Title: Human cerebral blood flow regulation: sex, mechanism, and stress differences

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Contents

I. Abbreviations/Acronyms

1. CBF Cerebral Blood Flow
2. CVC Cerebrovascular Conductance, CVC=CBF/BP
3. BP Blood Pressure
4. MABP Mean Arterial Blood Pressure
5. HR Heart Rate
6. SpO2 Oxygen saturation by pulse oximetry
7. ET CO2 end tidal CO2 (measure in exhaled breath)
8. Hypoxia, condition where participants breathe reduced amounts of oxygen (~11% vs 21%), resulting in lower oxygen levels in blood. Easily reversible when returned to room air breathing
9. Hypercapnia, condition where participants breathe increased amounts of carbon dioxide (5% vs >1%), resulting in higher levels in blood. Easily reversible when returned to room air breathing.
10. COX Cyclooxygenase enzyme that produces fatty acids that can cause vasoconstriction or vasodilation
11. Indomethacin, (Indo) COX inhibitor

II. Key Personnel

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1. PROJECT SUMMARY

Cerebrovascular disease is the third leading killer in the U.S. and contributes to decreased quality of life and increased long-term care spending. The risk of cerebrovascular disease is inversely associated with resting cerebral blood flow (CBF). Men exhibit a lower resting CBF and have twice the risk of cerebrovascular disease when compared to premenopausal women. The ability of cerebral vessels to respond to challenges is also inversely related to disease risk and may be useful in identifying at-risk patients pre-clinically. However, these studies are often confounded by aging and/or comorbidities, and the associations provide little insight into physiologic mechanisms responsible for sexually dimorphic cerebrovascular disease risk. Conversely, animal studies use supraphysiologic levels of hormone treatment in primarily young animals, which limits the translational relevance of animal CBF mechanisms. While there is general agreement that estrogen is protective in healthy adults, the basic impact of sex, and physiologic fluctuations in sex hormones, on mechanisms of CBF control remains unclear. The overall goal of this research program is to investigate the mechanisms which actively control cerebral blood flow (CBF) in humans, particularly how men and women differ in control mechanisms on a regional basis throughout the brain circulation; regional differences may help explain some of the pathophysiology of non-uniform brain diseases like stroke or Alzheimer's Disease. We propose to study CBF control mechanisms in healthy younger (18-40 yrs) adult men and women. The overall hypothesis is that female sex and sex hormones contribute to larger stress-induced increases in CBF, due to greater prostanoid (COX) and nitric oxide (NOS) dilation. A key technological innovation of this proposal derives from multi-mode, high-resolution, flow sensitive MRI to quantify CBF at macrovascular and microvascular levels, at rest, and in response to environmental challenges (stress test for the brain). Additionally, the research design allows us to quantify sex differences in two vascular control mechanisms across all brain regions. Our preliminary data demonstrate: hypoxic cerebral vasodilation is 60-100% higher in women compared to men, COX inhibition reduces dilation in women but *not* men. Those concepts will be tested in Aims 1-2 of the grant in this current proposal, covered in what we refer to as **Phase 1 (Aims 1-2)** using technical innovative MRI and pharmacologic tools to test potential sex specific mechanisms of CBF control. The conceptual innovation is planned in Aim 3 of the grant, what we refer to as **Phase 2 (Aim 3)**. We are introducing the concept of Phase 2 in this IRB submission to frame it in the context of Phase 1, but will seek full IRB approval in spring of 2023. Participants must complete Phase 1 studies to continue to Phase 2. Study procedures in Phases 1 and 2 are identical, but we conduct them twice: once in the context of sex hormone suppression, and a second time during a *single* hormone replacement (during suppression), to study the independent impact of testosterone (men) and estrogen (women) on CBF control mechanisms.

We have substantial preliminary findings that support our hypotheses, and have integrated physiologic, pharmacologic, and MRI approaches to test our hypotheses. This state-of-the-art approach will yield previously unattainable insight into not only maintaining basal CBF, but actively controlling it during physiologic demands for increased flow. These novel, high resolution, regionally-specific, sex-specific, and mechanism-specific findings will serve as a knowledge platform for designing sex-specific CBF studies in high risk disease populations (e.g. diabetes, hypertension, Alzheimer's) which exhibit strong sex-specific etiology and important vascular contributions.

2. SIGNIFICANCE

2.1. Synopsis

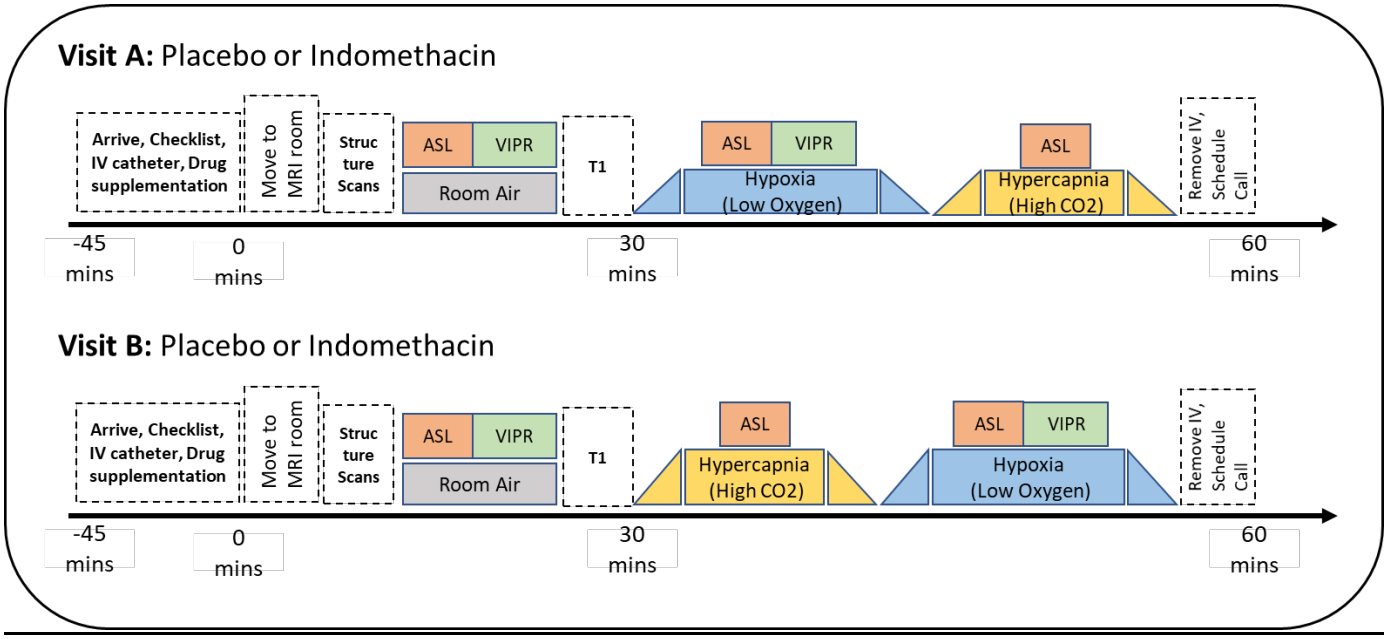
Objective: To determine cerebrovascular control mechanisms in humans and provide mechanistic knowledge to offer new *sex-specific* therapeutic options for cerebrovascular diseases. The **current objective** is to determine how sex and sex hormones influence CBF control in healthy young adults without confounds of age or disease. **Our central hypothesis** is men exhibit reduced cerebral vasodilator function due in part to differences in COX signaling compared to women. Comprehensive CBF data from

multi-modal MRI indicate the magnitude of sex differences—as well as the vasodilator mechanisms—are regionally distinct. We will address 2 specific aims: 1) Are cerebral vasodilator responses greater in women across other physiologic stressors? Do all cerebral vessels respond equally, or are there regional differences—by sex? 2) What mechanisms regulate the *increase* in CBF to stress; do these differ by sex, or brain region? This study will be conducted in compliance with federal investigational drug regulations (21 CFR 312) and Good Clinical Practice (GCP) guidelines, as well as state law and institutional policies.

Study Population: **Phase 1** includes 114 total participants (57 were enrolled in the previously approved protocol (pre-CP011) and 57 participants will be recruited for the updated (CP011) protocol (30 men and 27 women)). These subjects will complete both Aim 1 and Aim 2 who are ≥18 - ≤ 40 years old and considered healthy. Participants who completed the study pre-CP011 will be invited to complete the study again.

Approach: CBF testing will be performed in research-dedicated 3T magnets. Aims 1 and 2 studies the same 30 men and 27 women. Each sub-aim in Aim 2 requires 30 men and 27 women to interrogate a specific mechanism test or COX (Aim 2). Subjects will experience one hypoxia and one hypercapnia trial visit during one visit. Since prevalent sex differences exist to hypoxia, study design focuses on drug supplementation *during* one visit of hypoxia and hypercapnia to manipulate *active* vascular signaling during stress. We will use Indomethacin to test COX as a potential mechanism explaining sex differences in CBF control. To do this, we will conduct 2 MRI visits in a double-blind placebo controlled design. Studies in women will be menstrual cycle controlled (cycle days 1-7), such that both sexes will be studied in their native hormone state.

Phase 1: MRI visits A and B (Aims 1 and 2):
See figure below. Aim 1A is addressed by the first half of the panel (Hypoxia). Aim 1B is addressed by the second half of the panel (hypercapnia). Aim 2 is addressed during one of the randomized study visits when the subject orally takes the study drug to test COX (via indomethacin) mechanism.



Study Drugs:

- Phase 1
  - Indomethacin to inhibit cyclooxygenase (COX) in Aim 2

- By design, subjects will receive the study drug during a randomized study visit. The study drug will be indicated to subject during consent process.
- Subjects will be administered the study drug by a study team member ~45 mins prior to entering the MRI suite.
- In consultation with our study physician, Dr. Eldridge, we propose no direct MD oversight is needed during the study visit as the main risk for healthy adults for Indomethacin is GI upset/nausea. We regularly conduct visits with exposure to high CO_2 and/or low oxygen for the duration and magnitude experienced during these MRI visits in lab settings without MD oversight. Previously conducted studies in our lab have been approved by the IRB to utilize high CO_2 and low oxygen gas exposure during MRI visits and during regular laboratory visits.
    - Physician intervention was never utilized during the hypercapnia or hypoxia portions of the study. Physicians were on call or on site to administer the intervention drug through an IV and to monitor the subject from that point forward (post hypoxia gas being started). That requirement was due to the drug administration, not risks related to gas challenges.
    - In the current version of this protocol, the physicians were not on call or on site for hypercapnia study visits. The effects of the hypercapnia gas are safe, well tolerated, and easily reversible by switching to room air. The same holds for a hypoxia gas exposure.
    - Historically, the IRB has approved numerous study protocols for the Schrage Lab that have used hypercapnia or hypoxia. Neither have resulted in adverse events for these conditions.

<u>Methods and Materials:</u>

- Screening
- Study visits:
    - IV catheter
    - IV blood sampling
    - PC VIPR MRI scan sequences and ASL MRI scan sequences
    - 2D MRI scan sequences
    - Oral pill(s) of Indomethacin and Placebo. All other study visits are identical, apart from the study drug selection (made by study team). Subjects will be consented to receive the study drug.
    - Hypoxia (reducing SpO_2 to 80- 85%, normal CO2) – stressor used to increase CBF
    - Hypercapnia – stressor to increase arterial CO2 by 7-10 mmHg which increases CBF. Arterial CO2 will be estimated using End Tidal CO2 gas measurements, which track very closely to arterial levels.

<u>Subject participation:</u> Phase 1 involves ~4 laboratory visits for Aims 1 and 2:

1. 1 screening visit
2. 1 maximal fitness test and/or gas challenge familiarization visit (May be combined with screening visit, study visit, or made into separate visits depending on the subject's availability)
3. 2 MRI visits with randomized order of hypoxia and hypercapnia challenge. MRI study visit will be randomized with either Placebo or Indomethacin supplementation. Subjects will receive placebo during one of the MRI visits and the study drug (indomethacin) during the other MRI visit.

2.2. Detailed Rationale

Cerebrovascular disease is the third leading killer in the U.S., which exhibits a dramatic sex-specific risk pattern. Men exhibit lower resting cerebral blood flow (CBF) and two-fold greater risk of cerebrovascular disease compared to premenopausal women. Despite this sex-specific disease risk, the essential mechanisms controlling CBF in humans are not well-defined. A lack of physiologic insight is highlighted by the controversies in optimal sex hormone treatments—and adverse health outcomes—seen in important clinical populations including aging, PCOS, and gender reassignment. Herein we aim to address the gaps in knowledge of human cerebrovascular physiology prior to confounding factors like age and disease, in order to identify sex differences and similarities in how CBF is controlled.

Animal studies indicate cerebral arteries from male rats exhibit lower endothelium-dependent vasodilation compared to females. This is due to cyclooxygenase (COX) mediated vasoconstriction in male rats and greater nitric oxide synthase (NOS) and COX-mediated vasodilation in female rats. The translational relevance of animal data to men and women may be limited by several factors: 1) supraphysiologic sex steroid supplement levels, 2) using pharmacological rather than physiological vasodilator stimuli, 3) only studying *resting* CBF rather than during physiologic stressors, and 4) lack of consideration of regional differences in vascular control.

This proposal addresses these limitations in healthy men and women. First, we leverage state-of-the-art, 4D flow MRI technique (PC VIPR) paired with Arterial Spin Labeling (ASL). This dual MRI approach quantifies CBF responses with high spatial resolution, simultaneously in all major cerebral arteries, as well as microvascular perfusion of discrete brain regions. Second, we assess CBF under resting and controlled challenges with hypoxia and hypercapnia. Third, we test mechanistic control by acute pharmacologic inhibition.

2.3. Innovation

Our multidisciplinary team of investigators combines expertise in human integrative cardiovascular physiology, endocrinology, and MR imaging. The preliminary findings form a solid basis for applying these concepts to a larger number of subjects. Our exciting preliminary data clearly demonstrate sex differences in CBF control that are distinctly specific to a physiologic stressor, mechanism, and brain region. Data generated in these studies set the stage for designing mechanistic, sex-specific studies in focused disease populations who exhibit sex-specific etiology (e.g. diabetes, hypertension). Our comprehensive approach considers multiple stressors, mechanisms, and brain regions by generating high resolution, quantifiable data.

Multiple Stressors: All vascular tests are not created equal. Cerebrovascular reactivity of CBF (CVR_{CO2}) is an associative indicator of preclinical disease. Using only CVR_{CO2} might have missed critical information, as our hypoxia data indicate profound sex differences in healthy adults.

Multiple Mechanisms: Previous CBF research indicates CVR_{CO2} declines with age/disease and appears to be due to loss of COX dilation. While those studies make a clean story, our preliminary data indicate men and women use multiple COX-independent mechanisms, which are more interesting and complex than COX alone.

Regionally specific: Brain pathology is often region-specific. Analyzing the entire circulation allows us to test sex-specific cerebrovascular pathophysiology that may exhibit regional specificity.

Quantifiable: 2D, PC VIPR, and ASL complimentary approaches quantify CBF with reasonable temporal and high spatial resolution. Together, we are poised to detect sex structural and functional differences in CBF regulation.

Expected Findings: These studies will generate an important breadth and depth of new understanding of sex-specific CBF regulation because these data: 1) are obtained *in vivo* in humans, 2) demonstrate fundamental sex differences in stress responses, 3) quantify sex-specific, stressor-specific, and region-specific differences in mechanistic control, 4) demonstrate physiologic manipulation of endogenous sex hormones modifies *control*, and 5) demonstrate sex-specific CBF control mechanisms are established in young adults *without* cardiometabolic or aging risk factors. These studies will add vital insight to a field where unanswered questions await on: dose, delivery method, duration, and ideal age for hormone therapies in both men and women. Findings will form the basis of new strategies to prevent or treat cerebrovascular pathophysiology using a sex-specific approach.

3. SPECIFIC AIMS AND PRELIMINARY EVIDENCE

In previous IRB approved studies, we established an experimental setup where participants can be scanned with a head coil while wearing a mask to control inhaled gases in the MRI. CBF is measured in normoxic rest and during steady-state gas challenge (hypoxia and hypercapnia) in a research-dedicated magnet, with 4D PC VIPR or ASL techniques. Hypoxia reduced arterial O_2 (S_aO_2 = 80-85±1%) while maintaining CO_2 at normoxic levels (38±1 mmHg). Hypercapnia (breathing CO_2) increases end tidal CO_2 ($ETCO_2$) while maintaining S_aO_2 at resting levels. Our current study designs allow us to explore this exciting idea without increasing subject time burden or changing the overall benefit:risk ratio. Importantly, it falls under the scope of the grant to study stressor responses in CBF regulation.

Resting CBF was greater in women. In support of Aim 1A, hypoxia increased total CBF, and vasodilation was ~60% greater in women than men even when normalized to gray matter volume (not shown). In support of Aim 1B, hypercapnia increased CBF similarly between sexes. Even at rest, ASL data demonstrate resting microvascular CP is ~25% higher in females globally and regionally.

3.1. Aim 1: Sex differences in cerebral vasodilation vary by stressor

Test the hypothesis that healthy males exhibit reduced cerebral vasodilation compared to healthy females despite exhibiting similar vasodilation to hypercapnia.

Aim 1A: Vasodilation to hypoxia will be markedly lower in males.
Aim 1B: Vasodilation to hypercapnia will be lower in males.

3.1.1. Rationale

Females generally exhibit higher resting CBF and potentially greater CBF reactivity. Other than the role of COX in mediating resting CBF or hypercapnic dilation, human mechanistic data do not exist to assess sex differences. Despite intriguing studies in rats suggesting *in vitro* vascular control differences are driven by testosterone and estrogen, these mechanisms do not predict CBF in rats, and studies are limited by supraphysiologic hormone treatment, lack of regional comparisons, and lack of physiological stressors.

3.1.2. Preliminary Data

Resting CBF was greater in women. In support of Aim 1A, hypoxia increased total CBF, and vasodilation was ~60% greater in women than men even when normalized to gray matter volume (not shown). In support of Aim 1B, hypercapnia increased CBF similarly between sexes. Even at rest, ASL data demonstrate resting microvascular CP is ~25% higher in females globally and regionally (not shown).

3.2. Aim 2

Test the hypothesis that acute inhibition of COX will explain sex differences in hypoxia-mediated cerebral vasodilation.

Aim 2A: COX-signaling mediating hypoxic vasodilation is greater in females.
Aim 2B: COX-signaling mediating hypercapnic vasodilation is greater in females.

3.2.1. Rationale

Animal models indicate estrogen increases expression of COX vasodilation, related to increased vasodilator function. Rat data indicate the basilar artery is less NOS-dependent than MCA, such that we may find regional specific control mechanisms. Whether these same pathways are common in humans remains untested and may not occur.

3.2.2. Preliminary Data

Preliminary data suggest hypoxic vasodilation in men is largely COX-independent, in contrast to rat cerebral arteries.

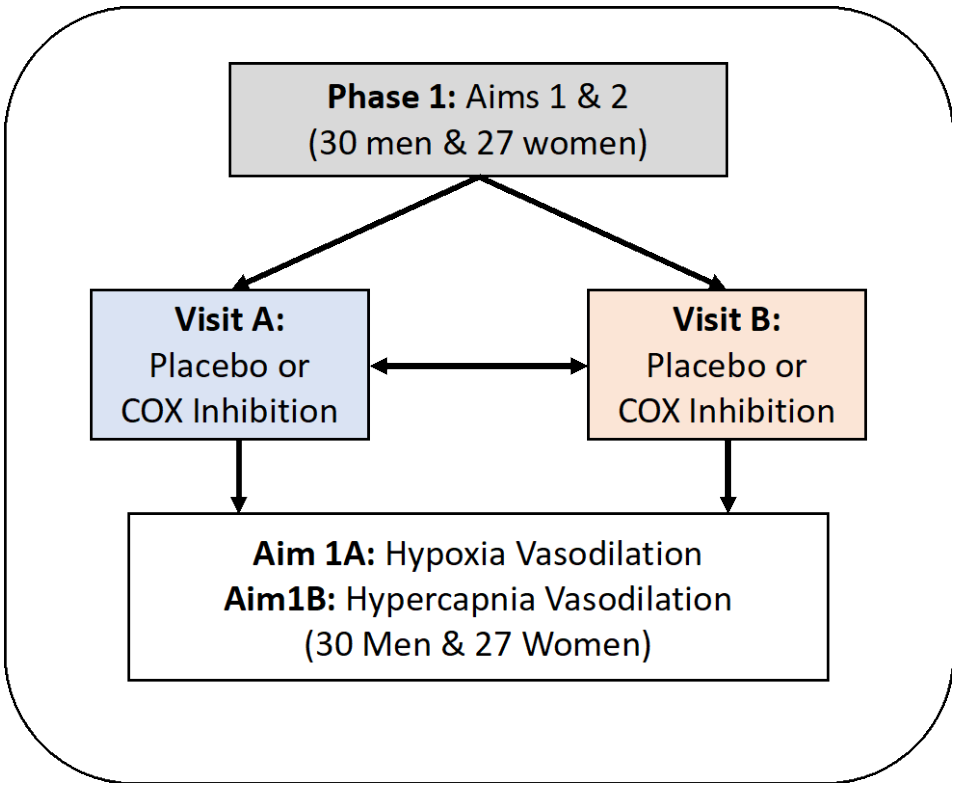
3.3. Expected Results

We expect findings in line with hypotheses. Women will demonstrate greater hypoxic vasodilation, but similar hypercapnic vasodilation (Aim 1). The difference in hypoxia responses will be due to COX dilation in women than men, and some COX vasoconstriction in men (Aim 2).

3.4. Study Duration

Expected start date 06/01/2020
Expected end date: 04/30/2025

4. RESEARCH DESIGN AND METHODS



4.1. Subject Population

Previous Protocol:
Fifty-seven (57) otherwise healthy adults between 18-40 years of age were recruited. These subjects completed either one or both Aims 1 and 2 of the previously approved protocol.

Updated Protocol (CP011):
Fifty-four (54) otherwise healthy adults between 18-40 years of age inclusive composed of 27 males and 27 females. These 54 participants will complete both Aims 1 and 2 (both Aims completed in 2 study visits).

Updated Protocol (CP017):
Fifty-seven (57) otherwise healthy adults between 18-40 years of age inclusive composed of 30 males and 27 females. These 57 participants will complete both Aims 1 and 2 (both Aims completed in 2 study visits). We have increased the number of males by 3 subjects, this was done to meet the recruitment requirements of our Phase 2 (2023-0548) study. Completion of Phase 1 (2020-0336) is required to participate in Phase 2. No females were added as the recruitment for Phase 2 females will fall within the 27 females who will have completed Phase 1 (2020-0336).

4.1.1. Inclusion Criteria

Subject will be included if they meet all the following criteria:

Age between ≥18 - ≤40 years inclusive

4.1.2. Exclusion Criteria

Subject will be excluded if they meet any of the following criteria:

- Hypertensive
  - >125 mmHg systolic blood pressure; or
  - >80 mmHg diastolic blood pressure
- BMI ≥25 kg/m^2
- Fasting blood glucose ≥100 mg/dl
- LDL cholesterol ≥130 mg/dl
- Triglycerides ≥150 mg/dl
- Current diagnosis or history of:
  - peripheral vascular disease
  - hepatic disease
  - renal disease
  - lung disease
  - gastrointestinal disorders/bleeding
  - hematologic disease
  - stroke
  - myocardial infarction
  - coronary heart disease
  - congestive heart failure
  - heart surgery
  - prediabetes
  - diabetes mellitus (type 1, type 2, MODY, or others)
  - sleep apnea
  - hypertension
  - some autoimmune diseases, such as inflammatory bowel disease or systemic lupus erythematosus (exclusion at discretion of reviewing MD)
- Current smoking, defined as the use of tobacco or nicotine products >5 times in the past 30 days.
- Cardiovascular medication use
- NSAID sensitivity
- Any contraindications of having an MRI
- Irregular menstrual cycle (females only)
- Medical conditions that can affect the menstrual cycle, such as hyperprolactinemia, prolactinoma, hypercortisolemia, and congenital adrenal hyperplasia (females only)
- Pregnancy, breast feeding, or plans to conceive within the next 3 months (females only)
- Polycystic ovary syndrome (females only)
- Hirsutism defined as unwanted and/or excessive hair growth on the face, chest, or back (females only)
- Levonorgestrel intrauterine device (IUD) (females only)
- Hormonal birth control will not be allowed in women, in order to control for high variability between type, dose, and route of therapy. However, in discussion with Dr. Davis (Co-I) and Dr. Laura Cooney M.D., our physician experts in medical and reproductive endocrinology and infertility, there are two broad exceptions to this birth control criteria:
  1. Copper intrauterine devices (IUDs) will be allowed as they do not change systemic sex hormone levels).

2. Women currently taking hormonal birth control (i.e. contraceptive pills, patch, ring) for contraception only (not for a medical condition such as those listed in exclusion criteria above) may consider temporarily stopping to become eligible for enrollment. Hormonal birth control must be stopped at least one month prior to Study Visit 1 to provide time for menstruation to resume. Then stoppage continues through the last planned study visit. Screening information will be reviewed by our endocrinology physicians to determine eligibility and timing on this issue (details below).

4.1.3. Subject Identification and Recruitment

Previous Protocol:
57 participants were recruited for Aim1-2 (completed at least one study visit) from the surrounding community by responding to our study postings.

Updated Protocol (CP011):
54 participants (27 per sex) for Aim1-2 will be studied (complete all assigned study visits) from the surrounding community by responding to our study postings. Participants who completed the study pre-CP011 will be invited to complete the study again. We will keep enrollment open until we reach our goal of 54 participants completing all assigned visits.

Updated Protocol (CP017):
Fifty-seven (57) otherwise healthy adults between 18-40 years of age inclusive composed of 30 males and 27 females. These 57 participants will complete both Aims 1 and 2 (both Aims completed in 2 study visits). We have increased the number of males by 3 subjects, this was done to meet the recruitment requirements of our Phase 2 (2023-0548) study. Completion of Phase 1 (2020-0336) is required to participate in Phase 2. No females were added as the recruitment for Phase 2 females will fall within the 27 females who will have completed Phase 1 (2020-0336).

All recruitment procedures will be reviewed and approved by the UW IRB. Subjects will be recruited through the University's email of faculty staff and students, online advertisements, newspaper advertisements, paper fliers, and by word of mouth. Additionally, postings may be put on the UW-Madison Department of Kinesiology webpage.

4.2. Research Sites

The following sites will be used for the following purposes:

- Medical Science Center
    - Consent
    - Screening questionnaires
    - Maximal exercise testing
    - Gas Challenge
- UW Human Exercise Research Core (HERC) in the UW Nursing School
    - Consent
    - Screening questionnaires
    - Maximal exercise testing
- Waisman Center
    - Consent
    - MRI
    - IV placement
    - Gas Challenge

4.3. Experimental Procedures

4.3.1. Pre-Screening Subjects

Determination of eligibility will be a two-step process. Subjects will be initially screened via a REDCap survey. If eligible based off of this survey, they will be invited in for a more in-depth in-person screen.

Delegated study team members will screen patients for eligibility for the study based on medical history, medication use, physical activity, and evaluate general safety to undergo MRI (e.g. claustrophobia, metallic implants, etc.). Consent/permission will be obtained following a description of the study and prior to survey questions either:

- Verbally for phone screen
- Agreeing to the REDCap survey

The same script/information sheet will be used for both the phone screening and REDCap survey (i.e. the REDCap survey will be discussed with the subject verbally and constitutes the “phone screen”).

These steps will be taken to minimize risk of participation. If an individual does not preliminarily meet all inclusion criteria or preliminarily meets any of the exclusion criteria, then the subject will be immediately eliminated from consideration for the study and their information will be destroyed (e.g. shredder or digital deletion). If the subject reapplies for the study due to a change in weight, medical history, or health status that has previously made them ineligible they are able to be re-screened for eligibility.

4.3.2. In-Person Screening

Duration: Approximately 1 hour

A screening visit reminder will be provided to potential subjects via phone or email at least 12 hours prior reminding them of the following.

- Location of visit (with general campus directions)
- Fasting directions

After informed consent is obtained, the following information will be collected during the screening visit:

- Health history questionnaire
- MRI screening questionnaire
- Female participants must first take a urine pregnancy test (commercial test provided by study team) to rule out pregnancy
- Height & weight
- Hip & waist circumference
- Resting Blood pressure
- Venipuncture blood sample
    - Glucose, lipid panel to test for inclusion/exclusion criteria

4.3.3. Exercise Test and Gas Challenge

Duration: Approximately 2 hours

These last procedures may occur on separate visits depending on subject availability, staff availability, and equipment availability on screen day. Subject does not need to be 8 hours fasted for these procedures.

1. Maximal exercise test to determine cardiorespiratory fitness
2. Hypoxia and/or hypercapnia breathing familiarization visit

Blood pressure, questionnaires, and venous blood sample will be used at screening to further determine eligibility.

All blood measurements taken during the screening procedures will be analyzed by Schrage lab staff.

The results of the screening procedure will be valid for study inclusion for 180 days. If the subject does not enroll in the study (actively start Aim 1 or Aim 2 visits), he/she must undergo screening procedures to assess eligibility.

4.3.4. Study Visits

Duration: Approximately 2 hours each (4-5 hours total over 2 study visits)

A visit reminder will be provided to potential subjects via phone or email at least 24 hours prior reminding them of the following:

- Location of visit
- Fasting directions
- Caffeine, NSAIDS, and exercise restriction
- Females: Menstrual cycle restriction

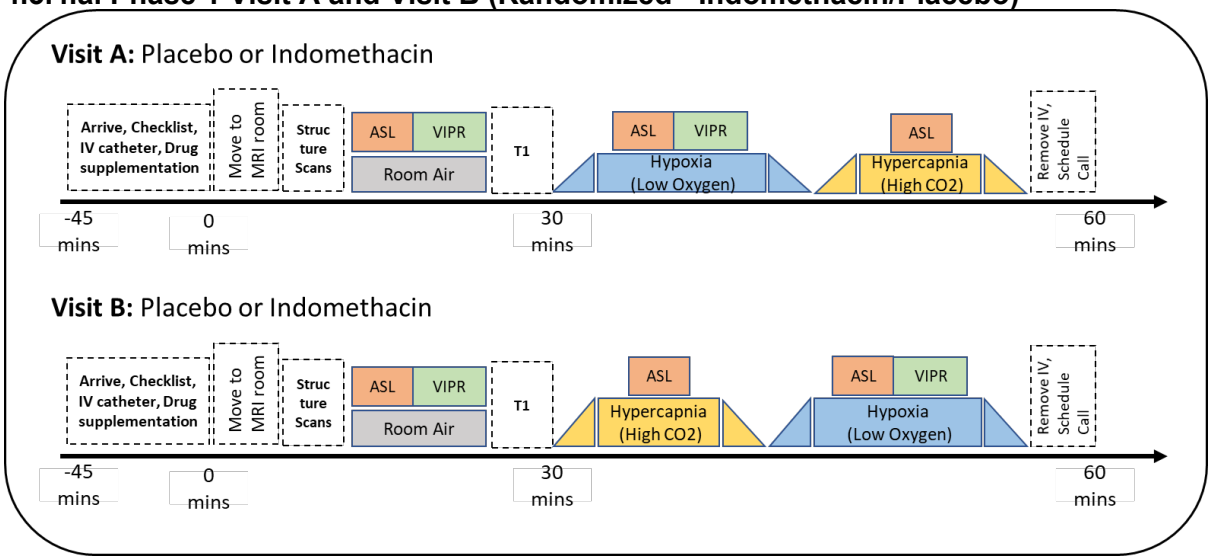
Due to the unpredictability of the menstrual cycle and MRI availability, if a MRI timeslot becomes available we will confirm with the subject that they meet the fasting and restriction directions listed above and schedule them for the MRI timeslot. On the day of the visit we will confirm the subjects have followed the protocol (CRFs 010 and 011.)

Study visits will be performed at the Waisman Center (1500 Highland Ave., Madison WI) and experience the following procedures:

- A study team member will verbally review the subject has:
  - Concomitant medication,
  - Fasted for a minimum of 8 hours prior to data collection for study visit (MRI scan),
  - Abstained from vigorous exercise and NSAIDS medication ≥24 hours prior to study visit.
  - Abstained from caffeine ≥24 hours prior to study visit
- Female participants must first take a urine pregnancy test (commercial test provided by study team) to rule out pregnancy. Study staff will determine and record the result on study form.
- MRI technicians will complete and review a MRI Safety screening with the subject.
- IV catheter and blood sampling: An intravenous (IV) catheter will be placed by qualified staff. Research laboratories will conduct the blood testing noted below. For example, we have a collaborator who is an expert in sex steroids in the cardiovascular system. Related to drug efficacy, stable plasma metabolites of COX can be assayed with simple 96 well plates. Our lab has conducted this previously using equipment in the UW School of Pharmacy. We will use the catheter to draw blood samples:
  - to assess sex hormones (typically near baseline)
  - to sample plasma markers of drug efficacy during hypoxia and during hypercapnia
- Prior to the study visit subjects will be randomized into the Visit A or Visit B protocols (gas order) for the duration of their time in the study. Randomization will consist of a study member utilizing a random number generator to sort subjects into Visit A (even number) or Visit B (odd number).

- Prior to the first study visit the order of drug supplementation (Placebo-Indomethacin or Indomethacin-Placebo) will be ramdomized. Randomization will consist of a study member utilizing a random number generator to determine the order of the drug supplementation for the MRI visits. Placebo for the first MRI visit, then Indomethacin for the second MRI visit will be an even number. Indomethacin for the first MRI visit, then placebo for the second MRI visit will be an odd number.

4.3.4.a. Phase 1 Visit A and Visit B (Randomized - Indomethacin/Placebo)



- **Isocapnic Hypoxia (Low Oxygen):** will be used to promote cerebral vasodilation under resting conditions. Based on previous research conducted in our lab, this amount of inspired oxygen should reduce arterial oxygen saturation to approximately 80-85% as measured by finger pulse oximetry. Details are found below under Hypoxia procedures.
- **Hypercapnia (High Carbon Dioxide):** An increase in blood CO_2 is a powerful signal to increase cerebral blood flow. After baseline data collection, subjects will breathe a gas mixture of:
    - Increased CO_2, 21% O_2 and balance nitrogen through a mouthpiece or facemask. This will raise the subject's $ETCO_2$ 7-10mmHg. *or*
    - 5% CO2, 21% O2, and balanced nitrogen through a mouthpiece or facemask.

  Subjects will breathe this mix of gas for about 15-20 minutes. After hypercapnia gas exposure, subjects will return to breathing room air. Details are found below under hypercapnia procedures.
- After baseline structural/localization scans, MRI scans will be paired (PC VIPR, ASL) at the following conditions:
    - Normoxia baseline
    - Steady-state hypoxia (roughly minute 3-20 of hypoxia)
    - Normoxia baseline post study drug
    - Steady-state hypercapnia (roughly minute 3-20 of hypercapnia)
- Order of the gas exposure will be randomized

4.3.5. Details of Procedures

4.3.5.a. Informed Consent

Informed consent/assent will be obtained from each participant outlining the potential risks. Consent will be obtained at the initial screening visit and will take place prior to any other procedures being performed. If written consent/assent is refused, the subject will no longer be considered for the study.

4.3.5.b. Fasting, Caffeine, NSAID, and Exercise Restrictions

Each subject will be asked to:

- fast for a minimum of 8 hours
- refrain from using NSAIDs (e.g. Ibuprofen) for a minimum of 24 hours
- refrain from caffeine for a minimum of 24 hours
  refrain from vigorous exercise for a minimum of 24 hours

4.3.5.c. MRI Screening Document

Each subject will be screened for MRI safety first during the pre-screening process. At the initial screening visit, the subject will fill out an MRI screening document provided by the research team to ensure their safety and comfort in the MRI scanner.

4.3.5.d. Maximal Exercise Test

Each subject will complete a maximal exercise test on a treadmill. Subjects will be fitted with a mask or mouthpiece to measure oxygen consumption (VO_2) and carbon dioxide production (VCO_2), and a Polar Heart Rate Monitor to measure heart rate. Following a 2-minute warm-up at 3.5 mph, exercise intensity in speed and grade every 2 minutes until the subject can no longer continue despite strong verbal encouragement. Average duration of maximal exercise testing is approximately 8-12 minutes (45, 48). If performed on different day than In-Person Screening Visit, the subject does not need to be 8 hours fasted.

4.3.5.e. Temporarily suspending hormonal birth control

Women who regularly take hormonal birth control (i.e. contraceptive pills, patch, ring) for contraception will be asked if they are willing to temporarily stop taking birth control until their MRI study visits are completed. Women who take hormonal birth control for medical conditions, such as PCOS, prolactinoma, or very heavy menstrual bleeding will not be allowed in the study as their hormonal birth control should not be stopped. Screening will determine the type of birth control, which will be reviewed by our collaborating physicians specializing in medical and reproductive endocrinology. The physicians will determine the appropriate number of days to withdraw birth control prior to MRI study visits. The resumption of menses as our primary marker that women have returned to “normal” hormone levels. However to ensure scientific validity, blood samples will be taken on one of the MRI study visits to assess hormone levels. Once the woman completes the MRI visits, she can choose to resume birth control. Resumption of birth control will also be coordinated with the collaborating physician to ensure it is done in an appropriate and safe manner.

4.3.5.f. Magnetic Resonance Imaging (MRI)

Images will be acquired on a MRI 3T system. The MRI is located at the Waisman Center Imaging Core. The research team will be administering the scans, as well as processing and analysis of the data. No contrast agent will be used. While in the scanner, subjects will be monitored for vital signs.

Application of Investigational Hardware and Software: Any or all of the pulse sequences (scanner software) may be custom pulse sequences developed by Dr. Alexander's lab or project collaborators. These custom research pulse sequences are not FDA approved; however, they are adapted from FDA approved GE product pulse sequences and follow all of the regulatory safety requirements for RF (radiofrequency) power and magnetic field switching (dB/dt). Custom research pulse sequences are commonly performed in MRI research using the EPIC pulse sequence software development tools from General Electric for research use on their MRI scanners. The safe use of these MRI pulse sequences is outlined in a letter from Andrew Alexander (as Waisman Brain Imaging Lab Core Co-Director). Image data acquisition may be performed using a 32-channel receive-only head coil from Nova Medical (Boston). The Nova coil is not an FDA approved device, but it was developed for safe use in humans and follows all of the regulatory requirements for human use. This coil is now widely used in neuroimaging research studies at the Waisman Center. As both the investigational hardware and software will operate within FDA guidelines, the MRI studies are considered nonsignificant risk. These technologies are not being evaluated for performance or safety in this study.

The software and head coil used in this study are not intended for use in the diagnosis or treatment of subjects, nor are they being assessed for safety or efficacy as part of this study. Study data will not be submitted to the Food and Drug Administration (FDA) in support of labeling changes for these devices.

Two phase contrast scans (PC VIPR and 2D PC) will be utilized to acquire volumetric data sets with three-directional velocity encoding and high spatial resolution in fairly short scan times. This technique has been developed over the last >10 years in the Departments of Medical Physics and Radiology, and has been used and validated in several hundred subjects and have been used with regularity by the medical imaging researchers collaborating on this IRB proposal (Oliver Wieben). We will also conduct ASL perfusion scans. These methods are well-characterized and used routinely during collaborative studies between Drs. Oliver Wieben and Schrage.

4.3.5.g. Venipuncture

Occurs only during screening visit. Venipuncture will usually occur in the antecubital fossa or hand by trained personnel using standard aseptic technique. If staff fail to obtain blood during screening, we will ask the participant to return to lab another day to attempt a new fasted blood draw.

4.3.5.h. Intravenous Catheter

An IV catheter will be placed by trained personnel in the antecubital fossa or hand using standard aseptic technique. Phlebotomy trained individuals, or higher study-associated clinical authority (e.g. physician) will insert an IV using standard medical procedures. The IV catheter will remain in place for approximately 2 hours.

4.3.5.i. Blood Sampling

<u>Screening visit:</u>
Up to 20 mL of blood will be obtained for blood chemistry values to determine eligibility and stored for determining sex hormone levels, and blood markers of COX activity or to be use in future studies.

<u>IV catheter on both MRI study visit days:</u>
Blood draws will be obtained via IV catheter. The specific time points are as follows: The volume taken on study day is expected to total ~100 mL before entering the MRI and ~10mL over 3 time points in the

MRI. However, in order to ensure complete blood sampling to address research aims, while keeping risk small, we request allowance of up to 150mL total.

1. Before entering MRI (for sex hormone levels, and blood markers of COX activity)
2. ~40 mins post indomethacin/placebo supplementation
3. During steady-state hypoxia, for blood markers of COX activity
4. During steady-state hypercapnia, for blood markers of COX activity

4.3.5.j. Randomized Drug Supplementation

Indomethacin:
The optimal dose is 1.5 mg/kg, as it appears to robustly inhibit COX with low risk of side effects (primarily GI discomfort). Unfortunately, oral liquid formulation that allows precise dosing is in short supply with a high price (~ $1900/ person) as opposed to pill indomethacin (pennies) that comes in 25 or 50 mg pills. Therefore, based on body mass our total dosing will be 1.3-1.7 mg/kg (Ex. 75mg for a 50kg person or 150mg for a 100 kg person and in between). We will not exceed a dosage of 1.7 mg/kg for a subject. Indomethacin is produced in the form of 25mg and 50mg pills. Per drug insert, this is within the recommended range of up to 3mg/kg/day. Indomethacin will be administered by a member of the study team. Subjects will also be given a dose of over-the-counter antacid to combat the gastrointestinal distress expected from Indomethacin.

Previous work by our lab (Kellawan et al. 2019) used an absolute dose of 100mg (which was a calculated relative dose of 1.4 ± 0.2 mg/kg). Additionally, a prior studies in our lab (Harrell et al. 2013, Peltonen et al. 2015, and Peltonen et al. 2016) and others (Fan et al. 2010), an absolute dose of indomethacin (100mg) resulted in a large decrease in circulating COX metabolites. The proposed dosage of 1.5mg/kg is similar to dosage used by Hoiland et al. 2015 (1.45 ± 0.17 mg/kg). There were no reports of GI stress/dropout in the studies.

Side effects or GI stress from Indomethacin are due to chronic dosing. Previous protocols approved by the IRB for the Schrage lab that utilized indomethacin with only 1 adverse event (nausea, which is an expected AE) during the 2 studies.

	N	**Dosage**	**Age (yrs)**	**BMI (kg/m^2)**
Kellawan et al. 2019	9	1.4 ± 0.2 (mg/kg)	28 ± 4	23 ± 1
Harrell et al. 2013	15	1.47 - 1.56 (mg/kg)	31 ± 2	23 ± 1
Fan et al. 2010	12	100 (mg)	30 ± 10	23 ± 2
Hoiland et al. 2015	22	1.45 ± 0.17 (mg/kg)	22.3 ± 2.4	22.5 ± 2.4
Peltonen et al. 2016	11	1.75 - 1.89 (mg/kg)	23 ± 1	21 ± 0
Peltonen et al. 2015	42	1.32 – 1.79 (mg/kg)	25 ± 2	22 ± 1

The mean half-life of indomethacin is estimated to be ~4.5 hours. Due to the short half-life of indomethacin, MRI visits can be scheduled to occur at a minimum of 24 hours apart for the same subject.

Indomethacin usage is IND exempt. Indomethacin is lawfully marketed in the United States. The research is not intended to be reported to the FDA as a well-controlled study in support of a new indication for use nor intended to be used to support any other significant change in the labeling for the drug (indomethacin). The research is not intended to support a significant change in the advertising for the product (indomethacin). The research does not involve a route of administration or dosage level or use in a patient population or other factor that significantly increases the risks (or decreases the acceptability of the risks) associated with the use of the drug product (indomethacin). The research is conducted in compliance with the marketing limitations described in 21 CFR §312.7.

Weight (kg)	1.5 mg/kg	Dose w/50mg (mg/kg)	Dose w/75 mg (mg/kg)	Dose w/100mg (mg/kg)	Dose w/125mg (mg/kg)	Dose w/150mg (mg/kg)
40	60.0	**1.25**				
42.5	63.8	**1.18**				
45	67.5		**1.67**			
47.5	71.3		**1.58**			
50	75.0		**1.50**			
52.5	78.8		**1.43**			
55	82.5		**1.36**			
57.5	86.3		**1.30**			
60	90.0			**1.67**		
62.5	93.8			**1.60**		
65	97.5			**1.54**		
67.5	101.3			**1.48**		
70	105.0			**1.43**		
72.5	108.8			**1.38**		
75	112.5			**1.33**		
77.5	116.3				**1.61**	
80	120.0				**1.56**	
82.5	123.8				**1.52**	
85	127.5				**1.47**	
87.5	131.3				**1.43**	
90	135.0				**1.39**	
92.5	138.8					**1.62**
95	142.5					**1.58**
97.5	146.3					**1.54**
100	150.0					**1.50**

Placebo:
Total dosing will be calculated to match the mg needed for the indomethacin study visit.
Subjects will also be given a dose of over-the-counter antacid to mimic the indomethacin study visit.

Antacid: Subjects will also be given a dose of over-the-counter antacid during both the Indomethacin Study Visit and the Placebo Study Visit. Over-the-counter antacid usage is IND exempt. Over-the-counter antacid is lawfully marketed in the United States. The research is not intended to be reported to the FDA as a well-controlled study in support of a new indication for use nor intended to be used to support any other significant change in the labeling for the drug (over-the-counter antacid). The research is not intended to support a significant change in the advertising for the product (over-the-counter antacid). The research does not involve a route of administration or dosage level or use in a patient population or other factor that significantly increases the risks (or decreases the acceptability of the risks) associated with

the use of the drug product (over-the-counter antacid). The research is conducted in compliance with the marketing limitations described in 21 CFR §312.7.

4.3.4.k. Hypoxia

Hypoxia will be used to promote cerebral vasodilation under resting, supine conditions. Subjects will breathe room air quietly while lying in the MRI for about thirty minutes during baseline data collection. After baseline data collection, subjects will breathe a gas mixture of ~11% O_2 and 89% nitrogen through a mouthpiece, reducing SpO_2 to 80-85%. We will also maintain a constant end-tidal CO_2. Subjects will breathe this mix of gas for 30-35 minutes. Based on previous research conducted in our lab, this amount of inspired oxygen should cause a fall in arterial oxygen saturation to approximately 85% as measured by finger pulse oximeter. After hypoxic breathing, subjects will be returned to breathing room air and monitored until all variables return to baseline levels (typically within 1 minute).

4.3.4.l. Hypercapnia

An increase in blood CO_2 is a powerful signal to increase cerebral blood flow. Subjects will breathe room air quietly while lying in the MRI for about two minutes. After baseline data collection, subjects will breathe a gas mixture of increased CO_2, 21% O_2 and balance nitrogen or 5% CO2, 21% O2, and balanced nitrogen through a mouthpiece or facemask. Subjects will breathe this mix of gas, during which end-tidal CO_2 will rise, but arterial oxygen saturation will not fall. We will measure the rise in end tidal CO_2 with an MRI compatible end tidal CO_2 analyzer. There is an MRI compatible subject monitor in the scanning room that analyzes expired gas and will allow us to monitor end tidal CO_2. We will collect expired air with a mouthpiece. We are using end tidal CO_2 as a reliable surrogate of arterial CO_2. We are not drawing arterial blood samples to measure CO_2. After 15-25 minutes of hypercapnia gas exposure, subjects will return to breathing room air, and we monitor them to ensure all variables return to baseline levels (typically within 1 minute).

4.3.4.m. Subject Monitoring

During the maximal exercise test, subjects will be monitored for:

- Heart rate (via electrocardiogram or heart rate monitor)
- Oxygen consumption (via mouthpiece or face mask)
- Adverse signs and symptoms
- The frequency of monitoring will occur during baseline and each stage of exercise. Visually, HR and expired gas variables are continuous readouts, but all are recorded each stage on CRFs. These are monitored by trained laboratory staff who are physiologists-typically graduate students or postdoctoral fellows who have significant experience monitoring these variables during exercise.

Throughout the MRI visits, subjects will be monitored for:

- Heart rate (via electrocardiogram or pulse oximeter)
- Blood pressure (via automated brachial artery auscultation)
- Blood oxygen saturation (via pulse oximeter)
- End-tidal carbon dioxide (via nasal cannula or mask)
- The frequency of monitoring is as follows: during baseline and each major MRI sequence (ASL or VIPR). Visually, HR, SpO_2 and $ETCO_2$ are continuous readouts on patient monitor. Practically speaking, all are recorded every 5-10 minutes on CRFs. These are monitored by trained

laboratory staff who are physiologists-typically graduate students, postdoctoral fellows, or PI. All recorded values are on CRFs with clearly stated stopping guidelines.

4.3.5. Summary of Study Visits and Procedures

Procedure	Screening Visits	MRI Study Visits
Fasting	X	X
Medical History	X	
Body Measurements	X	
Maximal Exercise Test	X	
Breathing Challenge Familiarization	X	
Blood Sample	X	X
Vital Signs	X	X
Females stop birth control temporarily (if applicable)	X	X
24 hour abstinence of Caffeine, vigorous exercise, NSAIDs		X
Pregnancy Test (females)	X	X
MRI Scan		X
Breathing Challenge		X
IV Catheter		X
Drug Supplementation (Placebo or Indomethacin)		X

4.4. Confidentiality Protections

Risks to confidentiality will be minimized by keeping copies of the documents linking study assignment number and the participant's unique identifiers with the participant's informed consent in locked cabinets of the offices of Dr. Bill Schrage. Only subject numbers will be used for group assignment, data processing, and analyses. All data will be stored in locked cabinets in a secure lab, and electronic files are all stored on password-protected databases and computers.

Personal information such as name, gender, date of birth, and medication history will be stored in a locked file cabinet in a locked office in the PI's laboratory. Subject information will be coded to remove any personal identifiers during data analysis or research publications. Research oversight and regulatory groups may review study records.

4.5. Remuneration

Subjects will be paid in the following manner:

Visit or procedure	Payment amount
Screening Visit	$20
Exercise and/or Gas Challenge Visit	$20
MRI study visits A and B	$30/hour; rounded to the nearest half hour

Completing both MRI study visits	Bonus $50
If you complete all procedures and visits	~$210 total

Payment will be provided at the end of the study visits. If participants choose to leave early or are taken off study for any reason, payment will be pro-rated. If any study visits are repeated for any reason (fitness test, gas challenge, failed blood draw, stopped MRI scan due to technical/scheduling errors), participants will be paid for additional study time based on rates above.

5. BANKING AND SHARING DATA

Blood analysis results and MRI images will be stored for future research use. Studies utilizing banked data and/or biospecimens will be approved by the IRB. Banked data will be stored on secure computer servers that are password-protected. Any data shared with personnel outside of the study team will be coded with a study ID number. Unless the staff is listed as key personnel on this study, they will not be given access to the key linking subject identifiers with their data or study ID number. The study PI will be responsible for the oversight of the data banking and will review all requests to utilize the data and images. The PI will be responsible for confirming that IRB approval or exemption has been granted prior to the release of any data. Future study results obtained from banked data will not be reported to subjects.

6. RISKS ASSOCIATED WITH PROCEDURES

6.1. Risk Summary

This protocol is minimally invasive with only one (1) IV catheter for blood sampling. MRI sequences are very safe. Short term gas challenges with hypoxia and hypercapnia are well-tolerated, and easily reversible. One of two drugs are FDA approved, and the other is a modified amino acid with a good safety record. Subject monitoring during study visit allows acute identification of AEs and subsequent action. Therefore, overall risk appears modest for IV catheter and drug administration, with minimal risk to healthy adults.

6.2. Detailed Risks

6.2.1. Fasting, and Caffeine, NSAID and Exercise Restriction

Risks include:

- feelings of hunger
- irritability
- fatigue
- light-headedness
- dizziness

There are no risks of NSAID restriction if NSAIDs are not prescribed by a physician for clinical care. There are no risks of acute abstention from exercise. These risks are considered minimal.

6.2.2. Maximal Exercise Test

Risks include:

- Feelings of exertion, fatigue, and breathlessness
- Discomfort due to VO_2 monitoring equipment (i.e., mouthpiece or mask)
- Serious complications including orthopedic injury, myocardial infarction, arrhythmia, hemodynamic instability, and death are rare (Takken 2016; van Brussel 2019)

These risks are considered minimal given the healthy population and age range, feelings of exertion, etc resolve quickly (seconds to minutes) after test termination.

6.2.3. Intravenous Catheter and Venipuncture

Risks include:
- bruise or clot formation
- infection
- pain at the site of catheter insertion/venipuncture
- hematoma after withdrawal
- soreness over the site

These should all be transient and resolve after several days.

6.2.4. Blood sampling

Risks include:
- Pain at site of blood draw
- Bleeding
- Infection
- Dizziness (unlikely since supine)

Poses no further risk than IV catheterization.

6.2.5. MRI

MRI uses a powerful magnetic field, not ionizing radiation, to create an image of the scanned area of the body. No contrast agent will be used.

Risks include:
- There are no known risks of MRI, aside from the standard risks associated with persons with certain metallic implants. If potential participants have a metallic implant, cardiac pacemaker, or are pregnant, they will be disqualified.
- Sensation of claustrophobia or anxiety, particularly in individuals susceptible to it.
- People with metallic implants, such as prostheses or aneurysm clips, or persons with electronic implants, such as cardiac pacemakers, the magnetic field generated by the magnetic resonance machine can cause a displacement or malfunctioning of these devices.
- We know of no risks or adverse effects from the radio signals used in this study.
- Some people have also reported tingling or tapping sensations, or muscle twitches in different parts of their body during the imaging procedure.
    - These sensations are not hazardous and should not cause discomfort to the participants.
- A small increase in risk may be associated with rapid gradient waveform switching times associated with fast MR imaging. In certain situations, the rapid switching of gradient waveforms has caused peripheral nerve stimulation in subjects.

  - Significant nerve stimulation, however, has not occurred as long as the imaging system has been programmed to stay within certain limitations of gradient strength and switching time (dB/dt).
- Occasionally, people who have clasped their hands tightly together during the study have reported a feeling of electrical shock in their hands and arms.
  - This is also not hazardous; however, to avoid any possible discomfort, participants will be instructed to not clasp their hands together during the study.
- Women who are pregnant must not participate in this study. The potential risks to a fetus from the MRI scan are not definitely known.
- The MR scanner produces loud tapping sounds during operation, which may reach somewhat objectionable levels.
- Anatomical abnormalities
  - Images will not be reviewed by neuroradiology
  - Any obvious abnormalities will be reported to the IRB upon discovery for assistance in determining how to proceed with potential reporting

6.2.6. Subject Monitoring

An automatic blood pressure cuff around the upper arm may feel uncomfortable while inflated, but this is temporary (30-60 seconds). Blood pressure measures are considered very safe.

6.2.7. Hypoxia

Hypoxia exposure will last approximately 35 minutes total. Acute exposure to hypoxia generally causes an increase in ventilation. Systemic symptoms are limited to possible slight discomfort, lightheadedness, or dizziness. These trials will be performed in the resting, supine position, maximizing subject comfort and minimizing risk of injury. Our lab has experience conducting hypoxia (arterial oxygen saturation = 80%) research in the proposed participant populations without serious adverse event. This level of hypoxia is slightly greater than people who travel to high altitudes of Colorado Mountains (12,000-14,000 feet elevation). It's also a level achieved with severe sleep apnea. Given our experience with this approach, hypoxia well-tolerated and there is minimal risk. Any symptoms can be rapidly reversed by switching to room air breathing.

6.2.8. Hypercapnia

Total exposure time to hypercapnia will typically last 15-20 minutes. Systemic symptoms are minimal to nonexistent, other than variable change in HR and moderate increase in BP. The most likely response to this condition is an increase in ventilation and possible slight discomfort or air hunger. These are not dangerous as we maintain oxygen saturation at normal levels (95-100%). These trials will be performed in the resting, supine position to maximize subject comfort and minimize risk of injury. Our lab has experience conducting hypercapnia research in the proposed participant populations without any serious adverse events. This level of CO2 is 20-30% higher than normal, and higher than typically associated with clinical diseases like COPD or sleep apnea. Similar exposures are conducted in hundreds of studies to test brain vascular reactivity of all ages without serious AE. Any symptoms can be rapidly reversed by switching to room air breathing.

6.2.9. Indomethacin

Possible Risks and Adverse Drug Reactions: The adverse reactions for Capsules INDOCIN listed below have been arranged into two groups: (1) incidence greater than 1%; and (2) incidence less than 1%. The incidence for group (1) was obtained from 33 double-blind controlled clinical trials reported in the literature (1,092 patients). The incidence for group (2) was based on reports in clinical trials, in the

literature, and on voluntary reports since marketing. The probability of a causal relationship exists between INDOCIN and these adverse reactions, some of which have been reported only rarely. The adverse reactions reported with Capsules INDOCIN may also occur with use of the liquid suspension. It is important to note that most of these adverse reactions are associated with chronic use rather than single use of Indocin.

Adverse reactions: The most common adverse reactions to indomethacin, as reported by clinical trials, with an incidence of greater than 1% are as follows with gastrointestinal and nervous system symptoms the most common:

- Gastrointestinal:
  - Nausea (with or without vomiting)
  - Dyspepsia (including indigestion, heartburn and epigastric pain)
  - Diarrhea
  - Abdominal distress or pain
  - Constipation
- Central Nervous System:
  - headache (11.7%)
  - dizziness
  - vertigo
  - somnolence
  - depression and fatigue (including malaise and listlessness)
- Special Senses:
  - tinnitus

Adverse reactions to indomethacin, as reported by clinical trials, with an incidence less than 1% are as follows:

- Gastrointestinal:
  - anorexia
  - bloating (includes distension)
  - flatulence
  - peptic ulcer
  - gastroenteritis
  - rectal bleeding
  - proctitis
  - Ulcerations
  - Gastrointestinal bleeding
  - Ulcerative stomatitis
  - Toxic hepatitis and jaundice
  - Intestinal strictures
- Central Nervous System:
  - Anxiety
  - Muscle weakness
  - Involuntary muscle movements
  - Insomnia
  - Muzziness
  - psychic disturbances including
    - psychotic episodes
  - mental confusion
  - drowsiness
  - light-headedness
  - syncope
  - paresthesia
  - aggravation of epilepsy and parkinsonism
  - depersonalization
  - coma
  - peripheral neuropathy
  - convulsion
  - dysarthria
- Special Senses:
  - blurred vision
  - diplopia
  - hearing disturbances, deafness
  - ocular — corneal deposits and retinal disturbances
- Cardiovascular:
  - hypertension
  - hypotension
  - tachycardia
  - chest pain
  - congestive heart failure
  - arrhythmia; palpitations
- Metabolic:
  - edema

    - weight gain
    - fluid retention
    - flushing or sweating
    - hyperglycemia
    - glycosuria
    - hyperkalemia
- Integumentary:
    - pruritus
    - rash; urticaria
    - petechiae or ecchymosis
    - exfoliative dermatitis
    - erythema nodosum
    - loss of hair
    - Stevens-Johnson syndrome
    - erythema multiforme
    - toxic epidermal necrolysis
- hematologic:
    - leukopenia
    - bone marrow depression
    - anemia secondary to obvious or occult
        - gastrointestinal bleeding
    - aplastic anemia
    - hemolytic anemia
    - agranulocytosis
    - thrombocytopenic purpura
    - disseminated intravascular
    - coagulation
- Hypersensitivity:
    - acute anaphylaxis
    - acute respiratory distress
    - rapid fall in blood pressure resembling
        - a shock-like state
    - angioedema
    - dyspnea
    - asthma
    - purpura
    - angiitis
    - pulmonary edema
    - fever
- Genitourinary:
    - Hematuria
    - vaginal bleeding
    - proteinuria
    - nephrotic syndrome
    - interstitial nephritis
    - BUN elevation
    - renal insufficiency, including renal failure
- Miscellaneous:
    - epistaxis
    - breast changes, including enlargement and tenderness, or gynecomastia

6.2.10. Antacid

Antacid is a drug used for the relief of acid indigestion, heartburn, sour stomach, upset stomach, and pressure and bloating (gas). Adverse reactions can occur if you have kidney disease or a magnesium-restricted diet. This product may interact with certain prescriptions and not allow them to be fully absorbed in the stomach.

6.2.11. Temporarily stopping birth control in women.

The largest risk of stopping birth control is pregnancy in sexually active women. We will reduce this risk by counseling patients to use a non-hormonal method (abstinence, condoms) while they are off birth control.

Additional risks of stopping hormonal birth control may include heavier, more painful, or more irregular periods and worsening of menstrual symptoms such as bloating, mood swings, or headaches. We will discuss these possible risks with subjects prior to their decision to discontinue hormonal birth control. Typically, if a woman did not have these type of menstrual symptoms before using hormonal birth control, they will not experience these symptoms with discontinuation. We will also exclude women who are taking hormonal birth control for specific medical conditions (such as PCOS, hyperprolactinemia, or very heavy menstrual bleeding) to prevent any medical risks of discontinuation.

7. DATA AND SAFETY MONITORING PLAN

7.1. Study Monitoring Service (SMS)

The SMS will conduct interim-monitoring visits (IMV) to ensure compliance and safety. The first IMV will be scheduled following the enrollment of the 5th subject or quarterly, whichever comes first. Subsequent IMVs will be scheduled to occur approximately quarterly. The schedule may vary based on study enrollment rate. Unscheduled visits may be conducted based on reports or evidence of potential noncompliance, significant increases in subject enrollment rates, or changes in protocol/personnel and training activities. SMS service agreement is attached to the ARROW application.

Monitoring may consist of full or partial review of study records, depending on risk level and observed compliance. As such, during their monitoring activities, UW ICTR SMS personnel plan to review all (100%) of the study-related subject records for approximately 50% of the enrolled subjects. The first two subjects in each cohort will be monitored in their entirety, with the additional number to be randomly selected from each of the subject cohorts. SMS personnel could increase the percentage of study or subject records to be reviewed if warranted by the ongoing monitoring findings. The study monitor(s) will work closely with the ICTR DMC statistician to conduct periodic central data reviews, with follow-up conducted by the study monitors for any data discrepancies identified.

When SMS monitoring reports are delivered to the research team, the research team will forward these reports to the IRB for review.

7.2. Data Monitoring Committee (DMC)

The ICTR DMC provides investigators with independent services to ensure appropriate measures are in place to promote subject safety, research integrity and compliance with federal regulations and local policies for individual clinical research protocols in need of DMC review as determined by the PI, the funding agency, the local Scientific Review Committee, or the local IRB, and for which no DMC exists. For these studies, the UW ICTR DMC will be the primary data and safety advisory group for the PI.

The DMC is supported in its mission of safety and compliance by experienced ICTR staff to provide administrative assistance, experienced members representing a diversity of backgrounds, skills and knowledge, and the use of REDCap which allows more efficient tracking of protocols and protocol subjects. In providing oversight for the conduct of this study, the ICTR DMC will meet on a annual basis throughout the study. Additional meetings may be scheduled as determined by the DMC or as requested by the PI. The DMC members will review protocol-specific reports created by statisticians that serve a non-voting member role on the DMC using data pulled from the REDCap. These standard reports will include an overview of study objectives, a review of actual and projected accrual rates, an evaluation of patient demographics for balance of randomization (if applicable), and a summary of the number and seriousness of adverse events. An interim analysis of study results may be performed and source documents may be reviewed to allow the DMC to independently judge whether the overall integrity and conduct of the protocol remain acceptable based on data provided and reported by the PI. The DMC will make recommendations to the PI that could include actions of continuation, modification, suspension, or termination

Data Type	Frequency of Review	Reviewers
Subject accrual (including compliance with protocol enrollment criteria)	Every 12 months	ICTR Data Monitoring Committee, PI

Subject demographics	Every 12 months	ICTR Data Monitoring Committee, PI
Status of all enrolled subjects as of date of reporting	Every 12 months	ICTR Data Monitoring Committee, PI
Primary outcome analysis	Every 12 months	ICTR Data Monitoring Committee
AEs and rates	Every 12 months	ICTR Data Monitoring Committee, PI
SAEs	Per occurrence	ICTR Data Monitoring Committee, PI

7.3. Adverse Event (AE) Reporting

This study will utilize the Common Terminology Criteria for Adverse Events (CTCAE) v5.0 for adverse event monitoring and reporting. This information can be downloaded from the CTEP home page (http://ctep.info.nih.gov/CTC4/ctc_ind_term.htm).

The severity of the event will be graded using the CTCAE. In addition, for comparison to the CTCAE, adverse events will be tabulated using a 3-level schema as defined below:

- Mild
  - Event may be noticeable to patient
  - Does not influence daily activities
  - Usually does not require intervention.
- Moderate
  - Event may be of sufficient severity to make patient uncomfortable
  - Performance of daily activities may be influenced
  - Intervention may be needed.
- Severe
  - Event may cause severe discomfort
  - Usually interferes with daily activities; patient may not be able to continue in the study
  - Treatment or other intervention usually needed.

Note the distinction between the severity and the seriousness of an AE. A severe event is not necessarily an SAE. For example, a headache may be severe (i.e. interferes significantly with patient's usual function) but would not be classified as serious unless it met one of the criteria for SAEs.

Next, it will be determined by the PI and study physician or clinical neuropsychologists whether the event is expected or unexpected and if the AE is related to the intervention or study procedures. With this information, it will be determined whether an AE should be reported as an expedited report in addition to submission via routine clinical data.

Expedited AE reporting may require e-mail notification, phone, or fax submission of a written report as directed below. All expedited AE reports will be submitted to the IRB of record, the UW Health Sciences Institutional Review Board (HS IRB) in the case of this study.

7.3.1. Assessment of Attribution

Attribution of AEs will be assessed in relation to the study procedures. When assessing whether an adverse event is related to a study procedure, the following attribution categories are utilized:

- Definitely Related
    - An AE categorized as definite is clearly related to study procedures. If the timing of the AE is definitely consistent with the exposure to the study related procedures and it is most likely that the AE was caused by the study procedures such as because a high occurrence of the AE was expected based on the study intervention materials. In this case, the PI may categorize the AE as definitely related.
- Probably Related
    - An AE that is likely related to study procedures. If the timing of the AE is consistent with the exposure to the study intervention and it is more likely that the AE was caused by the study procedures than not, the PI may categorize the AE as Probable.
- Possibly Related
    - An AE that may be related to study procedure. If the timing of the AE is reasonably consistent with the exposure to the study intervention, and there is another cause of the AE that could be equally likely, the PI may categorize the AE as Possible.
- Unlikely Related
    - An Unlikely AE is one that is doubtfully related to study procedures. The coincidence of the AE with the exposure of the investigational product or intervention should be assessed. An AE that continued while the intervention was interrupted or stopped, or if the AE resolved while the intervention continued, may be categorized as Unlikely. If there is another more likely cause of the AE, the PI may determine that the AE was unlikely related to the study intervention.
- Not Related/Unrelated

An Unrelated AE is one that is clearly NOT related to study procedures. An AE may be considered Unrelated if the subject did not receive the study intervention or if there is another obvious cause of the AE (for example, a car accident or other disease/condition).

7.4. Serious Adverse Event (SAE)

A serious adverse event (SAE) is an AE occurring during any phase of the study (i.e., screening, admission, treatment, or follow-up) that fulfils one or more of the following criteria:

- Results in death
- Is immediately life-threatening
- Requires in-patient hospitalization or prolongation of existing hospitalization (more than 24 hours)
- Results in persistent or significant disability or incapacity
- Is a congenital abnormality or birth defect
- Is an important medical event that may jeopardize the subject or may require medical intervention to prevent one of the outcomes listed above

7.5. Identifying, Reviewing, and Reporting Adverse Events and Unanticipated Problems to the IRB

Identifying

- To effectively identify all AEs (anticipated or unanticipated), Schrage lab staff will follow laboratory and protocol specific SOPs, complete AE reporting forms for each subject as they arise, and maintain a database of all reported adverse events for each protocol by entering all AEs into REDCap within a timely manner. All stopping events or subject reported complaints/concerns will be documented via study notes during the study. Participants will be encouraged to contact us with any questions or concerns following the study visit.

Reviewing

- The PI, study physician, and study team will also review AEs during internal audits of study documents in a cumulative fashion to identify any trends of safety concerns (see section 7.8 Internal Audits). In the event of an SAE, the PI, study physician, DMC, and IRB will review the event as soon as possible.

Reporting

- All adverse events will be recorded in REDCap in a timely manner consistent with reporting requirements. Schrage lab staff will log all AEs in REDCap, and report AEs and unanticipated problems to the DMC and to the IRB as indicated by IRB guidance on Knowledge Base.

7.6. Expedited Adverse Event Reporting Requirements

Serious Adverse Event— Reported Within 24 Hours

- Serious Adverse Events requiring expedited reporting within 24 hours will be reported to both the UW Health Sciences IRB and the ICTR DMC Manager within one working day as directed below. Confirmation that all appropriate parties were notified will be done at this time.

Serious Adverse Event – Reported within 15 Days

- Serious Adverse Events requiring expedited reports in writing within 15 working days will be sent by the PI and study team.

7.7. Protocol Deviations

To effectively identify deviations from the IRB approved protocol, Schrage Lab staff will maintain protocol checklists that are designed to not only minimize protocol deviations but to identify deviations when they do occur. Additionally, Schrage Lab staff will conduct regular internal audits (see section 7.8 Internal Audits) to identify deviations that may have been overlooked. All deviations will be logged on a Deviation Tracking Log and a database of deviations will be maintained in REDCap. The PI will review deviations as they are identified, and oversight committees will be notified according to their guidelines. The study team will implement corrective action plans when appropriate to address deviations.

7.8. Internal Audits

Regular internal audits will be conducted to ensure compliance and safety. Internal audits will occur on a quarterly basis. These audits could consist of full or partial review of study records, depending on observed past compliance. Audits will be documented on Internal Compliance Review forms modified specifically for the study and will include review of the following:

- Personnel (e.g., ensure all personnel have the appropriate training)
- Documentation of IRB submissions and correspondence
- Informed Consent/HIPAA Authorization Forms
- Laboratory Aspects of the Trial
- Equipment Maintenance
- Communication and Correspondence with oversight committees
- Study Conduct
- Completion of Case Report Forms
- Adverse Events
- Protocol deviations

These internal audits will identify any unanticipated outcomes or trends, and Schrage Lab staff can then notify the IRB of any new, common, or unanticipated adverse events, if applicable.

The results from internal audits (e.g., noncompliance, adverse event trends) will be discussed during regular lab meetings where team members can establish plans to prevent future issues.

7.9. Minimizing Research-Associated Risk

General Safety Overview: Our study has relatively low risk because of the age of the subjects (≥18-≤40 years) the minimal invasive procedures, and safe record of the study drug (indomethacin) in research settings. Gas challenges are also common, safe, easily reversed, and well-tolerated.

The procedures with the most potential for more than minimal risk are the venipuncture and intravenous catheter, the infusion of a (single) study drug, and MRI, but we minimize risks by employing:

- Rigorous screening to determine eligibility
- Rigorous screening of women taking hormonal birth control prior to temporary discontinuation with consultation of collaborating endocrinologists.
- Venipuncture or Intravenous catheterization by trained study staff
- Systematic documentation to ensure study drugs are mixed, verified, and delivered in an aseptic approach
- Clear study visit stopping guidelines for gas challenge or drug induced changes in hemodynamics.

Risks of venipuncture, or placing a venous catheter include bruise or clot formation and infection. The IV will be removed after ~1.5 hours. Minor risks associated with this procedure would be pain at the site of catheter insertion, bruising after withdrawal, and soreness over the site. These should all be transient and resolve after several days.

Subjects will always be monitored by research staff throughout the study for both hypoxia and hypercapnia visits.

Safety equipment:
MRI facility: Full-time staff along with local defibrillators are always accessible. The “code team” can be called from the UW Hospital.
PI Lab: Screening and maximal fitness testing occurs in PI lab or Human Exercise Research Core (HERC) at School of Nursing. A defibrillator is available in the hallway near our laboratory. 911 will be called in an emergency.

7.9.1. Initial Screening

Phone screen or internet screen: All subjects will be asked to complete a screening (via Redcap survey) documenting physical activity, medications, and personal and family history of cardiovascular disease and risks. The questions are designed to immediately eliminate those subjects meeting exclusion criteria, and most importantly identify and exclude subjects who are at increased risk for IV catheter problems, adverse drug reactions, or MRI risk.

If the individual is deemed ineligible, then their screen information will be destroyed. If an interested individual is deemed initially eligible based off of this pre-screen, they will be invited to come in for a more thorough in-person screen visit. To maximize the likelihood of studying only appropriate subjects for this study (eg no body metal implanted for MRI scanning), and minimize the risk of bleeding or bruising in at

risk subjects, a detailed relevant cardiovascular medical history will be performed prior to the catheterization procedure.

7.9.2. Subject Monitoring

Subject Monitoring: All testing will include subject monitoring of heart rate (HR) and blood pressure, to address subject stopping guidelines/safety and ensure data integrity. HR will be measured by Polar Heart Rate Monitor. Blood pressure will be monitored sphygmomanometry of the brachial artery. Oxygenation will be monitored on finger or ear SaO_2 monitor, and end tidal CO_2 by sampling of expired gases from mask/mouthpiece. With continuous monitoring, we can readily observe any adverse cardiovascular events and move to intervene immediately.

The frequency of monitoring is as follows: during baseline and each major MRI sequence (ASL or VIPR). Visually, HR, O_2 Sats and ETC02 are continuous readouts. Practically speaking, all are recorded every 5-10 minutes on CRFs. These are monitored by trained laboratory staff who are physiologists-typically graduate students, postdoctoral fellows, or PI.

7.9.3. Blood Sampling

Blood sampling (not venipuncture or IV placement) will be performed only by research personnel that have had phlebotomy training. This training has encompassed the insertion, maintenance, and use of intravenous catheters. These personnel all have at least a bachelor's degree or higher health and/or physiology-related education in terms of academic preparation. We feel education and training of personnel, and our positive track record over 10 years at UW performing similar invasive procedures, provide high confidence IV blood draws present a very low risk to subjects.

7.9.4. MRI

[Update depending on on-call / on-site determination]

MRI risks will be minimized by thorough MRI screening at screening visit, and by MRI technicians immediately prior to MRI visit. Second, all MRI sequences fall under FDA approved guidelines and carry very small risks like muscle tingling described under MRI risks. Subjects have verbal contact with MRI technicians and study team in control room, as well as a "panic button" they can press in case they feel any pain or discomfort.

7.9.5. Protecting the Confidentiality of Participant Data

Risks to confidentiality will be minimized by keeping copies of the documents linking study assignment number and the participant's unique identifiers with the participant's informed consent in locked cabinets of the offices of Dr. Bill Schrage. Only subject numbers will be used for group assignment, data processing, and analyses. All data will be stored in locked cabinets in a secure lab, and electronic files are all stored on password-protected databases and computers.

Personal information such as name, gender, date of birth, and medication history will be stored in a locked file cabinet in a locked office in the PI's laboratory. Subject information will be coded to remove any personal identifiers during data analysis or research publications. Research oversight and regulatory groups may review study records.

Data will be stored as both hard copies and electronic files. Hard copy data collection forms will be locked in a file cabinet in the restricted-access laboratory area. Electronic files that contain potential Protected Health Information (PHI) will be stored in REDCap and ONCORE, secure web-based data management systems, and/or a secure Box folder configured with the involvement of the UW-Madison HIPAA security officer. Electronic files will be HIPAA compliant and will use a subject ID instead of the subject's name. The raw MRI data will be stored on servers assigned to Dr. Wieben and managed by the Departments of Medical Physics and Radiology and/or on encrypted external hard drives.

Blood samples may be analyzed for various hormones and inflammatory markers externally to the study team (e.g. UW Primate Research Center). Samples analyzed at the UW Health Clinical Labs will be placed in the medical record of the subjects per UW Health policy. Samples analyzed at research labs (e.g. UW Primate Research Center) will remain coded such that the outside blood analysis team will not be provided any identifiers or means by which they could connect the blood samples to any individuals that have participated in the study.

7.10. Stopping Guidelines

7.10.1. Study Stopping Guidelines

This study may be temporarily suspended or prematurely terminated if there is sufficient reasonable cause. Written notification, documenting the reason for study suspension or termination, will be provided by the suspending or terminating party to investigator, funding agency, and regulatory authorities. If the study is prematurely terminated or suspended, the PI will promptly inform the IRB and will provide the reason(s) for the termination or suspension. Circumstances that may warrant termination or suspension include, but are not limited to:

- Determination of unexpected, significant, or unacceptable risk to subjects
- Insufficient compliance to protocol requirements
- Data that are not sufficiently complete and/or evaluable

The study may resume once concerns about safety, protocol compliance, and data quality are addressed and satisfy the applicable regulatory authorities.

7.10.2. Subject Stopping Guidelines

Each intervention (i.e. study visit) will be terminated based on the following specific guidelines:

1. If the subject wishes to stop
2. If the subject complains of serious pain or discomfort during study procedures
3. If the subject complains of severe anxiety or claustrophobia related to MRI
4. Any SAE occurring during study procedures

Subject Monitoring: Study visit will be stopped for day if any of the following occur:

1. If blood saturation falls below 75% (measured by pulse oximetry) over 10 minutes during hypoxia challenge.
2. If blood saturation falls below 65% *at any time* during the hypoxia challenge
3. If blood CO_2 levels (by end-tidal CO_2) increase to > 55 mmHg (during hypercapnia study visit)
4. If MABP (mean BP) decreases more than 20 mm Hg during hypoxia
5. If MABP increases more than 20 mm Hg during hypercapnia.

8. STATISTICAL CONSIDERATIONS

8.1. MRI Data Processing

Standard and validated tools will be used for image processing. The research team is expert in analyzing brain imaging data and have published several studies focused on MRI-derived blood flow-based measures.

Maps representing gray matter probability and cerebral blood flow will be generated using SPM, FSL, or similar software. Scans will be processed using the most up to date and recommended processing scheme, including methods previously used by our research group and collaborators.

We expect to use standard viewing software (Advanced Workstation 4.3, GE Healthcare) for magnetic resonance angiography and phase contrast data, in addition to

1) MIMICS and EnSight, commercial software packages commonly used in 4D flow visualization and
2) MatLab-based software for quantitative analysis of 4D data sets

Quantitative data from two 4D multi venc flow acquisitions will be available. The data includes:

1) Quantitative blood flow values (peak and mean flow, peak and mean velocity, pulsatility and resistance index) of 4D PC
2) Quantitative morphological data (vessel diameter) of:
    a. Standard magnetic resonance angiography techniques
    b. 4D PC

8.2. Statistical Summary

Previous Protocol:

Summary for statistical power

Power for our primary outcomes in each Aim is based on n=48/group, with an effect size of 1.01 for the sex effect. Power to detect sex (Aim 1, 2) differences in ΔCVC for any of five cerebral arteries/regions for the first of the sequential tests (alpha = .01) is 0.98, nearly unity. Our research design is powered from the bottom up, meaning that sub-groups of 24 for secondary outcomes, we have sufficient power (0.8) to detect a sex-specific drug effect on any one of five cerebral arteries (or more CP regions).

Updated Protocol (CP011):

Summary for statistical power

Power for our primary outcomes in each Aim is based on n=27/group. After consulting with our biostatistician, the revised enrollment numbers (accounting for 10% attrition) will provide good power (Power >0.8) for secondary outcomes (drug effects by brain region), and excellent power for sex differences to a given stressor (Power >0.9)

Updated Protocol (CP017):

Summary for statistical power

Power for our primary outcomes in each Aim is based on n=30 males and 27 females. After consulting with our biostatistician, the revised enrollment numbers (accounting for 10% attrition) will provide good power (Power >0.8) for secondary outcomes (drug effects by brain region), and excellent power for sex differences to a given stressor (Power >0.9)

9. DATA AND RECORD KEEPING

9.1. Data Management

Delegated and appropriately trained study team members will be involved in data collection. All personnel who will be involved in data collection have completed appropriate Human Subjects Protection training or will before they engage in contact with subjects and subject data.

The Research Electronic Data Capture (REDCap) system is used to manage the data for this study. REDCap is a largely self-service, secure, web-based application for building and managing data collection forms. REDCap provides data management functionality by allowing the development of instruments and surveys to support data capture for research studies.

The REDCAP system used for this study is managed by ICTR. The ICTR REDCap support team will work with the investigator, statistician, and study team to ensure the relevant, applicable study data are collected and managed with restricted access using the software electronic data collection form instruments.

9.2. Assured Confidentiality

The raw MRI data will be stored on servers assigned to Dr. Wieben and managed by the Departments of Medical Physics and Radiology. The UW-Madison School of Education houses the Schrage Lab server and all electronic files will be coded to maintain HIPPA compliance. Subject information and study tracking information will also be stored in REDCap, which is a secure site used for data management.

Any forms with PHI will be stored in locked cabinets inside the PI's secure laboratory. All data collected electronically will be password protected on a secure Box folder configured with the involvement of the UW-Madison HIPAA security officer. Electronic files will be HIPAA compliant and will use a subject ID instead of the subject's name. Only coded data will be used in data analysis. Any publications arising from this protocol will not include any personal identifying information or study code, thereby making the reported data completely de-identified.

9.3. Data Collection Methods

Data will be collected using REDCap, MRI, offline data collection software (e.g. LabChart), data analysis software, and data collection forms. These files will consist of electronic DICOM, .dat, LabChart, and Excel files, as well as hard copies of data collection forms for each subject.

All other study data referenced above will be collected using study visit checklists and data collection forms, most of which constitute both the Case Report Form (CRF) and the original source (i.e., the first and only place the data is manually written/recorded). These study specific forms will be developed and maintained with the assistance of the ICTR DMC for recording all necessary data for each subject. It is the PI's responsibility to ensure that these are properly, legibly, and fully completed and signed where appropriate. The CRFs are used to record study data and are an integral part of the study and subsequent reports. Therefore, the CRFs must be completed for each subject screened or enrolled according to the subject's source data on a per-visit basis. All study visit checklist and data collection forms will be retained in the subject research chart/file. All applicable data collected using the study visit checklists and data collection forms will be entered in to electronic Case Report Forms (eCRFs) within REDCap.

When the study is complete, the applicable CRFs must be signed by the investigator to attest that it is an accurate and complete record.

9.4. Retention of Study Records

In compliance with UW-Madison regulations, study records will be for a minimum retained for seven years following final project close-out.

10. DATA INTEGRITY

Justification for building in flexibility:

Increasing flexibility provides time for participants to get all MRI visits completed, and to overcome unexpected technical delays that briefly prolong the study visit (see below) without increasing risks.

Impact of building in flexibility on data integrity:
Flexibility will not impact data integrity. In fact, it should greatly reduce participants from dropping out of study, such that overall study risks will be reduced by reducing the total number of subjects enrolled to achieve primary outcomes.
Flexibility will enhance likelihood of collecting all primary data once a participant is enrolled.

Maximizing visit flexibility is important for meeting the competing influences of: female menstrual cycle timing, study team availability, and MRI scheduling availability.

No flexibility steps below significantly increase risk beyond those described in the main study design.

To build in flexibility while maintaining subject safety and data integrity. It will not be considered a deviation if:

1) Screening visit: it will not be considered a deviation if:
    a. Participants must return to lab to complete successful venipuncture
    b. Participants must return to complete successful maximal fitness test
    c. Participants must return to complete successful gas challenge familiarization visit
    d. Participants must return to complete successful pregnancy test
    e. Visit Reminder is sent less than 12 hours prior to gas familiarization or exercise visit
2) MRI Study Visit: it will not be considered a deviation if:
    a. We need to conduct extra scans (e.g. ASL and/or PC VIPR) as needed in order to complete primary outcome data collection. This is unlikely, but technical issues may cause the restart of any given scan.
    b. If hypoxia exposure lasts up to 60 minutes (planned for ~35) in order to complete all scans. This may happen due to technical issues with MRI (noted in a above), technical problems with gas delivery, fluctuating subject hemodynamics from steady state (namely S_aO_2 and ET CO_2).
    c. Study visit not completed due to technical (e.g. , error in gas challenge mixing, MRI failure, or practical reasons (scan time ended). Subject can be rescheduled for a complete study visit at on a future date.
    d. We extend hypercapnia duration up to 30 minutes. This may happen due to technical issues with MRI (noted in a above), or if subject hemodynamics appear to fluctuate from steady state (namely $ETCO_2$)