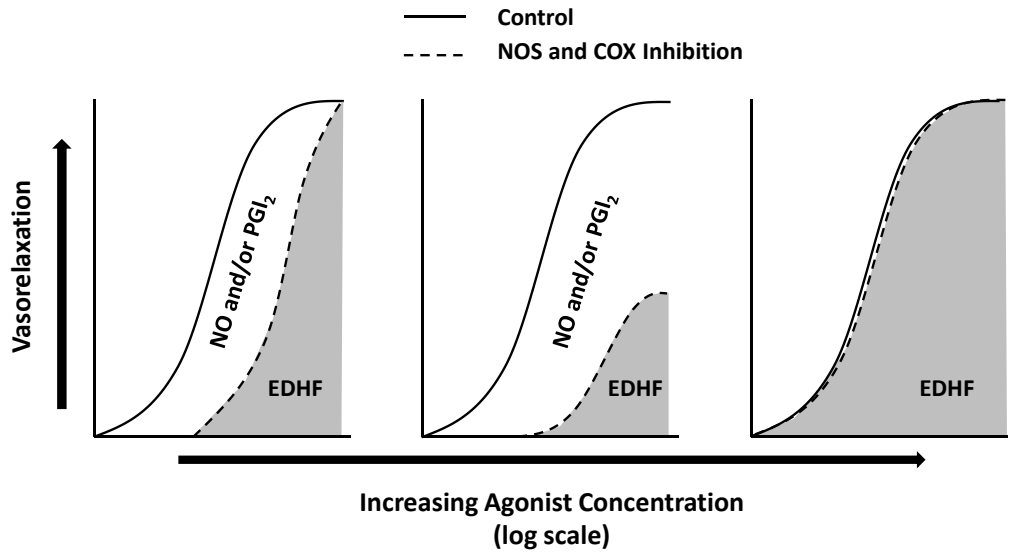

Figure 2. Graph indicating the ***proposed*** balance between NO, COX, and EDHF throughout different levels of agonist-induced vasorelaxation. Redrawn from Bryan (2005).

Cerebral Autoregulation

The Theoretical Model of Flow and Pressure

As forementioned, cerebral autoregulation is the intrinsic ability for the cerebral vasculature to maintain constant blood flow and perfusion pressure to the brain, despite changes in blood pressure (Ruland and Aiyagari, 2007). The first model of cerebral autoregulation, developed by Lassen in 1959, demonstrated that the brain's blood flow could only be maintained between a set range of blood pressures (between 50-175 mmHg), as seen in **Figure 3**. This model - termed the autoregulatory curve - has been the foundation of numerous studies, clinical teachings, and classroom lectures. Although Lassen's model gave a theoretical, basic understanding of the relationship between pressure and flow within the brain's vasculature, new research techniques has allowed scientists to improve upon Lassen's work and construct a more

defined autoregulatory model. Modern understanding of the cerebral autoregulation suggests that the autoregulatory range between lower and upper limits in pressure is far narrower than what was originally thought, ranging between 5 and 10mmHg - although this is highly variable between individuals (Tan, 2012; Rickards, 2015; Brassard et al., 2021). Additionally, the regulation between these smaller ranges of pressure is not a static "plateau" as once thought, but rather a gentle slope representing minor, passive changes in flow between these ranges. Researchers suggest that the magnitude of this slope may represent impaired CA, although this is up for interpretation (Rickards, 2015). Still, changes in blood flow above or below these upper and lower values demonstrate large, passive alterations in blood flow caused by perfusion pressure extremes (Lassen, 1959; Rickards, 2015; Tan, 2012). Evidence suggests that increases in sympathetic outflow caused by hypovolemia shifts the autoregulatory curve to the right, thus altering the lower and upper limit of the curve, without changing slope (Levine et al., 1994; Rickards, 2015). This shift would result in a compromised lower limit of autoregulation in the face of already decreasing pressures caused by hypovolemia (Levine et al., 1994).

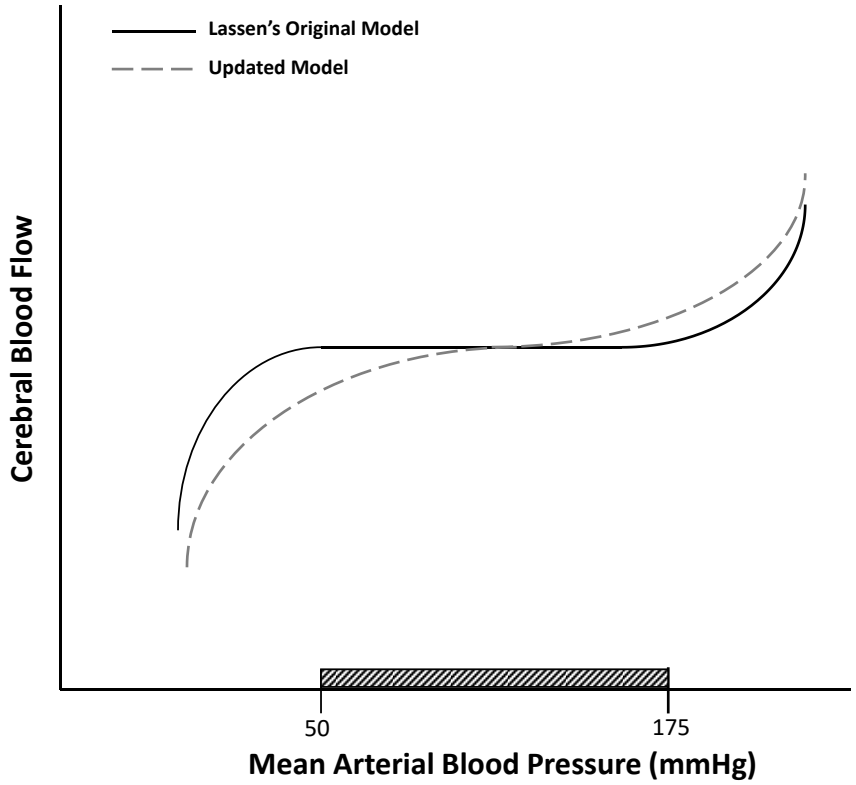

Figure 3. The classical cerebral autoregulation curve created by Lassen, 1959 with new, updated model. Redrawn from Rickards, 2015.

The Theoretical Components of Cerebral Autoregulation

Although CA's control of blood flow in the face of fluctuating blood pressures has been studied for decades, the intertwining, redundant mechanisms that comprise this intrinsic response are difficult to pinpoint. It has been thought that myogenic, neurogenic, endothelial-dependent, and metabolic factors play key roles in this process, however this is often debated (Rickards 2015; Faraci and Heistad, 1998; Silverman and Peterson, 2021). Rightfully, many scientists have begun to renounce the proposed metabolic control of cerebral autoregulation, stating that metabolic-mediated flow acts independently to alterations in blood pressure, and thus cannot be considered

apart of CA (Peterson et al., 2011, Silverman and Peterson, 2021, Rickards, 2015). However, given the difficulty to remove all metabolic stimuli from a subject and the often simultaneous, interrelated changes in $PaCO_2$ and blood pressure that occur during physiological stress, it is difficult to differentiate between the effects of metabolism and those caused by CA.

Retrospective analysis suggests that myogenic and autonomic-neurogenic control of autoregulation accounts for only 62% of the pressure-flow relationship observed, leaving 38% of responses unexplained (Hamner and Tan, 2014). Hamner and colleagues (2014) suggest that, while myogenic control of flow plays a role in autoregulation, it may only be as a neuroprotective measure against ischemia and hemorrhage. Feline animal models retained cerebral autoregulation following the denervation of both the sympathetic and parasympathetic nerves, while dog animal models demonstrated a complete loss of autoregulation observed following a similar protocol (Ainslie and Brassard, 2014). However, in human models, there is no direct evidence of the nervous system's effect on CA (Zhang and colleagues, 2002).

Sex Differences Within the Cerebral Circulation

Being critically understudied, understanding cerebral vascular sex differences has become a high priority within research given emerging epidemiological differences in stroke and cardiovascular disease between men and women (Mosca et al., 1996). Although not completely understood in humans, animal models have shown numerous variations in the anatomy of the brain's vasculature between both sexes. Female rats have shown more elastin, paired with less contractile capabilities within the MCA – resulting in higher basal tone and wall stress than their male counterparts (Wang et al., 2020). In human studies, female subjects often show better

regional and global flow throughout the brain than males, where female cerebral blood flow is 11-15% larger than that of males (Gur et al., 1982; Rodriguez et al., 1988; Gur and Gur, 1990).

During lower-body negative pressure, men and women show little differences in cerebral hemodynamics. Rosenberg et al., (2021) found that decreases in mean arterial pressure, stroke volume, MCAv, and cerebral oxygen saturation (ScO2) were not statistically significant between the sexes, however end-tidal CO_2 levels at presyncope were different, suggesting that CO_2 reactivity between the sexes may be different.

It is not completely understood why such a stark difference in blood flow and cardiovascular health between the sexes exist, however it is possible that hormonal variations contribute. Geary and colleagues (1998) found that circulating estrogen within the blood of mice significantly lowered myogenic tone of cerebral arteries by enhancing NO synthesis. However, researchers have found that the pressure-flow relationship is maintained all throughout menstrual cycles of young women and that acute changes in hormones do not play a role in sex differences within autoregulation (Favre and Serrador, 2018). Still, the same study determined that females have significantly more efficient autoregulation mechanisms, where MCAv decreased less than that of men's during sit-to-stand maneuvers – agreeing with previous findings (Favre and Serrador, 2018).

EDHF

EDHF's Role in the Vasculature

The role of endothelium-derived hyperpolarization factor is not well known within peripheral or cerebral circulation. Originally thought to be a vasodilatory molecule, new

evidence suggests that EDHF may be an entirely new process or mechanism independent of NO or COX pathways (Golding et al., 2002). Increasing number of studies has shown that EDHF may act as a redundancy mechanism within the body, being most active when NO and COX pathways are attenuated (Luksha et al., 2009; Ozkor et al., 2010; Nishikawa et al., 2000). With the ambiguity of what EDHF may be, some researchers have chosen to identify EDHF as any unknown metabolite or pathway that: 1) requires an intact endothelium, 2) is still active despite NO and COX inhibition, 3) actively hyperpolarizes vascular smooth muscle, and 4) utilizes calcium-activated potassium channels (Golding et al., 2002).

Like nitric oxide, EDHF is believed to be released in response to shear stress and pulsatile stretch within the periphery (Shimokawa and Matoba, 2001). However, unlike NO, EDHF has often been found to play a larger role in smaller resistance arteries rather than conduit arteries within the brain and periphery, resulting in a more crucial role in determining vascular resistance (Shimokawa and Matoba, 2001; Golding et al., 2002; Luksha et al., 2009). EDHF may also be upregulated following stroke or traumatic brain injury, causing increasing physiological interest (Andresen et al., 2006).

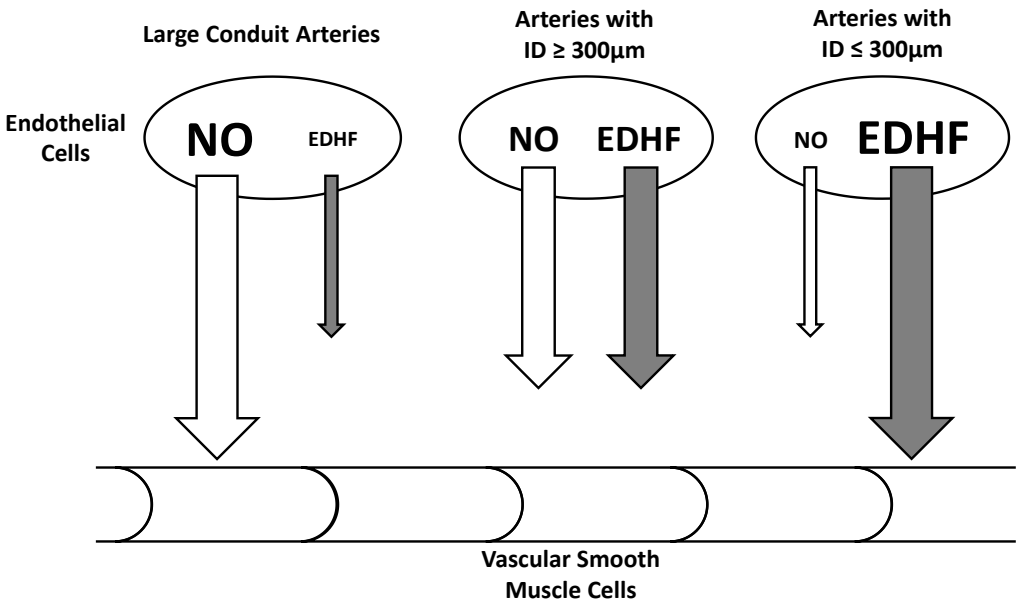

Figure 4. Proportional activation of EDHF and NO with regards to vessel diameter. Recreated from Luksha et al., 2009.

Mechanisms of Action

There is debate on the pathway that EDHF takes to form vasorelaxation, however it is believed that CYP-450 (a product of some arachnoid acids) may serve as EDHF's molecule of action or even its key messenger (Luksha et al., 2009). Data has shown that CYP-450-dependent, EDHF-mediated responses are present within coronary, mammary, forearm, and skeletal arteries of humans (Luksha et al., 2009). Studies have also observed CYP-450 within brain tissue of rats and within the postmortem human brain – however it is unclear if they are related to EDHF-mediated responses within these tissues (Gervasini et al., 2004). It is speculated that CYP-450 may regulate Ca^{2+} entry into the endothelium, activate KCa^{2+} channels, and allow for communications between gap junctions via the protein kinase C pathway (Luksha et al., 2009). An increase in CYP-450 within the endothelium may activate calcium-dependent potassium

channels located within the membrane of vascular smooth muscle via the formation of epoxyeicosatrienoic acids (EET), thus hyperpolarizing and relaxing the smooth muscle cell leading to vasodilation.

ATP's Possible Role with EDHF Pathways

Adenosine 5-triphosphate (ATP) is a well-documented vasomodulary molecule within the circulatory system that is released from numerous tissues, each promoting unique responses within the vasculature (Lohman et al., 2012). ATP released from the sympathetic nerve terminals induces vasoconstriction within the periphery and brain, while ATP delivered in the vessel lumen results in dilation via increases in NO synthase (Lamont et al., 2006; Buvinic et al., 2002; You et al., 1997). Often, ATP used by the endothelium to cause increases in relaxation is released from the endothelium itself in response to a rise in shear stress (Lohman et al., 2012). However, hypoxic conditions can also cause erythrocytes to excrete ATP (Bergfeld and Forrester, 1992; Lohman et al., 2012).

Although ATP-mediated activation of NO pathways commonly elicits increases in vessel diameter, ATP's role within EDHF is not well established. However, it is known that ATP-sensitive potassium channels (K_{ATP}) are partially responsible for the vasorelaxation effects of vascular smooth muscle hyperpolarization (Zhang et al., 2002). This gives rise to the possibility that EDHF responses may – in part – be affected by ATP concentrations within the body. Additionally, studies have found that ATP infusion following the blockade of NO, COX, Sodium-Potassium-ATPase, and inwardly rectifying potassium channels (K_{IR}) prior to alpha-agonization continue to result in a vasodilatory response within the forearm vasculature (Hearon et al., 2017). This suggests that ATP is utilized within an unknown pathway independent of NO, COX, Na+/K+-ATPase, and K_{IR} channels – possibly EDHF (Hearon et al., 2017).

Inhibition of EDHF with Fluconazole

Fluconazole is an antimicrobial agent that has been implemented by numerous research teams to block EDHF activation within the circulation (Trinity et al., 2021; Petterson et al., 2021; Bellien et al., 2008). This agent prevents EDHF activation by inhibiting CYP epoxygenase 2C9, which synthesizes EETs to induce vascular smooth muscle hyperpolarization and therefore, relaxation (Bellien et al., 2006). Previous studies have administered 150-200mg doses to subjects at least 2 hours prior to testing (Petterson et al., 2021). This is because fluconazole is highly absorptive regardless of food, antacid, or H_2-rececptor pretreatment, where its bioavailability is above 90% within 1-2 hours of ingestion (see **Figure 5**) (Grant and Clissold, 1990). When taken in 100mg doses, plasma concentrations can be near 1.9mg/L (Grant and Clissold, 1990). The agent has been found to even distribute itself to all tissues without the need for protein binding, including the nervous system, within laboratory mice (Grant and Clissold, 1990). The pharmaceutical is excreted through urine, where nearly 80% of the drug is found unchanged (Grant and Clissold, 1990). It has a half-life of between 27 and 37 hours (Grant and Clissold, 1990).

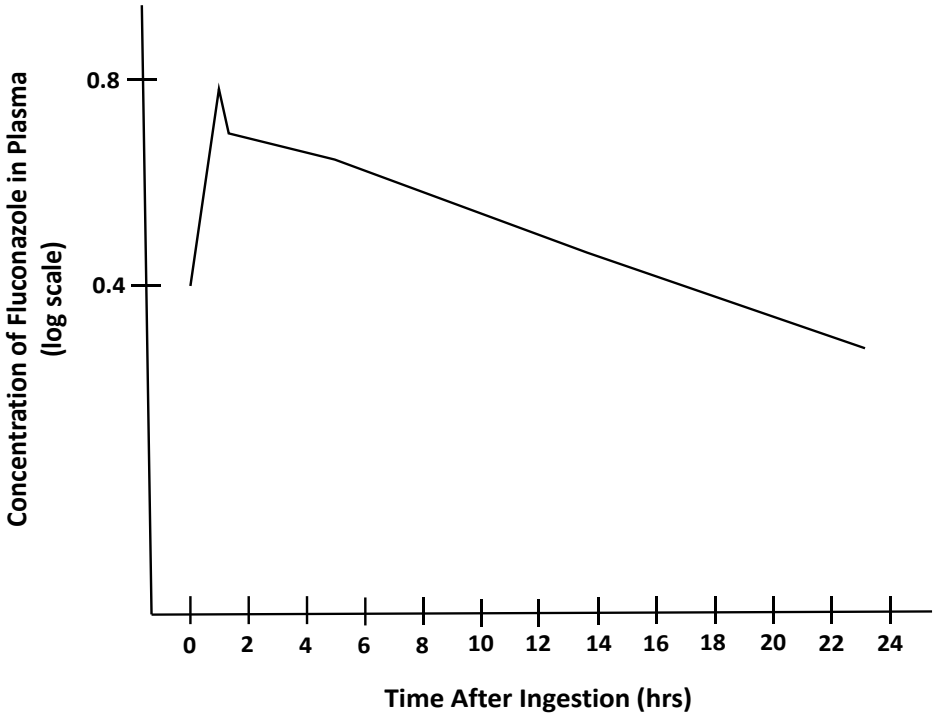

Figure 5. Fluconazole's plasma concentration level plotted against time since ingestion. Redrawn from Grant and Clissold (1990).

Responses to Reductions in Central Blood Volume & Pressure

Central Cardiovascular Responses to Hypovolemia

Due to hypovolemia's relation to numerous physiological stressors (dehydration, blood loss, acceleration, orthostasis, etc), changes in central blood volume and pressure are intently studied and are of high clinical interest. Classification of hypovolemia and cardiopulmonary responses due to loss in vascular volume are summarized using the Advanced Trauma Life Support system (ATLS) as seen in **Table 1.** Compensatory phases (class I & II) show increases in heart and breathing rate; however, blood pressure remains constant (Gutierrez et al., 2004; Rickards 2015). This is indicative of an increase in sympathetic output leading to elevations of heart rate and total peripheral resistance to maintain blood pressure (Rickards, 2015). Class III results in

hypotension and bradycardia due to presumed decreases in sympathetic activation and vasodilation (Rickards 2015). Lastly, class IV results an increase in heart rate, continued loss of pressure, along with extreme vasoconstriction (Rickards, 2015).

Table 1 **Classification of Shock**

Classification of Hemorrhagic Shock

Parameters	**I**	**II**	**III**	**IV**
Blood Loss (% total volume)	≤ 15	15-30	30-40	>40
Heart Rate (beats/min)	↔↑	↑↑	↓↓	↑
Blood Pressure (mmHg)	↔	↔	↓	↓↓↓
Respiratory Rate (breaths/min)	14-20	20-30	30-40	>35
Cerebral Blood Flow	↔	↔	↓	↓↓↓
Mental Status	Normal/slightly anxious	Mildly anxious	Anxious, confused	Conducted, lethargic, loss of consciousness

Modified from Rickards, 2015.

Cerebrovascular Responses to Hypovolemia

Numerous sources, including the ATLS guidelines, report that cerebral blood flow remains constant during the compensatory phases of hypovolemia (Rickards 2015; Rickards et

al., 2015; Gutierrez et al., 2004). However, any hypovolemia above 30% is expected to reduce cerebral blood flow (Rickards, 2015; Gutierrez et al., 2004). Although blood flow does not falter during initial phases, reports have indicated that the oxygenation of the brain may change during all stages of hypovolemia. Torella et al. (2002) found that the removal of 6% of estimated total blood (class I) in 40 volunteers resulted in a significant decrease in cerebral sinus oxygenation when using near-infrared spectroscopy. Still, this value may represent a higher extraction of oxygen rather than decrease in oxygenation due to the NIRS's measurement of mixed-venous oxyhemoglobin (HbO_2) and deoxyhemoglobin (dHb) rather than arterial levels. (Rickards 2015).

Systemic vascular resistance has been shown to increase within the initial stages of LBNP (class 1), where increases in sympathetic output via baroreceptor activation supports cerebral perfusion pressure, despite lowering of blood volume levels (Goswami et al., 2019). Studies have also suggested that a rise in cerebral vascular resistance during lower body negative pressure occurs due to perceived increases in vasoconstriction of small cerebral vessels (Giller et al., 1992). Additionally, Zhang et al. (1998) reported a significant increase in gain at -50mmHg LBNP, however, coherence was not significantly different in any individual (Zhang et al., 1998). In the same study, gradual degreases in central blood volume via LBNP in healthy individuals resulted in a positive phase shift between blood pressure and middle cerebral artery velocity (Zhang et al., 1998).

Lower-body Negative Pressure – A Non-Invasive Method of Inducing Temporary Hypovolemia

Lower-body negative pressure has been used for decades as a unique cardiovascular stressor that displaces central blood volume to simulate hypovolemia. With the use of a sealed air

chamber positioned around subject's legs, negative pressure can be exerted onto the lower limbs - causing central blood volume to shift towards the feet (Rickards 2015). This movement of blood from the center of the body decreases venous return and preload of the heart, resulting in cardiovascular reflexes like those experienced during acute hypovolemia (Cooke et al., 2004; Rickards 2015; Hinojosa-Laborde et al., 2014; Goswami, 2019).

Volume – Pressure Displacement. When using LBNP as a simulation of hemorrhage, it was commonly believed that applying -10-20 mmHg, -20-40 mmHg, and -40-60 mmHg of negative pressure was equivocal to the simulated loss of ~400-550 ml, ~500-1000 ml, and >1000 ml of blood, respectfully (Cooke et al., 2004). However, more recent studies have estimated new values. Rickards et al. (2015) compared LBNP of -15, -30, and -45 mmHg to bloodletting of -333, -667, and -1000 ml and found that LBNP caused greater reductions in central blood volume than actual blood loss. It should be noted, however, that Rickards et al. (2015) failed to remove relative blood values from subjects – suggesting that cardiovascular responses may be under-expressed or over-expressed in larger subjects or smaller subjects, respectfully. Another study, which did account for total blood volume, suggests that – for a 70 kg human – an estimated -450, -1000, and -1600 ml of blood is shifted during -30, -60, and -90 mmHg of lower body negative pressure (Hinoja-Laborde et al., 2014). Computational models using Cooke et al.'s study (2004) by Summers and colleagues (2009) suggests that a loss of -486, -664, and -938 ml of blood can be predicted when using -15, -30, and -60mmHg, respectfully.

Considerations when using LBNP. When performing a traditional LBNP protocol, an interval length of 5 minutes at a given pressure is most common (Rosenberg et al., 2021; Rickards et al., 2015). This duration of stress is ideal for acute autonomic responses within the body, while longer intervals - such as ≥ 20 minutes - can be used to incite hormonal changes

within a subject (Goswami, 2019). When setting up LBNP, location of the sealing process can change outcomes. Commonly, the seal on the chamber is placed at the iliac crest to prevent the squishing of the splanchnic regions – causing inaccurate blood redistribution (Goswami, 2019).

Transfer Function Analysis – A Means to Quantify Cerebral Autoregulation

Currently, there is no standardized procedures for measuring cerebral autoregulation, however the use of the transfer function analysis (TFA) technique has gained immense popularity among cardiophysiologists to help quantify cerebral autoregulation (Rickards, 2015; Claassen et al., 2016). This analysis, which transforms time-domain fluctuations of arterial pressure and cerebral blood flow into numerous oscillatory frequencies, is used to evaluate the relationships between pressure and flow in terms of changes in magnitude, timing, and linearity (Rickards, 2015; Tzeng et al., 2012; van Beek et al., 2008). This allows researchers to quantify the modulatory effects of autoregulation between the input signal (blood pressure) and the output signal (cerebral blood flow) (van Beek et al., 2008). TFA has three parameters that are used to interpret the characteristics of CA, which are oscillatory gain (gain), oscillatory phase shift (phase), and coherence.

Oscillatory gain, also known more simply as gain or magnitude, represents the diminishing effects of autoregulation on waveforms between the input and output variables. A large gain is typically associated with impaired cerebral autoregulation, indicating that a large autoregulatory effect within the vessel at a given time point (Rickards, 2015; Tzeng et al., 2012, van Beek et al., 2008). Inversely, a smaller gain is interpreted to signify a more efficient cerebral autoregulatory process (Rickards, 2015; Tzeng et al., 2012, van Beek et al., 2008).

Oscillatory phase shift, also referred to as phase or timing, represents the displacement of one waveform to another and is commonly expressed in degrees of change (0° - 360°) or radians (0 - 2π) (van Beek et al., 2008). Researchers can transform the degrees of shift into a time-shift, using the following equation:

$$\Delta\tau = \frac{(\frac{deg}{360})}{f}$$

Δτ = time-shift (s), deg = phase shift in degrees, f = frequency

Due to cerebral blood flow's ability to recover from alterations in cerebral blood flow velocity faster than that of blood pressure, cerebral blood flow waveforms are often shifted to the right, preceding blood pressure waveforms. This shift represents an intact cerebral autoregulatory response within the vasculature (van Beek et al., 2008; Kuo et al., 2003). If the phase shift is near zero, it is often link to patients with a complete loss of cerebral autoregulation, while a larger phase shift represents an intact autoregulatory response (van Beek et al., 2008).

Coherence within the TFA is used to determine the relationship between blood pressure oscillations and cerebral blood flow oscillations, as well as determine how well the data collected fit the TFA model. A coherence near zero represent no relationship between each variable, while a larger coherence represents a greater linear relationship is present (van Beek et al., 2008). This measure, however, does present limitations. Coherence can be altered by excessive noise within the data, and as such, proper care must be made to ensure accuracy of this value (van Beek et al., 2008). It is recommended that for any band frequency of interest, that coherence is valued at .5 or higher, ensuring that the data fits the TFA model.

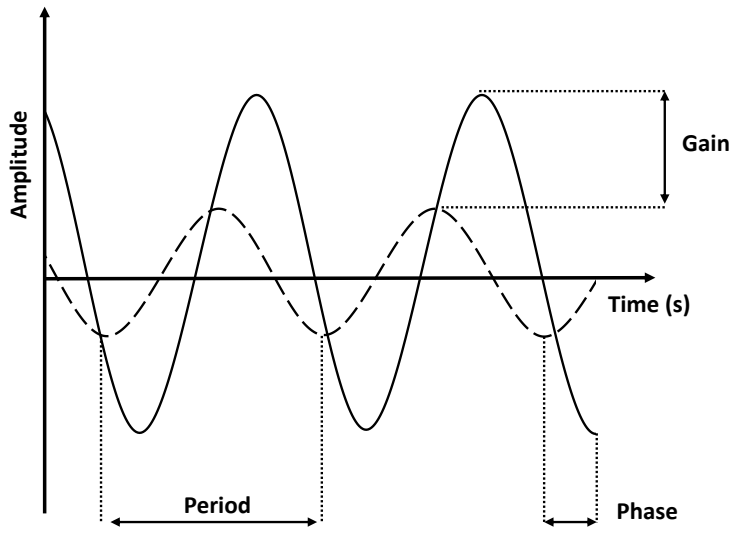

Figure 6. Example of gain and phase, redrawn from van Beek et al. (2008).

Traditionally, a lower phase paired with high coherence and gain is believed to represent impaired cerebral autoregulation, where flow is more largely determined by pressure (Rickards, 2015; Tzeng et al., 2012; van Beek et al., 2008). The opposite is also assumed, where a high phase accompanied by low gain and coherence is believed to show enhanced cerebral autoregulation (Rickards, 2015; Tzeng et al., 2012; van Beek et al., 2008).

It should be noted that, while transfer function analysis is commonly used, there is currently no standardized procedure to perform this analysis. Because of this, comparison of results between studies is hindered and the clinical application of this protocol is diminished. However, Panerai and colleagues (2022) have produced recommended guidelines for the standardization of transfer function analysis. Additionally, although it is recommended that only

data that has a coherence value ≥ 0.5 be reported, some studies report data regardless of meeting coherence thresholds (Worley et al., 2022).

Conclusion

Precise regulation of cerebral blood flow is essential for an individual's health and well-being and without it, disease, injury, and death can occur. The exact mechanisms that account for the automatic control of flow at ranging pressures is not fully understood, however, the evidence implicates an integration of myogenic, metabolic, neurogenic, and endothelial-dependent factors are involved. Of these factors, an endothelium-dependent pathway (e.g. EDHF) that acts independently of NO or COX mechanisms has gone largely under investigated in humans. EDHF, a broad term for a pathway or molecule that causes hyperpolarization of the vascular smooth muscle, has a largely undefined role in human cerebral vaso-regulation and may be integral to advancing our understanding of cerebrovascular health. LBNP shifts central blood volume towards the lower extremities causing a physiologically relevant stimulus that elicits a baroreflex-mediated sympathetic response (Rickards, 2015). 5 minutes of LBNP has been shown to elicit these acute neuronal responses, simulating a wide level of volume depletion depending on pressure and protocol chosen. With the limited investigations into EDHF's contribution to cerebral autoregulation and cerebral control of blood flow, alongside the clinically relevant stressor of simulated hypovolemia, this study can give way to knowledge that can change the way we perceive and understand autoregulation and hypovolemia.

Chapter III

Methodology

Hypovolemic stress can occur during a variety of physiologically relevant stressors, traumas, and illnesses (dehydration, blood loss, acceleration, orthostasis etc.). This loss of blood volume affects numerous homeostatic processes in the body, including the ability to supply adequate blood flow to the brain. The maintenance of perfusion pressure and flow to the brain is vital to survival, where loss of oxygen and glucose delivery to neuronal brain tissue results in decline in cognition, increase in pathology, and death. Cerebral autoregulation, which ensures proper flow is met to the brain regardless of large fluctuations in blood pressure, is not fully understood in any condition and the exact factors that form this physiological process are illusive. EDHF, a mysterious endothelial-derived vasorelaxant, has shown great promise as a contributor of autoregulation and the control of blood flow to the brain, but no study has directly confirmed its role within these processes. This study aims to be the first to directly determine EDHF's role within the cerebrovascular during sympathetic stress in health, young adults.

Research Design

This research design was a true experimental, placebo controlled, crossover study. By using a crossover design, we were able to limit participation to a smaller sample size and more fully understand the effects of EDHF inhibition within single individuals. Further, this study design also strengthened internal and external validity by reducing effects of history, maturation, selection, and more.

Participants

A convenient sample was recruited at the University of Oklahoma by use of flyers, advertisements, email, and word-to-mouth referrals. After which each participant was scheduled for their first session where informed consent and inclusion/exclusion data was collected.

All subjects were healthy young adults, ages 18-30 years old. No participant had any diagnosed or overt evidence of cardiovascular or metabolic disease, chronic illnesses, history of smoking, use of prescription medications or have a BMI > 30 kg/m^3. All females were tested during their follicular phase (days 1-5) to prevent estrogen-linked changes in endothelium-dependent vasodilation (Hashimoto et al., 1995; Mannon et al., 2020).

Inclusion Criteria	Exclusion Criteria
• 18-30 years old. • No cardiovascular or metabolic diseases. • No chronic illnesses. • Systolic pressure < 130mmHg and diastolic pressure < 85mmHg. • BMI < 30 kg/m^2.	• History of smoking • Prescription medications • Pregnancy • Systolic pressure ≥130mmHg and diastolic pressure ≥ 85mmHg. • BMI ≥ 30 kg/m^3

Experimental Protocol

Day 1 - Screening Visit

Subjects were required to attend two experimental visits and one screening visit. During the first visit, informed consent, HIPAA authorization and subject screening was conducted to ensure that all participants met the inclusion criteria and none of the exclusion, as well as to familiarize participants with the experimental protocol. Participants completed a medical history survey, physical activity questionnaire, medication log, and a demographics (e.g. sex assigned at birth, height, weight, ethnicity/race, emergency contact, etc) document. Following the completion of all paperwork, subjects had their anthropometric data and vital signs collected, and were fitted for proper equipment size and positioning. After all inclusion and exclusion criteria had been determined and the subject was deemed eligible, a DXA scan was performed to collect body composition data. Subjects were then scheduled for their second visit.

Days 2 and 3 - Experimental Visits

Experimental visits were identical in protocol except for which treatment the subject received. Placebo and fluconazole treatments were randomly assigned and counter balanced, where, on the third visit, each subject received the opposite treatment that they received on visit 2. Each participant laid in a supine position on an examination table and had their lower body sealed into the lower-body negative pressure (LBNP) chamber at the iliac crest. Participants were then instrumented. Participants were instructed to relax and breath normally in at dark, temperature controlled (22-24^{o}C) laboratory setting. Following rest, baseline measurements were collected for 5 minutes, followed by 5 minutes of LBNP at -20 mmHg, similarly to White et al., 2000. After LBNP, participants were allowed to recover for 5 minutes.

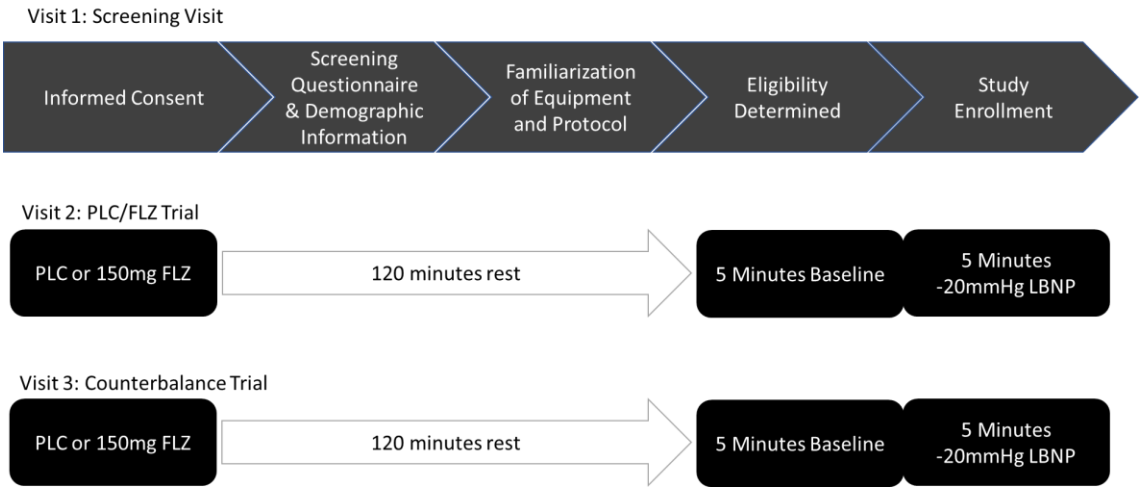

Figure 7. Protocol Outline. Placebo (PLA), Fluconazole (FLZ), Lower Body Negative Pressure (LBNP)

Pharmaceuticals and Placebos

The use of fluconazole has shown to antagonize EDHF effects within the vasculature (Petterson et al., 2021; Trinity et al., 2021; Billien et al., 2006). In this regard, 150mg of fluconazole was used to inhibit EDHF within the cerebral vasculature by inhibiting CYP epoxygenase 2C9. Additionally, 250mg of microcrystalline cellulose was given as placebo.

Measurements

Heart rate (HR) and Breathing frequency (Bf). HR was measured by wireless 2-lead ECG and Bf was measured by chest expansion using the Equivital life monitor system (EQ life monitor, Equivital Limited, Cambridge, United Kingdom) (Liu et al., 2013).

Mean arterial pressure (MAP), Cardiac Output (Q), Stroke Volume (SV), and Total Peripheral Resistance (TPR). MAP, Q, SV, and TPR was measured continuously using a finometer

(Finapres® NOVA, Finapres Medical Systems B.V, Enschede, Netherlands), where pressure was measured at the brachial artery and within the tip of the middle digit of the dominant arm. Participants were instructed to relax their hands during protocol to ensure accurate data from this device. This form of measuring blood pressure has been deemed valid and reliable for tracking mean arterial pressure and has been used in other LBNP protocols (Silke and McAuley, 1998; Waldron et al., 2018; Reisner et al., 2011).

Transcranial Doppler Ultrasonography. Changes in middle cerebral artery velocity (MCAv) were assessed using robotic transcranial doppler ultrasonography (TCD) (2 MHz pulse-wave Robotic TCD probe; Nuerovision Transcranial Doppler Ultrasound, Multigon Industries, Elmsford, New York). Each robotic probe was placed onto the temporal window of the skull with ultrasound gel and affixed to an adjustable headband prior to data collection. Participants were asked to limit movement to ensure that robotic probes accurately maintained the signal required. TCD has been shown to be an accurate surrogate for determining ΔMCA blood flow (Bishop et al., 1986).

Near-infrared Spectroscopy. Cerebral oxygenation was assessed using a small, portable near-infrared spectroscopy device (Portalite, Artinis Medical Systems, Elst, Netherlands) attached to the forehead. A blackout headband was worn to ensure little-to-no light interfered with data collection. NIRS data has been found to accurately measure changes in cerebral oxygenation during brain activation (Villringer et al., 1993). *Oximetry. Arterial* oxygen saturation levels (SpO_2) were automatically measured via earlobe oximeter (Oximeter pod, ADInstruments, Sydney, Australia).

Lower-body Negative Pressure

Using a sealed chamber (Lower Negative Pressure Chamber 1000, Technavance, Austin TX, USA) around the lower extremities, -20mmHg of pressure was removed from the legs for 5 minutes following 120 minutes of rest after ingestion of fluconazole or placebo. This equipment required a seal around the iliac crest to ensure that the instrument does not compromise splanchnic blood flow to the body (Goswami et al., 2019). Following the advice of Claassen et al.'s (2016) white paper, as well as recommendations from Burma and colleagues (2021), LBNP was applied to each subject for a minimum of 5 minutes to allow for adequate data collection to use in transfer function analysis. This form of sympathetic activation is considered a valid protocol to assess hypovolemia within baboons and is largely used in human studies (Hinojosa-Laborde et al., 2014).

Data Acquisition and Analysis

Data was collected throughout the 2021-2023 academic year. The principal investigator for this study was J. Mikhail Kellawan, Ph.D. Other investigators who were responsible for data collection were Jacob Matney, Alexander Buelow, John Ashley, Jiwon Song, Sarah Skillett, Chris Mixon, Amir Akbari, Nathan McKenzie, Tylar Lason, and Jongjoo Sun, Ph.D. All data was collected and stored in the Human Circulation Laboratory and the University of Oklahoma's Department of Health and Exercise Science.

Central cardiovascular and cardiopulmonary variables were collected within LabChart software using PowerLab (ADInstruments, Colorado, USA) at a frequency of 1KHz and averaged beat-to-beat for data processing. Prefrontal cortex oxygenation was collected via

portable NIRS device (Portalite, Artinis Medical Systems, Elst, Netherlands) at a frequency of 10 Hz and was exported as a separate text file for processing. Central cardiovascular and pulmonary were then processed into three second time bins for statistical analysis within Excel (Microsoft Corporation, Redmond, WA, USA). Raw MCAv data was passed through a low-pass filter using SigmaPlot (Systat Software, San Jose, CA, USA) at a filtering of 99.8% of high frequencies, then processed into three second bins. This form of filtering has been shown to significantly lower the amount of standard error in MCAv measurements (Ferreira et al., 2006). Additionally, NIRS data was processed into three second time bins in the same manner. Transfer function analysis of MAP and MCAv data was performed using Ensemble R (Elucimed, Wellington, New Zealand) to produce changes in gain, coherence, and phase between the input variable (blood pressure) and the output variable (MCAv) oscillations. Calculation to determine each patient's cerebrovascular index (CVCi) was completed using the following equation:

$$CVCi = \frac{MCAv}{MAP}$$

All data analysis was performed during the last 30 seconds of rest and last 30 seconds of LBNP

SPSS v.26 (Chicago, IL, USA), Microsoft Excel, and SigmaPlot were used for statistical analysis. Ensemble-R (Elucimed, Dunedin, New Zealand) was used to calculate transfer function analysis variables (gain, coherence, and phase). A Shapiro-Wilks test was performed to check for normality of the data. Repeated measures 2x2 ANOVAs were used to determine the difference between CVCi levels, MCAv, TSI, SV, HR, TPR, and Q. Additionally, Bonferonni post-hoc testing was used to determine any differences in CVCi, MCAv, and TSI from rest to LBNP. Wilcoxon related samples tests were performed to test the significant differences between CVCi

levels, MCAv, TSI, before and after LBNP. One sample t-tests were performed to determine significance from zero for all cerebrovascular measures. Pearson's correlations were performed to determine any significant relationships between IPAQ scores/Relative Drug Dosage and changes in cerebrovascular variables. If data was non-normally distributed a single sample ranks test was performed instead. In the case of single sample signed rank testing or Wilcoxon testing, effect size was calculated using the following equation:

$$r = \frac{Z}{\sqrt{n_1} + \sqrt{n_2}}$$

Alpha was set at .05.

Chapter IV

Results

Subject Demographics and Characteristics

A total of 24 subjects (11 males, 13 females) completed all three study visits, where 16 subjects (7 males, 9 females; 22 ± 3.88 years-old; BMI of 24.19 ± 2.85 kg/m^2) were included for final analysis. One subject was excluded due to lack of MCAv signal, while seven subjects' data were excluded due to technical difficulties during data collection. All subjects were considered healthy, young (≤ 30 years of age), non-obese (BMI < 30kg/m^2) individuals who had not used nicotine products within the previous six months nor used prescription medications. All female data was collected during the first five days of their pre-follicular phase.

Table 3. Participant Characteristics

	Total *(n = 16)*	**Male** *(n=7)*	**Female** *(n = 9)*
Age (yrs)	22 ± 3.88	23.29 ± 4.07	21.00 ± 3.64
Height (cm)	170.44 ± 9.30	177.43 ± 8.36 *	165 ± 5.81
Weight (kg)	70.38 ± 10.34	76.79 ± 9.48 *	65.40 ± 8.31
BMI (kg/m^2)	24.19 ± 2.85	24.34 ± 1.90	24.08 ± 3.53
SBP (mmHg)	114.38 ± 10.30	123 ± 5.26 *	107.67 ± 7.91
DBP (mmHg)	66.94 ± 11.13	62.71 ± 15.26	70 ± 5.45
Waist Circ. (cm)	82.33 ± 8.69	82.31 ± 5.84	82.33 ± 10.77

Hip Circ. (cm)	100.00 ± 6.25	98.5 ± 5.27	101.17 ± 6.99
iPAQ (MET/min/wk)	4050.19 ± 3286.10	5493.36 ± 3911.38	2787.43 ± 2139.75

*Values shown as mean ± SD. * represents significant different from females, p < 0.05. BMI = Body Mass Index, SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure, iPAQ = International Physical Activity Questionnaire*

Central Cardiovascular and Pulmonary Variables

There was no difference in any central cardiovascular or cardiopulmonary variables between treatments (**Table 4**, $p > 0.05$). HR significantly increased in response to -20 mmHg LBNP activation in both conditions (**Table 4,** PLC 60.73 ± 7.51 *vs.* 68.00 ± 10.38 bpm, $p = 0.002$, $\eta_p^2 = -0.50$; FLZ 61.04 ± 5.49 *vs.* 68.31 ± 9.21 bpm, $p = <0.001$, $\eta_p^2 = 0.53$). SV significantly decreased following -20 mmHg LBNP activation in both treatments (**Table 4,** PLC 84.58 ± 20.50 *vs.* 73.33 ± 22.03 mL/beat, $p = <0.001$, $\eta_p^2 = 0.72$; FLZ 88.83 ± 21.97 *vs.* 78.30 ± 22.56 mL/beat, $p = 0.012$, $\eta_p^2 = 0.35$). Furthermore, within the placebo visit, $P_{ET}CO_2$ was significantly reduced from baseline to -20mmHg LBNP (**Table 4,** 41.64 ± 4.98 *vs.* 40.74 ± 4.98%, $p = 0.016$, $\eta_p^2 = 0.33$).

Table 4. Central Cardiovascular and Pulmonary Variables (*n = 16, 7 males, 9 females*)

	Baseline	**LBNP (-20mmHg)**
Q (L/min)		
PLC	5.36 ± 1.29	5.14 ± 1.36
FLZ	5.75± 1.76	5.64 ± 1.63

SV (mL/beat)		
PLC	84.58 ± 20.50	73.33 ± 22.03 †
FLZ	88.83 ± 21.97	78.30 ± 22.56 †
HR (bpm)		
PLC	60.73 ± 7.51	68.00 ± 10.38 †
FLZ	61.04 ± 5.49	68.31 ± 9.21 †
TPR		
PLC	118.67 ± 28.70	124.76 ± 27.98
FLZ	119.53 ± 41.43	122.02 ± 46.19
$P_{ET}CO_2$ (mmHg)		
PLC	41.64 ± 4.98	40.74 ± 4.98 †
FLZ	41.74 ± 4.28	40.37 ± 5.88
MAP (mmHg)		
PLC	99.41 ± 8.97	99.11 ± 8.84
FLZ	100.04 ± 8.58	100.38 ± 9.31

*Values shown as mean ± SD. * represents significant differences between treatment groups, $p < 0.05$. † represents significant differences between baseline and LBNP. PLC = Placebo Treatment, FLZ = Fluconazole Treatment, LBNP = Lower-body Negative Pressure at -20mmHg, Q = Cardiac Output, SV = Stroke Volume, HR = Heart Rate, TPR = Total Peripheral Resistance, $PETCO_2$ = Partial Pressure End-tidal CO_2, MAP = Mean Arterial Pressure*

Cerebral Vascular Variables

No treatment-timepoint interaction within MCAv, CVCi, and TSI were observed (**Table 5,** $p = 0.176$, $p = 0.409$, $p = 0.385$, respectfully**).** However, there was a main effect of time for MCAv and CVCi variables ($p < 0.001$, $p < 0.001$, respectfully). In both treatments, TSI was found to persist with the application of -20 mmHg LBNP (PLC $p = 0.452$, FLZ $p = 0.681$). MCAv and CVCi were found to decrease (-6.7% and -8.3%, respectfully) with application of -20mmHg only within FLZ when compared using 2x2 ANOVA and Wilcoxon testing with medium-to-large effect size (**Table 5 and Table 6**, ANOVA MCAv $\eta_p^2 = 0.56$, ANOVA CVCi $\eta_p^2 = 0.52$ / Wilcoxon MCAv $r = 0.62$, Wilcoxon CVCi $r = 0.55$). Only MCAv and CVCi within the FLZ treatment were found to show a significant decrease from zero with medium to large effect when using single samples testing (**Table 5**, MCAv $r = 0.62$, CVCi $r = 0.55$). No variable showed any significant differences between FLZ or PLC at baseline conditions (**Table 5 and Table 6**). No variable showed any significant differences between FLZ or PLC at -20 mmHg LBNP (**Table 5 and Table 6**). Lastly, there was no correlations between subject's relative dosing to body weight or IPAQ score on SV or any cerebrovascular variables (**Table 7 and Table 8**).

Table 5. Cerebral Vascular Variables (*n = 16, 7 males, 9 females*)

	Baseline	**LBNP (-20mmHg)**	**Δ Baseline-LBNP**
MCAv (cm/s)			
PLC	76.29 ± 12.89	73.18 ± 12.70	-3.11 ± 7.04
FLZ	77.79 ± 11.58	71.18 ± 11.17 †	-6.61 ± 6.00 ψ

CVCi (cm/s/mmHg)			
PLC	0.78 ± 0.15	0.75 ± 0.16	-0.04 ± 0.09
FLZ	0.78 ± 0.13	0.72 ± 0.12 †	-0.06 ± 0.06 ψ
TSI (%)			
PLC	67.90 ± 5.84	68.07 ± 5.52	0.19 ± 0.92
FLZ	66.55 ± 7.64	66.41 ± 7.50	-0.12 ± 1.33

*Values shown as mean ± SD. * represents significant differences between treatment groups, p < 0.05. † Represents significant differences between baseline and LBNP. ψ represents significance from a zero change. PLC = Placebo Treatment, FLZ = Fluconazole Treatment, LBNP = Lower body Negative Pressure at -20mmHg, MCAv = Middle Cerebral Artery Velocity, CVCi = Cerebrovascular Conductance Index, TSI = Total Saturation Index*

Table 6. Non-parametric Wilcoxon Test Results for Cerebrovascular Variables (*n = 16, 7 males, 9 females*)

	Baseline	**LBNP (-20mmHg)**	**P- Value (Baseline-LBNP)**
MCAv (cm/s)			
PLC	76.29 ± 12.89	73.18 ± 12.70	*0.148*
FLZ	77.79 ± 11.58	71.18 ± 11.17 †	***<0.001***
P-Value	*0.756*	*0.836*	
CVCi (cm/s/mmHg)			

PLC	0.78 ± 0.15	0.75 ± 0.16	*0.163*
FLZ	0.78 ± 0.13	0.72 ± 0.12 †	***0.002***
P-Value	*0.836*	*0.438*	
TSI (%)			
PLC	67.90 ± 5.84	68.07 ± 5.52	*0.796*
FLZ	66.55 ± 7.64	66.41 ± 7.50	*0.836*
P-Value	*0.469*	*0.501*	

Values shown as mean ± SD. † Represents significant differences between baseline and LBNP

Table 7. Correlations Between Cardiovascular Variables and Relative Dosage of FLZ (*n = 16, 7 males, 9 females*)

	r	**P-Value**
ΔMCAv (cm/s)	0.073	0.787
ΔCVCi (cm/s/mmHg)	-0.171	0.526
ΔTSI (%)	-0.212	0.430
SV at Rest (mL/beat)	-0.492	0.053
SV at -20mmHg LBNP (mL/beat)	-0.379	0.148

Table 8. Correlations Between Cardiovascular Variables and IPAQ Results (*n = 16, 7 males, 9 females*)

	r	P-Value
ΔMCAv (cm/s)		
PLC	0.051	0.857
FLZ	0.102	0.717
ΔCVCi (cm/s/mmHg)		
PLC	0.201	0.474
FLZ	0.060	0.833
ΔTSI (%)		
PLC	-0.329	0.231
FLZ	0.116	0.680
SV at Rest (mL/beat)		
PLC	0.197	0.482
FLZ	0.085	0.764
SV at -20mmHg LBNP (mL/beat)		
PLC	0.228	0.414
FLZ	0.380	0.162

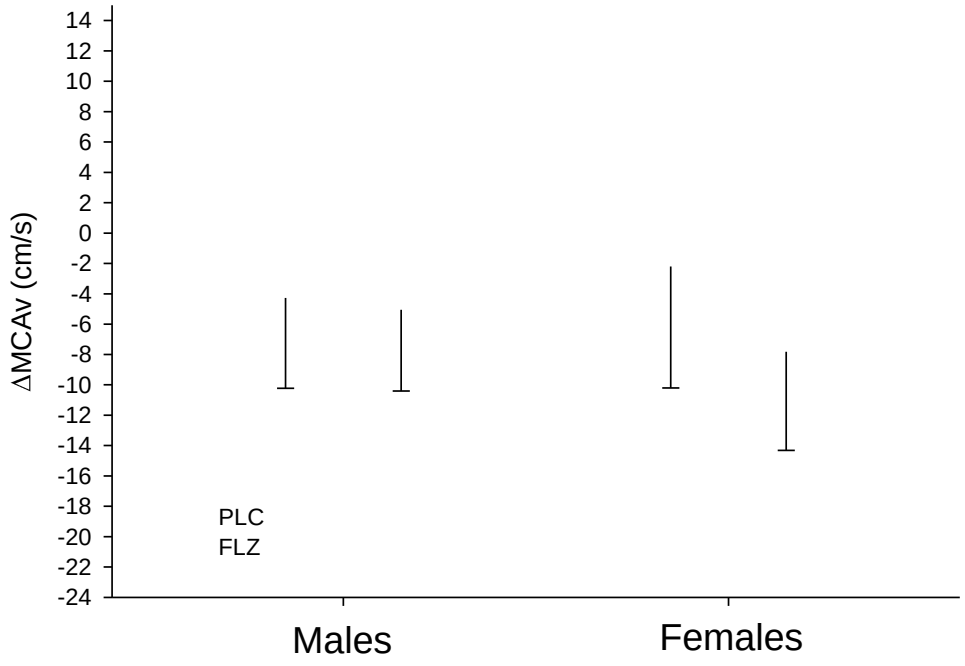

Figure 8. Individual and averaged data (mean ± SD) representing alterations in middle cerebral artery velocity (MCAv) from rest to LBNP at -20mmHg between EDHF-blockade via fluconazole (n = 16) and placebo (n = 16). ΔMCAv was calculated by subtracting the averaged last 30 seconds of baseline by the averaged last 30 seconds of LBNP at -20mmHg. Alpha was set at $P < 0.05$. No significant difference in ΔMCAv was seen between groups, however, only FLZ was observed to have a decrease in ΔMCAv.

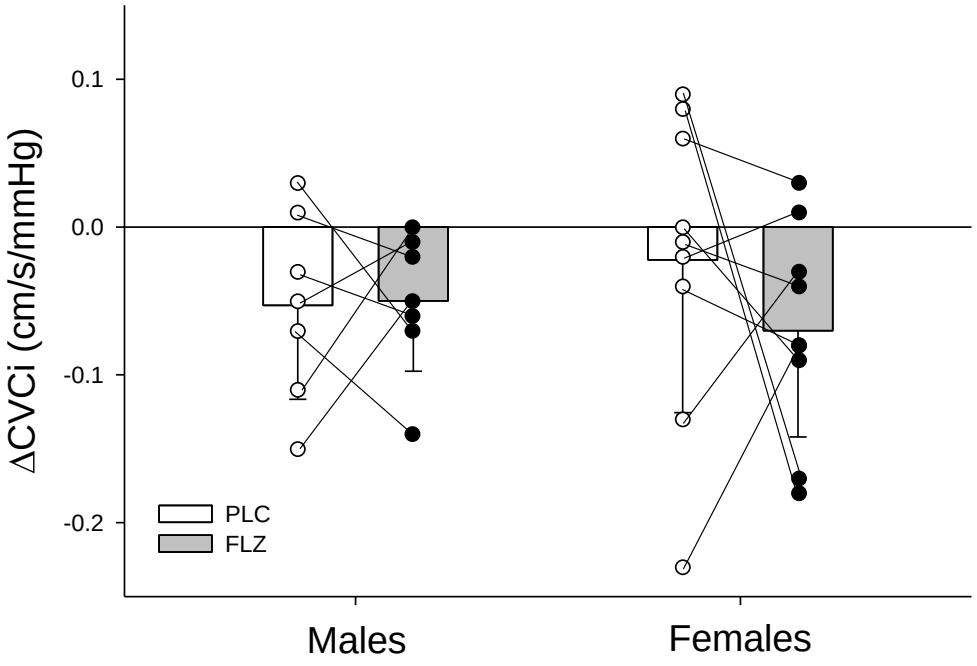

Figure 9. Individual and averaged data (mean ± SD) representing alterations in cerebral vascular conductance index (CVCi) from rest to LBNP at -20mmHg between EDHF-blockade via fluconazole (n = 16) and placebo (n = 16). ΔCVCi was calculated by subtracting the averaged last 30 seconds of baseline by the averaged last 30 seconds of LBNP at -20mmHg. Alpha was set at $P < 0.05$. No significant difference in ΔCVCi was seen between groups, however, only FLZ was observed to have a decrease in ΔCVCi.

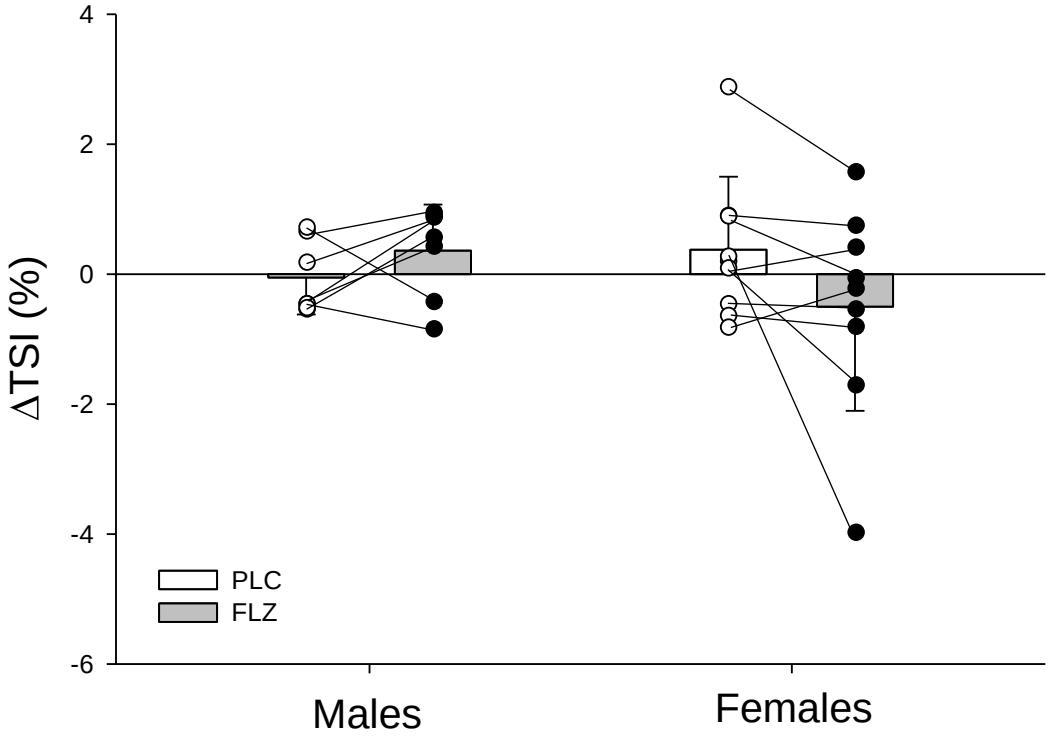

Figure 10. Individual and averaged data (mean ± SD) representing alterations in total saturation index (TSI) from rest to LBNP at -20mmHg between EDHF-blockade via fluconazole (n = 16) and placebo (n = 16). ΔTSI was calculated by subtracting the averaged last 30 seconds of baseline by the averaged last 30 seconds of LBNP at -20mmHg. Alpha was set at $P < 0.05$. No significant difference in ΔTSI was seen between either group. Neither PLC or FLZ were different than zero.

Transfer Function Analysis

Due to low coherence for many subjects, statistical testing of transfer function analysis was not able to be performed. However, TFA results can be found in the appendix.

Chapter V

Discussion

The purpose of the current study was to investigate EDHF's contribution to cerebral hemodynamics in healthy, young subjects. In partial agreement with the first hypothesis of the study, the results indicated that EDHF blockade using fluconazole did not alter resting MCAv or CVCi – but did attenuate blood flow responses during sympathetic activation by -20 mmHg of lower body negative pressure. Our second hypothesis, which used transfer function analysis to assess autoregulatory control, could not be interpreted due to the subject's low coherence levels at rest and during lower body negative pressure activation. Thirdly, the data disagrees with the third hypothesis of the study, where prefrontal cortex brain oxygenation was found to persist at rest and during sympathetic activation, regardless of treatment.

Cerebral Hemodynamics and Endothelial-dependent Factors

This study found that resting, steady-state cerebral hemodynamic variables were unchanged with the inhibition of EDHF. This is the first time, to our knowledge, that MCAv, CVCi, and TSI have been measured during in vitro human EDHF-blockade studies during rest. However, in protocols that measure flow within the periphery, projects have shown that EDHF does not play a significant role in resting hemodynamics – where blockade of EDHF using fluconazole did not significantly alter forearm blood flow at rest in healthy adults (Ozkor et al., 2010). Our data indicates similar findings in cerebral vasculature. Additionally, EDHF has been observed to provide a redundancy mechanism within the body's vessels at rest (Golding et al., 2002). In the same experiment, Ozkor and colleagues (2010) observed that EDHF-inhibition only

further significantly altered blood flow after the removal of NO synthesis. Because of this, it would stand to reason that our result may also represent this finding in the cerebral vasculature, where resting blood flow was preserved during EDHF-knock out only. Inhibition of nitric oxide production, one of the most researched vasomodulatory molecules in the body, has been shown to decrease cerebral blood flow and autoregulatory capacity in healthy subjects (Preckel et al., 1996; White et al., 2000; Carter et al., 2021). Similarly, cyclooxygenase pathway blockade, which causes endothelial-derived alterations in vessel tone by the production of prostaglandins, also plays a large role in the control of blood flow to the brain (Peltonen et al., 2015; Shoemaker et al., 2021; Kellawan et al., 2020).

Although clearly major determinants of brain hemodynamics, COX and NO-mediated dilation do not account for all endothelial-dependent vasorelaxation within the cerebral vasculature (Golding et al., 2002; Petersson et al., 1995). Petersson and colleagues (1995) demonstrated that, following NO blockade and COX blockade, human pial vessels were still capable of relaxation, attributing the remaining vasodilatory reserve to EDHF. Even so, research indicating whether EDHF is a strong effector of brain hemodynamics and oxygenation within in vitro humans is virtually non-existent. The current study displayed that FLZ did not affect resting MCAv or CVCi values. Interestingly, however, we saw an attenuated MCAv and CVCi response within EDHF-inhibited cerebral vessels during the onset of -20 mmHg of LBNP. This suggests that EDHF plays an important role in resisting sympathetic-mediated vasoconstriction during light simulated hypovolemia rather than maintaining flow at steady state conditions. Additionally, EDHF may prove to be an endothelial-component of autoregulation, where CYP450-blockade resulted in decreased ability to maintain proper flow during bouts of

hypovolemic stress. This finding provides further evidence of EDHF's existence within human cerebral arteries and shows that they do play a role in the control of blood throughout the brain.

Pre-frontal cortex oxygenation differences between the FLZ and PLC were insignificant during rest and during -20mmHg LBNP. Moreso, Δ TSI Rest-LBNP was also similar between each treatment group, suggesting that pre-frontal cortex oxygenation was not altered by EDHF blockade or by slight hypovolemia. This is unsurprising, as neither treatment nor time point (rest or LBNP activation) required excessive increases in pre-frontal cortex activation, eliminating the need for increased uptake of oxygen for metabolic processes (thus limiting neurovascular coupling). Furthermore, since our finding suggests that MCAv responses are similar between treatments, it is likely that neither group would present different oxygenation outcomes.

Central Cardiovascular Effects of Lower Body Negative Pressure

This protocol used lower body negative pressure to increase sympathetic outflow to the body, a method used by numerous research teams to simulate orthopedic stress and hemorrhage (Goswami et al., 2019; Levine et al., 1994; Rickards et al., 2015; Rickards et al., 2015; Zhang et al., 1998; Worley et al., 2023; Rosenberg et al., 2021). The methods through which lower body negative pressure elicits increases in sympathetic outflow are multifaceted. Firstly, LBNP pressure shifts central blood volume towards the legs, lowering blood pressure and activating the cardiac baroreflex response, resulting in increases in HR to maintain blood pressure (Goswami et al., 2019; Cooke et al., 2004; Rosenberg et al., 2021; Rickards, 2015; Rickards et al., 2015; Levine et al., 1994). Our data coincides with this, where subject heart rate significantly increased following activation of -20mmHg of LBNP for both treatments. Moreover, HR at rest or during -

20 mmHg of LBNP were not different between treatments. Secondly, LBNP activity lowers central perfusion pressure in subjects – diminishing preload of the heart - which results in a sympathetically-mediated vasoconstriction response to increase venous return (Goswami et al., 2019; Cooke et al., 2004; Rosenberg et al., 2021; Rickards, 2015; Rickards et al., 2015; Levine et al., 1994). The current data supports this as well, as FLZ and PLC were found to have significant decreases in stroke volume following -20 mmHg of LBNP. Additionally, SV at rest or during -20 mmHg LBNP was not different between treatments, as well.

This experiment confirms that lower body negative pressure enhanced sympathetic outflow for both treatments in this study and that subjects received approximately the same level of sympathetic activation regardless of treatment visit. We found no central cardiovascular or cardiopulmonary differences between FLZ and PLC visits, suggesting that systemically administered EDHF-blockade did not greatly influence baroreflex mediated responses to LBNP from rest.

Implications of the Current Study

The results of this study indicate that EDHF does not play a role in the maintenance of MCAv, CVCi, or TSI during resting conditions (**Table 5**). This is likely due to unhindered NO and prostacyclin synthesis, where EDHF most likely plays a more valuable role as a redundant mechanism at rest – contributing to resting blood flow only when NO or COX is inhibited (Ozkor et al., 2010; Nishikawa et al., 2000). Currently, no study has examined this effect within the cerebral vasculature in humans. Because of this, future studies should examine cerebral vascular response to EDHF+NO/COX-blockade to verify that the vessels in the brain respond

similarly to the vessels in the periphery following EDHF knock out. If future research collaborates with our findings, while also observing NO/COX-mediated EDHF inhibition, it could provide valuable insight into the understanding and treatment of diseases with endothelial disfunction (such as dementia, aneurysm, stroke, and other cerebrovascular disease) (Wang et al., 2018; Sheinberg et al., 2019; Cosentino et al., 2001).

Additionally, this experiment strengthens the claim that EDHF is present in the human cerebrovasculature and plays a role during active vascular control like during a sympathetic stress via mild hypovolemia. Our data suggests that dynamic vascular control of blood flow during sympathetic activation mediated by cardiac baroreflex control is attenuated during EDHF-inhibition (**Table 5, Figure 9**)**.** Moreso, this interpretation is evident by the attenuated CVCi response during -20mmHg LBNP **(Table 5, Figure 10)**. Again, this gives credibility to EDHF's control of blood flow during sympathetic stress, like other common endothelial-derived molecules (Preckel et al., 1996; White et al., 2000; Carter et al., 2021; Peltonen et al., 2015; Shoemaker et al., 2021; Kellawan et al., 2020).

Due to the present study being the first to study MCAv and CVCi responses during EDHF-blockade in vitro within humans, our findings lay the groundwork for future EDHF-related cerebrovascular studies – specifically during disease states and during other physiological stresses like exercise, altitude, and injury. Future studies should investigate the effects EDHF during postural changes, exercise and during cognitive functioning to determine if inhibition alters commonly performed tasks. This would allow us to obtain a better understanding of EHDF's role in commonly experienced, multifaceted increases in sympathetic activity that are typically weakened with aging, injury, and disease.

Limitations and Methodological Considerations

We recognize that there were several limitations in our experimental design for this study. Firstly, due to the use of questionnaires and verbal reporting, we had to rely on subjects to be truthful when reporting their physical activity, health status, menstrual phase, medication use, and pre-study requirements prior to each visit.

Secondly, we systemically administered fluconazole to subjects orally through 150mg tablets. This method of drug administration has the possibility to invoke and alter unwanted cardiovascular reflexes during the protocol, however, our data suggests that these alterations are unlikely seeing as none of the central cardiovascular or pulmonary variables were significantly altered during baseline and steady state sympathetic activation between treatment visits (**Table 4**). Additionally, due to the invasive, often dangerous, and difficult task of administering pharmaceuticals directly to the brain's circulation, researchers tend to avoid the risks involved and opt to use systemic methods of drug administration in order to protect the subject's health (Kellawan et al., 2020; Peltonen et al., 2015; Shoemaker et al., 2021). Furthermore, like previous research protocols, this study gave each subject the same absolute dose of fluconazole (150mg), regardless of body mass variation (Petterson et al., 2021). However, when the drug is prescribed to treat fungal infections, it is commonly prescribed between the dosage of 100mg and 200mg regardless of body size – because of this, we are confident that our dosage was adequate to see physiological responses (Grant and Clissold, 1990).

Third, this experiment used transcranial doppler ultrasonography to measure blood flow velocity through the MCA, not flow itself. Because of this, absolute changes in blood velocity are not able to indicate absolute changes in blood flow through the MCA. However, assuming MCA diameter remains constant, changes in blood flow velocity have been shown to be a strong

indicator of changes in cerebral blood flow when using TCD ($r = 0.849$, $p = < 0.001$), allowing us to make accurate conclusions on EDHF's effect on flow (Bishop et al., 1986). Further, recent studies have that MCA diameter is sensitive to arterial content of CO_2. However, our data finds that $PetCO_2$ (common surrogate for arterial CO_2 content) remained constant, with less than 1.4 mmHg change from rest to lower body negative pressure. A range in which MCA diameter has been found to be stable using high-field MRI (Verbree et al., 2015). Moreso, TCD was recorded on separate visits (PLA vs FLZ). However, like other research teams, we recorded the location, depth, and gate of subject's MCA locations to ensure each visit's data collection was closely matched (Worley et al., 2023).

In addition, we measured pre-frontal cortex oxygenation rather than global oxygenation using near-infrared spectroscopy. However, due to the lack of stimuli during the study, which would increase neural metabolic demand as well as the MCAv being located within the pre-frontal cortex, we believe that our measure of oxygenation is representative of global oxygenation because any alterations seen at up-stream locations would permeate to other locations of the brain.

Fourth, we assumed that -20mmHg of lower-body negative pressure shows the same effect on everyone, regardless of size or sex. This is a common assumption when using LBNP primarily due to the volatility in responses between individuals and the inconsistency of blood redistribution estimates (Rickards et al., 2015; Rickards et al., 2015; Rosenberg et al., 2021; Levine et al., 1994; Goswami et al., 2019). However, due to the nature of the current study's design, each participant is in its own control – allowing for interpersonal differences in relative LBNP to be limited due to the high intrapersonal reliability of LBNP (Goswami et al., 2019).

Lastly, the use of TFA on healthy young subjects without forced oscillations in blood pressure is not recommended due to tight autoregulatory capacity. Without forced oscillations in blood pressure using oscillatory LBNP or sit to stand maneuvers, transfer function analysis is unable to detect such minute changes in autoregulation in this population. These oscillations display an artificially enhanced coherence level, which as stated, determines the trustworthiness of all other TFA measures and have been done by a large portion of TFA research (Rickards et al., 2015; Rickards, 2015; Smirl et al., 2014). Furthermore, without these artificial increases, coherence can become so low that other variables are not able to be calculated at all, resulting in NA values.

Chapter VI

Conclusion

The purpose of the current study was to identify the contribution of EDHF to cerebral hemodynamics and oxygenation during simulated hypovolemia in young, healthy adults.

Main Findings

1. We partially accept the first hypothesis of the study that EDHF-blockade would result in decreases in MCAv and CVCi, as well as increases in MAP at rest and during simulated hypovolemia in healthy, young subjects.
2. We are unable to evaluate the second hypothesis that EDHF-blockade would result in impaired cerebral autoregulation (which would manifest as an increase in gain and decrease in phase) due to coherence being too low in healthy, young subjects.
3. We reject the third hypothesis that EDHF-blockade results in a maintained cerebral oxygenation at rest and attenuated oxygenation during simulated hypovolemia in healthy, young subjects.

In conclusion, we found the EDHF does not play a significant role in the maintenance of resting steady state MCAv, CVCi, or pre-frontal cortex oxygenation. This suggests that EDHF plays a non-obligatory, redundant role in determining blood flow at rest. However, due to the significant alterations in MCAv and CVCi from rest to LBNP following EDHF-blockade, paired with significant differences from zero within FLZ only, it is likely that EDHF plays a role in the dynamic, possibly autoregulatory, response caused by simulated hypovolemia. Future research should investigate the alterations in cardiovascular kinetics between rest and hypovolemia within EDHF-blunted subjects.

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Appendix

			Coherence			
Sub_ID	P_Base	P_LBNP	Δ	F_Base	F_LBNP	Δ
101	0.1831	0.2473	0.0642	0.2876	0.2573	-0.0303
105	0.3691	0.2946	-0.0745	0.0334	0.223	0.1896
106	0.1923	0.4803	0.288	0.243	0.3216	0.0786
108	0.2272	0.4825	0.2553	0.5098	0.2883	-0.2215
201	0.6326	0.2713	-0.3613	0.7691	0.8779	0.1088
203	0.6568	0.4603	-0.1965	0.5422	0.2471	-0.2951
205	0.494	0.3701	-0.1239	0.5516	0.219	-0.3326
206	0.72	0.2771	-0.4429	0.1691	0.5203	0.3512
207	0.2679	0.2074	-0.0605	0.5931	0.5546	-0.0385
210	0.5711	0.5783	0.0072	0.3142	0.3532	0.039
212	0.914846	0.752102	-0.16274	0.568854	0.377668	-0.19119
220	0.58012	0.319703	-0.26042	0.625957	0.601916	-0.02404
221	0.630462	0.833293	0.202831	0.52431	0.481957	-0.04235

			Phase			
Sub_ID	P_Base	P_LBNP	Δ	F_Base	F_LBNP	Δ
101	NaN	NaN	#VALUE!	-0.0238	2.9715	2.9953
105	-1.6029	-3.073	-1.4701	NaN	NaN	#VALUE!
106	NaN	-2.917	#VALUE!	NaN	-2.436	#VALUE!
108	NaN	-0.566	#VALUE!	-1.418	-1.662	-0.244
201	-1.558	-0.202	1.356	-1.405	-0.278	1.127
203	-1.615	-2.416	-0.801	0.894	-0.129	-1.023
205	-0.512	NaN	#VALUE!	0.172	NaN	#VALUE!
206	0.014	NaN	#VALUE!	NaN	-0.216	#VALUE!
207	NaN	NaN	#VALUE!	-2.871	-1.173	1.698
210	0.337	-1.259	-1.596	-2.347	NaN	#VALUE!
212	-0.87675	-2.14162	-1.26487	-1.88721	-1.7307	0.15651
220	-0.31755	NaN	#VALUE!	2.01356	1.94801	-0.06555
221	-1.5036	-2.01008	-0.50648	-0.65955	-1.23195	-0.5724

			Gain				
Sub_ID	**P_Base**	**P_LBNP**	**Δ**		**F_Base**	**F_LBNP**	**Δ**
101	NaN	NaN	#VALUE!		2.224	0.351	-1.873
105	0.688	0.686	-0.002		NaN	NaN	#VALUE!
106	NaN	0.762	#VALUE!		NaN	0.789	#VALUE!
108	NaN	1.002	#VALUE!		0.755	0.731	-0.024
201	0.684	1.193	0.509		0.747	1.046	0.299
203	0.92	0.768	-0.152		1.089	2.2	1.111
205	0.847	NaN	#VALUE!		0.849	NaN	#VALUE!
206	0.404	NaN	#VALUE!		NaN	0.507	#VALUE!
207	NaN	NaN	#VALUE!		NaN	1.842	#VALUE!
210	2.444	1.514	-0.93		1.995	NaN	#VALUE!
212	0.72748	0.989986	0.262506		0.66469	0.59681	-0.06788
220	0.980997	NaN	#VALUE!		1.91031	0.919894	-0.99042
221	1.40448	0.363715	-1.04077		2.04667	0.620999	-1.42567

			nGain				
Sub_ID	**P_Base**	**P_LBNP**	**Δ**		**F_Base**	**F_LBNP**	**Δ**
101	NaN	NaN	#VALUE!		4.446	0.682	-3.764
105	1.05	1.099	0.049		NaN	NaN	#VALUE!
106	NaN	1.055	#VALUE!		NaN	1.195	#VALUE!
108	NaN	1.78	#VALUE!		1	1.1	0.1
201	0.785	1.42	0.635		0.988	1.401	0.413
203	1.446	1.393	-0.053		1.51	3.345	1.835
205	1.078	NaN	#VALUE!		0.942	NaN	#VALUE!
206	0.519	NaN	#VALUE!		NaN	0.619	#VALUE!
207	NaN	NaN	#VALUE!		1.714	1.282	-0.432
210	2.972	1.857	-1.115		2.331	NaN	#VALUE!
212	1.12403	1.38854	0.26451		0.941124	1.09862	0.157496
220	1.08496	NaN	#VALUE!		2.06559	0.997847	-1.06774
221	1.70692	0.459595	-1.24733		2.59391	1.0448	-1.54911

			BP Power				
Sub_ID	P_Base	P_LBNP	Δ		F_Base	F_LBNP	Δ
101	9.294	76.139	66.845		9.397	20.652	11.255
105	2.569	7.176	4.607		593.382	29.943	-563.439
106	188.566	8.502	-180.064		4.404	5.967	1.563
108	10.576	10.646	0.07		9.231	32.947	23.716
201	6.336	15.186	8.85		129.097	35.85	-93.247
203	5.643	55.939	50.296		3.816	17.985	14.169
205	58.648	69.562	10.914		17.838	28.817	10.979
206	834.313	128.894	-705.419		45.423	38.82	-6.603
207	107.039	24.371	-82.668		18.467	34.482	16.015
210	8.034	3.782	-4.252		2.728	3.225	0.497
212	78.1625	79.0478	0.8853		8.93136	23.296	14.36464
220	29.0454	53.1603	24.1149		368.998	164.135	-204.863
221	3.67103	440.143	436.472		37.1834	67.4278	30.2444

			FV Power				
Sub_ID	P_Base	P_LBNP	Δ		F_Base	F_LBNP	Δ
101	5.876	3.426	-2.45		3.426	47.289	43.863
105	2.773	3.609	0.836		75.731	12.03	-63.701
106	28.874	7.164	-21.71		2.002	3.81	1.808
108	4.716	9.977	5.261		8.266	21.381	13.115
201	4.241	18.685	14.444		79.15	41.151	-37.999
203	7.925	33.627	25.702		5.063	108.94	103.877
205	62.477	119.368	56.891		22.186	90.862	68.676
206	172.286	19.736	-152.55		40.092	20.857	-19.235
207	545.941	71.987	-473.954		74.806	86.654	11.848
210	48.714	8.979	-39.735		7.692	23.552	15.86
212	44.0243	94.9503	50.926		5.65177	16.5515	10.89973
220	33.3004	33.9342	0.6338		2536.1	134.386	-2401.71
221	10.095	68.5569	58.4619		240.412	38.9206	-201.491

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**

AUTHORIZATION TO USE or SHARE
HEALTH INFORMATION[1] THAT IDENTIFIES YOU FOR RESEARCH
An Informed Consent Document for Research Participation may also be required.
Form 2 must be used for research involving psychotherapy notes.

Title of Research Project: **Endothelial Derived Hyperpolarization Factor and regulation of cerebral and muscle blood flow.**

Leader of Research Team: **J. Mikhail Kellawan, Ph.D**

Address:

Department of Health and Exercise Science

University of Oklahoma

1401 Asp Ave., Room 112

Norman, OK 73019

Phone Number**: (405) 325-9028**

If you decide to sign this document, University of Oklahoma Health Sciences Center (OUHSC) researchers may use or share information that identifies you (protected health information) for their research. Protected health information will be called PHI in this document.

PHI To Be Used or Shared. Federal law requires that researchers get your permission (authorization) to use or share your PHI. If you give permission, the researchers may use or share with the people identified in this Authorization any PHI related to this research from your medical records and from any test results. Information used or shared may include all information relating to any tests, procedures, surveys, or interviews as outlined in the consent form; medical records and charts; name, address, telephone number, date of birth, race, government-issued identification numbers, and exercise testing information such as cerebrovascular and cardiovascular measurements.

Purposes for Using or Sharing PHI. If you give permission, the researchers may use your PHI to evaluate risk of adverse effects occurring during testing and to assess if there are physiological

[1] **Protected Health Information includes all identifiable information relating to any aspect of an individual's health whether past, present or future, created or maintained by a Covered Entity.**

differences in cerebrovascular and cardiovscular responses to exercise and sympathetic stress after EDHF blockade when compared to a placebo.

Other Use and Sharing of PHI. If you give permission, the researchers may also use your PHI to develop new procedures or commercial products. They may share your PHI with other researchers, the research sponsor and its agents, the OUHSC Institutional Review Board, auditors and inspectors who check the research, and government agencies such as the Food and Drug Administration (FDA) and the Department of Health and Human Services (HHS), and when required by law. The researchers may also share your PHI with no one else.

Confidentiality. Although the researchers may report their findings in scientific journals or meetings, they will not identify you in their reports. The researchers will try to keep your information confidential, but confidentiality is not guaranteed. The law does not require everyone receiving the information covered by this document to keep it confidential, so they could release it to others, and federal law may no longer protect it.

YOU UNDERSTAND THAT YOUR PROTECTED HEALTH INFORMATION MAY INCLUDE INFORMATION REGARDING A COMMUNICABLE OR NONCOMMUNICABLE DISEASE.

Voluntary Choice. The choice to give OUHSC researchers permission to use or share your PHI for their research is voluntary. It is completely up to you. No one can force you to give permission. However, you must give permission for OUHSC researchers to use or share your PHI if you want to participate in the research and, if you cancel your authorization, you can no longer participate in this study.

Refusing to give permission will not affect your ability to get routine treatment or health care unrelated to this study from OUHSC.

Canceling Permission. If you give the OUHSC researchers permission to use or share your PHI, you have a right to cancel your permission whenever you want. However, canceling your permission will not apply to information that the researchers have already used, relied on, or shared or to information necessary to maintain the reliability or integrity of this research.

End of Permission. Unless you cancel it, permission for OUHSC researchers to use or share your PHI for their research will never end.

Contacting OUHSC: You may find out if your PHI has been shared, get a copy of your PHI, or cancel your permission at any time by writing to:

Privacy Official University of Oklahoma Health Sciences Center PO Box 26901 Oklahoma City, OK 73190	or	Privacy Board University of Oklahoma Health Sciences Center PO Box 26901 Oklahoma City, OK 73190
If you have questions, call: (405) 271-2511	or	(405) 271-2045.

Access to Information. 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 is completely finished. You consent to this temporary restriction.

Giving Permission. By signing this form, you give OUHSC and OUHSC's researchers led by the Research Team Leader permission to share your PHI for the research project listed at the top of this form.

Patient/Participant Name (Print): _____

_____ _____
Signature of Patient-Participant Date
or Parent if Participant is a minor

Or

_____ _____
Signature of Legal Representative** Date

**If signed by a Legal Representative of the Patient-Participant, provide a description of the relationship to the Patient-Participant and the authority to act as Legal Representative:

_____
OUHSC may ask you to produce evidence of your relationship.

A signed copy of this form must be given to the Patient-Participant or the Legal Representative at the time this signed form is provided to the researcher or his representative.

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

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:** _____