Cerebral Blood Flow Regulation during Hypoxia in Healthy Adults

By

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*"The joy of life comes from our encounters with new experiences, and hence there is no greater joy than to have an endlessly changing horizon, for each day to have a new and different sun."*

--Christopher McCandless

*"And in the end, it's not the years in your life that count. It's the life in your years."*

--Abraham Lincoln

# Contents

# Introduction

The brain comprises ~2% of body mass but accounts for ~20% of total body oxygen ($O_2$)

consumption and cardiac output at rest (42). This extraordinary oxygen demand, driven by a

high metabolic rate and almost total reliance on oxidative metabolism, necessitates tight

regulation of cerebral blood flow (CBF) across a wide range of environmental and metabolic

perturbations. Despite adequate CBF regulation being critical to neuronal integrity, mechanisms

of cerebrovascular control remain relatively unknown in humans, while even less is known about

how sex, menstrual phase, or regional specificity may influence cerebrovascular control.

Consider stroke, the most common cerebrovascular disease in the United States

accounting for over $73 billion in annual medical and disability costs. Stroke is known to be

both sex-specific and regionally distributed within the brain. Thus, elucidating sex-, menstrual

phase-, and region-specific mechanisms of cerebrovascular control during physiologic stress,

such as hypoxia, may help identify novel therapeutic targets aimed at preventing or reducing the

impact of cerebrovascular disease.

With this in mind, the aims of this dissertation were to investigate sex-, menstrual phase-,

and region-specific cerebrovascular control in young, healthy adults. We utilized the

physiological stress of hypoxia to elicit a robust increase in CBF and administered a drug that

allowed us to test the mechanistic contribution of cyclooxygenase (COX) to hypoxic cerebral

vasodilation. This research sought to identify potential sex-, menstrual phase-, and region-

specific differences in CBF regulation during in hypoxia, as well as the mechanistic contribution

of COX. These findings contribute to our growing understanding of the cerebral circulation and

may advance our search for novel therapeutic targets aimed at slowing or preventing the development of cerebrovascular disease.

Specifically, we aimed to:

1.) Determine the sex-specific contribution of COX to hypoxic vasodilation in the middle cerebral artery (MCA).

2.) Determine the menstrual phase-specific contribution of COX to hypoxic vasodilation in the MCA.

3.) Determine the region-specific contribution of COX to hypoxic vasodilation by examining eleven major cerebral arteries.

The first and second projects of this dissertation addressed Aim1 and Aim 2, determining the sex- and menstrual phase-specific contribution of COX to hypoxic vasodilation in the MCA. The third project of this dissertation explored the region-specific contribution of COX to hypoxic cerebral vasodilation by concurrently examining eleven major cerebral arteries including the basilar artery (BA), and left (L) and right (R) internal carotid (ICA), anterior cerebral (ICA), middle cerebral (MCA), posterior cerebral (PCA), and vertebral (VA) arteries.

Based upon these aims we generated the following hypotheses. First, we hypothesized that hypoxic vasodilation in the MCA will be greater in women than men, due in part to a greater contribution of COX. Second, we hypothesized that hypoxic vasodilation in the MCA will be greater during the late follicular versus early follicular phase of the menstrual cycle, due in part to a greater contribution of COX. Third, we hypothesized that hypoxic vasodilation will be

greater in the posterior circulation (VA, BA, and PCA) compared to the anterior circulation

(ICA, MCA, and ACA), due in part to a greater contribution of COX.

# Background

## Cerebral Vasculature

The cerebral vasculature consists of both the macrovasculature and microvasculature. The macrovasculature is comprised of arteries that arise from the neck (ICA, VA, and BA) and feed into the Circle of Willis, providing redundancy (also known as collateral) of blood flow within the cerebral circulation (10, 143). From the Circle of Willis the macrovasculature extends outward into the ACA, MCA, and PCA. Distally, the macrovasculature then branches, decreases in size, and eventually transitions into the microvasculature. The microvascular includes pial arteries, penetrating arterioles, and ultimately capillaries where gas and nutrient exchange occurs (10, 143).

The cerebral vasculature is a highly regulated vascular bed ensuring relatively constant cerebral perfusion. The macrovasculature contributes to the maintenance of CBF, as these vessels are sensitive to severe alterations in arterial blood gases and perfusion pressure (33, 34, 116, 143). However, microvascular pial arteries are commonly considered the primary sites of resistance within the cerebral circulation (132, 143).

Pial vessels are located within the subarachnoid space, exposing them to both local and global metabolic conditions (via cerebral spinal fluid arterial blood gases), making them ideal resistance vessels (143). When exposed to vasodilatory stimuli such as hypoxia the smooth muscle surrounding pial arteries relax causing an  increase vessel diameter and subsequent increase in cerebral blood flow (132). These small changes in vessel diameter (radius) can have a profound influence of blood flow, as indicated by the Hagan-Poiselle Equation (Appendix A, *Hagan-Poiselle Equation*) flow is proportional to the radius raised to the fourth power. Further

upstream, blood flow within the macrovasculature increases secondarily to the downstream vasodilation of microvascular pial arteries (143). These secondary macrovascular changes in blood flow, driven by downstream vasodilation of the microvasculature, can be quantified noninvasively with several methodologies including transcranial Doppler ultrasound (TCD) (1) and magnetic resonance imaging (MRI) (139).

## Hypoxic Cerebral Vasodilation

It is relatively easy and cost effective to quantify CBF with TCD (38). Despite this methodology, the regulatory mechanisms of cerebrovascular control remain underexplored. Control of CBF is complex and involves many overlapping mechanisms of regulation. Principle regulators include, but are not limited to, arterial blood gases (carbon dioxide – $CO_2$ and oxygen – $O_2$), cerebral metabolism, autoregulation, and the autonomic nervous system (143). Although each contributes to the regulation of CBF under given conditions, for the purposes of this dissertation, I will be focusing on cerebrovascular control during alterations of arterial blood gases. Specifically, I will be investigating CBF regulation during hypoxia. Elucidating mechanisms of CBF control during hypoxia is relevant to sojourns to altitude and clinically relevant to sleep apnea, which is associated with poor cerebrovascular outcomes (28). Furthermore, unraveling general mechanisms of cerebrovascular control may provide therapeutic targets for the treatment and prevention of cerebrovascular disease.

Hypoxia is defined as a reduction in ambient $O_2$ tension that causes a decrease in arterial $O_2$ content. The body is able to sense a decrease in arterial $O_2$ content, with $O_2$ delivery subsequently restored through multiple homeostatic adjustments. Systemically, the reduction in arterial $O_2$ tension is sensed by the peripheral chemoreceptors. Specifically, the carotid chemoreceptors are responsible increasing ventilation during hypoxia, classically known as the

hypoxic ventilatory response. Increased ventilation during hypoxia serves to limit the reduction in arterial $O_2$ by increasing alveolar $O_2$, while simultaneously causing a reduction in arterial $CO_2$ (hypocapnia). Both $O_2$ and $CO_2$ are potent regulators of CBF, a reduction in $O_2$ mediates vasodilation and increases CBF, while a reduction in $CO_2$ induces vasoconstriction and decreases CBF. Therefore, the regulation of CBF during hypoxia is comprised of a balance between hypoxic vasodilation and hypocapnic vasoconstriction, with vasodilation overcoming vasoconstriction as indicated by the increase in total CBF during hypoxia exposure (3). Cerebral metabolism during hypoxia, at least in humans, remains unchanged (68, 141) which is important to consider given the tight coupling between metabolism and CBF (113).

On a local level cerebral vessels adjust to hypoxia by increasing vessel diameter (vasodilating), serving to maintain neuronal cell integrity by providing adequate $O_2$ and nutrient delivery while efficiently removing metabolic waste products such as $CO_2$. The precise mechanism by which hypoxia is sensed by cerebral vessels is not completely known. It has been theorized that a drop in arterial $O_2$ content elicits tissue hypoxia that decreases extra-vascular pH (anaerobic metabolism) and induces a retrograde vascular stimulus from neurons (neurovascular coupling), both of which cause vascular relaxation and an increase in CBF (143). Hypoxia may also have direct effects on the vasculature, including the endothelium, which can contribute to hypoxic vasodilation and increases in CBF (143). For example, hypoxia causes vasodilation of isolated cerebral vessels (108) which appears to be mediated by ATP sensitive K+ channels (132). More recently, it has been postulated that hemoglobin within erythrocytes serves as the primary $O_2$ sensor that governs cerebral vascular tone. Products released from erythrocytes such as adenosine triphosphate may influence the formation and release of additional signaling molecules, such as COX, that are critical to the vascular response during hypoxia (52).

Regardless of the specific $O_2$ sensor in cerebral vessels, the cerebral vasculature is not equally responsive to all severities of hypoxia. Generally speaking, hypoxia can be expected to increase CBF 0.5% - 2.5% per every 1% reduction in arterial $O_2$ saturation (143). However, hypoxic sensitivity appears to increase as arterial $O_2$ saturation approaches ~85% (123) or when the arterial partial pressure of $O_2$ is reduced from ~100 mmHg to ~50 mmHg (143). Varying levels of arterial $CO_2$ can also influence cerebral vessel sensitivity to hypoxia. If arterial $CO_2$ is above basal levels, a condition known as hypercapnia, sensitivity to hypoxia is increased. In contrast where there is lower than basal levels of $CO_2$, a condition known has hypocapnia, hypoxic sensitivity is decreased (85). Therefore, when conducting studies examining cerebrovascular response to hypoxia, it is important to clamp arterial $CO_2$ in order to limit variability and avoid confounding interpretation.

## Role of Cyclooxygenase in Hypoxic Cerebral Vasodilation

Maintenance of CBF across a variety of physiological conditions, such as hypoxia, is critical for neuronal function and integrity. Despite this importance, vascular mechanisms mediating hypoxic increases in CBF are poorly understood. Uncovering these mechanisms of cerebrovascular control will provide potential therapeutic targets aimed at preventing, reducing, or improving the lives of those with cerebrovascular disease. With the aims of this dissertation in mind, one mechanism that may mediate hypoxic vasodilation is COX.

COX is an enzyme that is essential for the metabolism of arachidonic acid (a 20-carbon fatty acid) to the vasoactive compounds prostacyclin ($PGI_2$) and thromboxane ($TXA_2$; Appendix A, *Cyclooxygenase Pathway*), with prostaglandin $G_2$ and prostaglandin $H_2$ serving as intermediates (27, 35, 124). There are two isoforms of COX, COX 1 and COX 2. COX 1 is a constituently active isoform of COX and has been shown to play a role in resting CBF responses

as well as hypercapnic responses (98).  COX 2 is inducible and is known to mediate the functional hyperemia associated with neuronal activity (97) and become activated during hypoxia (12, 59, 93).  Both isoforms of COX assist in the production of prostaglandins, essential bioactive messengers that vary widely in function, including $PGI_2$ (prostacyclin) and $TXA_2$ (thromboxane).

$PGI_2$ is known for its beneficial vascular effects including prevention of platelet aggregation, thrombosis formation, and vascular smooth muscle proliferation, while concurrently decreasing vascular tone and mediating an increase blood flow (27, 35, 124).  It has been suggested that the COX-2 mediates greater production of vasodilatory $PGI_2$ compared to COX-1(117), which may explain the increased cardiovascular risk associated with COX-2 specific inhibitors.  The $PGI_2$ mediated reduction in vascular tone occurs through $PGI_2$ receptors located on vascular smooth muscle cells.  $PGI_2$ receptors are G-coupled proteins that activate cyclic AMP (cAMP) and Protein Kinase A (PKA).  When activated, PKA decreases myosin light chain kinase activity (via phosphorylation), subsequently decreasing phosphorylation of vascular smooth muscle myosin light chain, and inducing vascular relaxation (35).  Alternatively, PKA can mediate hyperpolarization induced vascular relaxation through the activation of $K_{ATP}$ and $K_{Ca,}$ channels (35).

From a vascular perspective, $TXA_2$ can be considered the opposite of $PGI_{2.}$  $TXA_2$ promotes platelet aggregation and smooth muscle cell proliferation while concurrently increasing vascular tone and decreasing blood flow (27, 35, 124).  Endothelial $TXA_2$ mediates its vascular actions through G-protein coupled $TXA_2$ receptors located on vascular smooth muscle cells.  $TXA_2$ receptors mediate their actions through phospholipase C mediated stimulation of the IP3/DAG pathway.  IP3 is able to increase $Ca^{2+}$ release from the endoplasmic reticulum, which

activates myosin light chain kinase (via $Ca^{2+}$/calmodulin), and causes vascular smooth muscle contraction via the phosphorylation of regulatory myosin light chains (35). Additionally, DAG is able to activate protein kinase C (PKC). Once activated, PKC inhibits $K_{Ca}$ channels, inducing vascular depolarization and a subsequent increase in cytosolic $Ca^{2+.}$ The elevated cytosolic $Ca_{2+}$ actives myosin light chain kinase (via $Ca^{2+}$/calmodulin), causing vascular smooth muscle contraction via myosin light chain phosphorylation (35).

Given the contrasting vascular effects of $PGI_2$ and $TXA_2$, the relative contribution of each prostanoid determines whether COX can act to increase or decrease vascular tone and therefore positively or negatively regulate CBF. Studies in healthy adults indicate that COX positively contributes to the regulation of basal CBF as the non-selective COX inhibitor indomethacin reduces basal CBF ~30% (8, 31, 49-51, 54, 79). COX has also been shown to contribute to hypercapnic vasodilation as COX inhibition reduces the hypercapnic mediated increases in CBF by ~55% (7, 49).

Despite the importance of COX in the regulation of basal CBF and hypercapnic increases in CBF, the contribution of COX to hypoxic mediated cerebral vasodilation remains controversial. Animal data indicate a role for COX in hypoxic vasodilation (23, 37) whereas human data are equivocal (31, 49, 51). To date there is one study in humans suggesting that COX mediates a portion of hypoxic cerebral vasodilation (51), while there are two studies suggesting no role of COX (31, 49).

One potential explanation for this discrepancy, in the contribution of COX to hypoxic cerebral vasodilation in humans, may due to a sex- and/or menstrual phase-specific contribution of COX. During reproductive years, females are exposed to chronic cyclic oscillations in female sex hormones, including estrogen. Estrogen increases the production of the vasodilatory

prostanoid $PGI_2$ by upregulating $PGI_2$ synthase (103, 129) and increasing $PGI_2$ receptor density (135). In contrast, the predominant male sex hormone is testosterone which upregulates $TXA_2$ synthase, increasing formation of the vasoconstrictor prostanoid $TXA_2$ (44). Despite the contrasting roles of estrogen and testosterone mediating prostanoid formation, no study to date has examined the sex-specific or menstrual phase-specific contribution of COX to hypoxic vasodilation. Some worthwhile investigations would include comparing men to women during the early follicular phase of the menstrual cycle, when female sex hormones are most similar to males and therefore avoiding the acute effects of elevated estrogen. Additionally, comparing the early and late follicular phases of the menstrual cycle to explore the acute effect elevated estrogen on hypoxic cerebral vasodilation would be valuable. These studies would serve to reveal whether the current interpretation on the contribution of COX to hypoxic cerebral vasodilation is confounded by the influence of sex hormones. Needless to say, sex- and menstrual phase-specific studies are needed to increase our understanding of the complex role of COX in hypoxic vasodilation.

## Hypoxic Cerebral Vasodilation: Role of Sex

Cerebrovascular disease is sex-specific as men have an earlier onset and incidence of stroke (4, 111). However, after menopause when sex-hormones dissipate women experience greater rates and burden of stroke (4, 111). Increased cerebrovascular disease risk may be due to a reduced ability of the cerebral vasculature to vasodilate. For example, cerebrovascular reactivity is inversely associated with stroke risk (146) and cerebrovascular reactivity is lower in men (65) and post-menopausal women (63) when compared to pre-menopausal women. Despite these strong epidemiologic observations, there is a lack of mechanistic human studies

investigating potential sex differences in CBF regulation, which may be contributing to sex-specific cerebrovascular disease risk.

Available animal data indicate sex differences in CBF regulation. Isolated cerebral arteries from female rats exhibit larger diameters than males (40). The reduced cerebrovascular tone in female animal models is due, in part, to COX mediated production of the vasodilatory prostanoid, $PGI_2$ (70, 102, 103). Estrogen is known to increase the production of $PGI_2$ through upregulation of $PGI_2$ synthase while additionally increasing $PGI_2$ receptors (103, 129, 135). In contrast, testosterone promotes increased production of the vasoconstrictor prostanoid $TXA_2$ (37, 44). However, when considering animal studies it should be noted that most use supra-physiologic sex hormone supplementation after gonadectomy, which does not accurately represent the internal environment in young, healthy humans.

Consistent with observations in animals there is evidence suggestive of sex-specific CBF regulation in humans. Compared to men, women display greater resting global CBF (30, 115) and higher CBF velocities (75, 89, 101, 133, 138). Pre-menopausal but not post-menopausal women also appear to have greater hypercapnic cerebrovascular reactivity than men (63), which is suggested to be mediated by COX metabolites (64). Importantly, these studies did not control for menstrual phase or account for sex hormones, which are known to acutely alter CBF and cerebrovascular responsiveness (14, 71-74, 95). Without accounting for menstrual phase, it is difficult to differentiate whether greater CBF responsiveness in women is due to a fundamental sex difference, or acute fluctuations in sex hormones during the menstrual cycle.

To our knowledge, no studies to date have examined sex differences in hypoxic cerebral vasodilation. Animal studies suggest COX contributes to hypoxic CBF regulation (23, 37), whereas in humans it appears that COX may (51) or may not (31, 49) play a substantial role in

mediating hypoxic cerebral vasodilation.  Importantly, neither animal nor human studies have addressed potential sex differences in COX mediated regulation of hypoxic cerebral vasodilation. Human studies with physiologically relevant hormone levels, in addition to accounting for menstrual phase, may provide direct translational insight into sex-specific regulation of CBF (60).

Exploration of the sex-specific mechanisms responsible for the regulation of CBF is warranted to develop strategies that will slow or prevent the onset of cerebrovascular disease in both men and women.  Therefore, the primary purpose of Aim 1 was to investigate potential sex differences in CBF regulation during hypoxia and determine if the contribution of COX to the regulation of hypoxic CBF differs between the sexes.  Although not the primary purpose of this dissertation, we will also investigated potential sex-specific differences in basal CBF and hypercapnic vasodilation, as well as the contribution of COX to each of these measures.  We hypothesized young, pre-menopausal women will exhibit greater basal CBF, greater vasodilation to hypoxia and hypercapnia, and a greater contribution of COX.  Our rationale focused on comparing women during the early follicular phase of the menstrual cycle (when sex hormones least different from men) to understand fundamental sex-differences in CBF regulation without the confounding effects of acute elevations in sex hormones.  Results from this study will lay the framework for follow-up studies comparing across the menstrual cycle.

## Hypoxic Cerebral Vasodilation: Role of Menstrual Phase

Circulating estrogen is associated with reduced stroke risk and favorable stroke outcomes in women. Increasing levels of estrogen lowers cerebrovascular impedance (72) and resistance (74), and is positively associated with CBF velocity (71).  Accordingly, is has been shown that the highest CBF velocities occur during the late follicular phase of the menstrual cycle when

estrogen is at its highest (71). Conditions such as pregnancy (9), ovarian stimulation (95, 126), and post-menopausal hormone replacement therapy (100, 126) where estrogen is elevated are also associated with increased resting CBF. Despite these notable associations between estrogen and CBF, human research studies have not rigorously tested mechanisms responsible for increased CBF between menstrual phases.

Mechanistically, animal data indicate that estrogen enhances the production of the vasodilatory COX metabolite $PGI_2$, by upregulating endothelial COX and $PGI_2$ synthase (102, 103). Ultimately this reduces cerebrovascular tone, albeit in ovarectomized rats exposed to supra-physiologic levels of estrogen (102). Human data is limited but isolated endothelial cells from umbilical veins also suggest that estrogen increases $PGI_2$ production (129) and therefore may be mediating, in part, the beneficial effects of estrogen on CBF. However, to our knowledge no human studies have addressed the mechanism(s) mediating the greater CBF associated with cyclic oscillations of endogenous estrogen across the female menstrual cycle.

In addition to the regulation of basal CBF, the influence of menstrual phase on active CBF responses to environmental stressors such as hypoxia or hypercapnia is underexplored. Cyclic oscillations in estrogen across the menstrual cycle are positively associated with the ability of the cerebrovasculature to vasodilate (reactivity) when exposed to the physiologic stress of hypercapnia (73). From a clinical perspective, this is important as poor hypercapnic cerebrovascular reactivity is predictive of cerebrovascular disease risk (55, 87). Hypoxic cerebrovascular reactivity may hold even greater clinical relevance as hypoxia is a characteristic of sleep disordered breathing and experienced during sojourns to altitude. Mechanistically, COX has been shown to play a large role in hypercapnic mediated cerebral vasodilation (7, 49, 64) while the role of COX in hypoxic vasodilation is equivocal (31, 49, 51). Importantly, the

menstrual phase specific mechanisms of hypoxic and hypercapnic cerebrovascular reactivity remain to be tested.

In context of the limited animal and human studies noted above, exploring the influence of menstrual phase differences in CBF regulation are needed to unravel the fundamental role of physiologic estrogen fluctuations within the cerebrovasculature in healthy humans. Therefore, the overall goal of Aim 2 was to compare the cerebrovascular responses to hypoxia during the early and late follicular phases of the menstrual cycle and test whether the contribution of COX to hypoxic cerebral vasodilation is different between phases. Although not the primary aim of this dissertation, we will also examined potential phase differences in basal CBF and hypercapnic vasodilation as well as examine the contribution of COX to basal CBF and hypercapnic vasodilation between menstrual phases. We hypothesized that women in the late follicular phase will exhibit greater basal CBF and greater vasodilation to hypoxia and hypercapnia, due to a greater contribution of COX. To test this hypothesis we compared the natural low versus high estrogen phases of the menstrual cycle, in the same female subjects, while progesterone remains low, providing novel insight into CBF control during cyclic hormonal fluctuations associated with the menstrual cycle.

## Hypoxic Cerebral Vasodilation: Regional Specificity

Despite it being well known that hypoxia induces a robust increase in global CBF (11, 16, 80), relatively little is known about region-specific increases in CBF. Current human data indicate that hypoxia may cause heterogeneous increases in macrovascular blood flow (80, 99, 140). For example, following 60, 210, and 360 minutes of poikilocapnic hypoxia ($F_IO_2=11\%$) eliciting an $S_PO_2\sim80\%$, the relative increase in VA flow (~37%) was greater when compared to ICA (~18%). Accordingly, hypoxia increased VA diameter by ~12% whereas ICA diameter

only increased ~9% (80).  Similarly, during sequential 15-minute isocapnic hypoxia bouts

eliciting $S_PO_2$ of ~90%, ~80%, and~70 % respectively, the relative increase in VA blood flow

was ~50% greater than that of the ICA, MCA, or PCA (140).  Furthermore, 15-minutes of

poikilocpanic hypoxia ($F_IO_2$=12%) eliciting an $S_PO_2$~90% increased VA blood flow while ICA

blood flow remained unchanged (99).  Considered collectively, the more primitive posterior

brain regions, including the brain stem, supplied by the VA and BA appear to be more sensitive

to hypoxia than anterior brain regions supplied by the ICA, MCA, and ACA (80, 140).

In addition to examining hypoxia induced regional increases in macrovascular blood

flow, there have been several studies exploring hypoxia-induced regional increases in

microvascular perfusion.  In one study investigating 50 separate brain regions during  isocapnic

hypoxia, eliciting an $S_PO_2$ ~80% ($F_IO_2$=10%), relative increases in regional cerebral perfusion

varied from +9.9% in the occipital lobe to +28.9% in the nucleus accumbens (11).  Overall, there

appeared to be greater increases in blood flow to phylogenically older regions of the brain

associated with autonomic function including the  brain stem, nucleus accumbens, putamen,

pallidum, caudate nucleus and thalamus when compared to newer regions of the brain such as the

cerebral cortex (Appendix A, *Regional Blood Flow Distribution During Hypoxia*) (11).  In

agreement with these findings, a 20-minute exposure to a simulated altitude of 4500 m eliciting

an $S_PO_2$~74% resulted in a disproportionate increase in hypothalamic and cerebellar flow when

compared to cortical brain regions (16).

It is thought that that heterogeneous cerebral blood flow response to hypoxia, mainly a

larger increase in flow to phylogenically older areas of the brain, serves to preserve vital

homeostatic functions at the expense of cognitive ability (11).  This is in line with the idea that

neuropsychological function associated with anterior brain regions (cerebral cortex) is impaired

during hypoxic exposure (137, 145), while posterior brain regions (i.e. brain stem) housing the respiratory and cardiovascular control centers become more active to increase ventilation and cardiac output to maintain homeostasis (80).

Epidemiologic data also support the idea of heterogeneous CBF regulation with greater hypoxic responsiveness in the poster circulation. Anterior brain regions fed by the ICA, MCA, and ACA have twice the incidence of stroke compared to posterior brain regions fed by the VA, BA, and PCA (96). Thus, cerebral vessels located in the posterior circulation may have a greater ability to vasodilate during homeostatic disturbances compared to anterior vessels, potentially preventing or reducing the incidence and impact of stroke in posterior brain regions. Likewise, in animal models during sudden anemia, older regions of the brain associated with homeostatic functions survive longer than younger, cortical regions (130), suggesting greater maintenance of blood flow and $O_2$ delivery to older, posterior brain regions.

There are also data suggesting the opposite, that the anterior circulation may have greater hypoxic sensitivity than the poster circulation. When comparing the MCA and BA in humans during a stepwise reduction in inspired $O_2$ ($F_IO_2$ % = 13, 11, 10, 9, 8 and 7), blood flow velocity increased in the MCA at less severe level of hypoxia than the BA. Although a comparison was not directly made, it also appears that the relative change in blood flow velocity was less in the BA than MCA (58). However, these data require cautious consideration as $CO_2$ fell precipitously throughout the protocol, making it impossible to determine whether these regional differences in blood flow are a result of regional differences in hypoxic or hypocapnic sensitivity. These data however, are in agreement with findings in the microvasculature indicating that poikilocapnic hypoxia ($F_IO_2$ % = 12) increased frontal cortical grey matter perfusion while perfusion decreased in areas associated with the default mode network (77).

Animal data also support that idea of greater anterior hypoxic sensitivity as chronic hypoxia in fetal sheep results in greater vasodilation of the ACA compared to the MCA and PCA (26).

It has also been suggested that there are no regional differences in hypoxic increases in CBF. For instance, 15-minutes of isocapnic hypoxia ($F_IO_2$=12%) eliciting an $S_PO_2$~90% resulted in similar increases in ICA and VA blood flow (99), while 30-minutes of poikilocapnic hypoxia eliciting an $S_PO_2$~89% resulted in similar white and grey matter blood flow responses (29). However, the mild level of hypoxia utilized in these studies may limit interpretation as CBF does not begin to significantly increase until an $S_PO_2$~85% equating to an arterial $O_2$ tension of ~50 mmHg (142).

Utilizing a more severe hypoxic stimulus, 10-minutes of isocapnic hypoxia eliciting an $S_PO_2$~80%, showed no difference in relative cerebrovascular reactivity between the MCA and PCA, as well as no difference in reactivity when comparing the ICA and VA (51). Similarly, sequential 5-minute stages of isocapnic hypoxia eliciting an $S_PO_2$ ~90%, ~80%, and ~70% also showed no difference in relative cerebrovascular reactivity between the MCA and PCA, and no difference in reactivity between the ICA and VA (53). It is important to note that these prior two findings come from the same group who previously reported a ~50% greater relative increase in VA flow compared to ICA, MCA, and PCA (140).

Likewise, during severe poikilocapnic hypoxia ($S_aO_2$~56%), there was found to be no major blood flow redistribution in the cerebral circulation aside from a small increase in the motor cortex and basal ganglia (104, 105). However, the lack of regional differences in this study may be contributed to the severity of hypoxia ($S_aO_2$~56%) and variability in the hypoxic ventilatory response. Specifically, inter-subject variability in the hypoxic ventilatory response resulted in variable arterial $CO_2$ and $S_aO_2$ responses. Given that the cerebral blood flow response

during hypoxia is dependent upon the balance between hypoxic vasodilation and hyperventilation induced hypocapnic vasoconstriction, the variable $S_aO_2$ and $CO_2$ responses severely limit interpretation.

Despite these contradictory and variable findings amongst studies, there is a reasonable amount of evidence suggesting heterogeneity in the hypoxic CBF response. Specifically, there is evidence to indicate the posterior circulation, which supplies the cardiorespiratory centers in the brainstem, may demonstrate greater hypoxia induced increases in blood flow compared to the anterior circulation. This heterogeneous CBF distribution during hypoxia may serve to preserve autonomic function in older areas of the brain at the expense of newer cortical brain regions. However, it is important to note that these conclusions are based upon studies utilizing varying degrees of hypoxia and employing poikilocapnic hypoxia, making it impossible to isolate the effects of hypoxia from hyperventilation-induced hypocapnia. Clamping end-tidal $CO_2$ is especially important given regional specificity in hypercapnic vasodilation (56, 119, 125). Moreover, many of these studies utilized cerebral blood flow velocity as an index of CBF rather than quantification of blood flow and only compared responses in a small number of cerebral vessels. To date, there has not been a comprehensive study examining the CBF responses to hypoxia in multiple anterior and posterior arteries, while clamping end-tidal $CO_2$ in order to isolate the effects of hypoxia from hyperventilation-induced hypocapnia.

With this in mind, as part of Aim 3 of this dissertation we aimed to investigate regional hypoxic cerebral vasodilation by concurrently quantifying blood flow in eleven major cerebral arteries that comprise the anterior and poster macrovascular circulation during hypoxia while simultaneously clamping end-tidal $CO_2$ as basal levels.

## Regional Specific Contribution of COX to Hypoxic Vasodilation

The evidence suggesting regional differences in hypoxic cerebral vasodilation may also indicate that mechanisms mediating hypoxic responses are regionally distinct. However, regional specific mechanisms of hypoxic vasodilation remain relatively unknown. To date, there is only one study in humans that has proposed vessel specific differences in the contribution to COX to hypoxic cerebral vasodilation (51). In this study, COX has been shown to mediate hypoxic vasodilation in both the MCA and VA but not in the ICA and PCA (51). This suggests that COX signaling may be regionally specific and has the potential to explain the heterogeneous cerebral blood flow responses to hypoxia.

With regional cerebrovascular specificity in mind, as part of Aim 3 of this dissertation we investigated the regional contribution of COX to hypoxic cerebral vasodilation by concurrently quantifying blood flow in eleven major cerebral arteries, that comprise the anterior and poster macrovascular circulation, during hypoxia while clamping end-tidal $CO_2$.

## Hypercapnia

Although cerebrovascular reactivity to hypercapnia is not the focus of this dissertation we have included hypercapnia into two of our study designs and understand the importance of clamping end-tidal $CO_2$ during hypoxia to isolate the effects of hypoxia. Hypercapnia is important and clinically relevant as cerebrovascular reactivity is predictive of cerebrovascular risk (87, 146). Therefore, I will review hypercapnia briefly, and incorporate approaches in the research design to allow parallel study of hypoxia and hypercapnia in two of my three aims.

Cerebral vessels are highly sensitive to changes in arterial carbon $CO_2$, with increases in $CO_2$ eliciting robust vasodilation (2, 7, 48, 49). It appears that all vessels along the cerebrovascular arterial tree are sensitive to changes in arterial $CO_2$ including the macro-

vasculature of the neck (140) cranial arteries (7, 48, 140), and microvascular pial and penetrating arterioles (143). There is evidence indicating that there may (56, 119, 125) or may not (106) be regional differences in hypercapnic responses, with anterior regions being more sensitive than posterior brain regions.

During hypercapnia arterial $CO_2$ increases and a portion of this $CO_2$ diffuses across the blood brain barrier. This $CO_2$ then mediates a reduction in extravascular space pH, via carbonic anhydrase, that elicits robust vasodilation (24, 69). In general, CBF increases ~3-6% for each 1 mmHg increase in arterial $CO_2$, while there is a ~1-3% decrease in blood for each 1 mmHg decrease in arterial $CO_2$ (143).

Mechanistically, COX has been shown to mediate a significant portion of hypercapnic mediated cerebral vasodilation (7, 49). Inhibition with the non-selective COX inhibitor indomethacin reduces hypercapnic mediated vasodilation by ~55% (7, 49). Greater cerebrovascular reactivity to hypercapnia in women has been suggested to be mediated by COX (64) while mechanisms mediating changes in cerebrovascular reactivity throughout the menstrual cycle remain unknown (73). However, inadequate consideration of female sex hormones limit the interpretation of existing data and further study is required.

With our current study designs, we were able to examine sex and menstrual phase-specific hypercapnic cerebrovascular reactivity, in addition to identifying sex and menstrual phase-specific contributions of COX to hypercapnia

# Aims and Hypotheses

With the aforementioned background in mind, this dissertation involved a total of three studies and three aims that are as follows:

1. **Determine the sex-specific contribution of COX to hypoxic vasodilation in the MCA in young, healthy men and women.**

We hypothesized hypoxic cerebral vasodilation in the MCA will be greater in women than men, due in part to a greater contribution of COX.

2. **Determine the menstrual phase specific contribution of COX to hypoxic vasodilation in the MCA in young, healthy women.**

We hypothesized that hypoxic cerebral vasodilation in the MCA will be greater during the late follicular compared to the early follicular phase of the menstrual cycle, due in part to a greater contribution of COX.

3. **Determine the region-specific contribution of COX to hypoxic vasodilation by examining eleven major cerebral arteries.**

We hypothesized that hypoxic vasodilation will be greater in the posterior circulation (VA, BA, and PCA) compared to the anterior circulation (ICA, MCA, and ACA), due in part to a greater contribution of COX.

# General Methods

## Subjects

### Aims 1 and 3

Subjects were free of disease, otherwise healthy, and not taking medication with the exception of birth control as determined by health history questionnaire.  Low to moderate physical activity was be permitted and assessed by a physical activity questionnaire.  Women were not be pregnant (urine pregnancy test) and studied during the early follicular phase of the menstrual cycle (cycle days 1-5) or the low hormone phase of birth control.  Subjects reported to the laboratory on all study days after completing a $\geq$ 10-hour (Aim 3 $\geq$ 4-hour) fast and having abstained from exercise, alcohol, caffeine, and non-steroidal anti-inflammatory drugs for $\geq$ 18-hours (Aim 3 $\geq$ 24 hours).  The experimental protocol conformed to the standards set forth by the Declaration of Helsinki and be approved by University of Wisconsin-Madison Institutional Review Board.  The nature, purpose, and risks of the study were provided to each subject before written informed consent is obtained.

### Aim 2

All aforementioned criteria pertain to subjects taking part in Aim 2, except for the following.  Based upon the objectives of Aim 2, only women who were not on any form of hormonal birth control were allowed to participate in the study.  Additionally, all subjects were studied twice each during the early (cycle days 1-5) and late follicular phases (cycle days 12-16) of the menstrual cycle.

## Transcranial Doppler Ultrasound – TCD

*Aims 1 and Aims 2*

The location of cerebral vessels within the enclosed cranium makes in vivo study of the cerebrovasculature difficult. Utilization of TCD technology for the measurement of in vivo cerebral blood flow velocity in the early 1980s was a crucial advancement in cerebrovascular physiology (1). TCD allows for the measurement of CBF velocity through three acoustic windows within the cranium. These acoustic windows include the transtemporal, transforaminal, and transorbital window, where naturally thin cranial areas allow penetration of the ultrasound beam. The transtemporal window can be utilized to measure CBF velocity in the ACA, MCA, and PCA and the anterior and posterior communicating arteries. The transforaminal window allows for the determination of CBF velocity in the VA and BA, while the transorbital window allows for the investigation of the cavernous carotid and ophthalmic arteries (38).

For the purposes of this dissertation MCA velocity was measured via the transtemporal window with a 2-MHz transcranial Doppler ultrasound probe (TCD, Neurovision model 500M, Multigon Industries, Inc.; Yonkers, NY, USA). The MCA is the easiest of all vessels to insonate with a mean diameter of ~3 mm and a length of 16.2 mm (38). Sample depths of the MCA typically range from 30-60 mm, demonstrate a positive flow or flow towards TCD probe, and have velocities of $55\pm12$ cm s$^{-1}$ (38). After identification of the MCA, an optimal signal was obtained and an adjustable headband secured the TCD probe. The position and settings of the TCD probe was documented and maintained for subsequent visits.

## 4-Dimensional Flow Magnetic Resonance Imaging – 4D Flow MRI

*Aim 3*

Although TCD is a non-invasive, cost effective methodology providing an index of cerebral blood flow, it is limited in the assumption of vessel diameter remaining unchanged. Prior studies have suggested that MCA diameter may (144) or may not change in response to hypoxia (112, 122). If vessel diameter does increase in response to hypoxia, this will result in underestimation of hypoxic increases in blood flow. An additional limitation of TCD is that only one or two cerebral arteries can be insonated simultaneously, therefore limiting its usefulness in the investigation of region-specific CBF responses during hypoxia.

With this in mind, for Aim 3 of this dissertation we utilized 4D Flow MRI or more specifically Phase Contrast-Vastly Under Sampled Isotropic Projection Reconstruction (PC-VIPR). PC-VIPR is a methodology that measures three-dimensional flow across a fourth dimension, time. PC-VIPR is known to provide accurate blood flow measures within cerebral arteries (47, 139). All study imaging was completed on a 3T MRI (Discovery MR750, GE Healthcare, Waukesha, WI, USA) with an eight-channel head coil. The following scan parameters were utilized: imaging volume = 22 x 22 x 22 $cm^3$, $(0.69 \text{ mm})^3$ acquired isotropic resolution, scan time = 5 min 30 seconds, velocity encoding (Venc) = 100 cm/s, flip angle = $20^0$, TR/TE = 6.7/2.8 ms, 20 reconstructed cardiac time frames using retrospective cardiac gating and temporal view sharing (83). Appendix B, *4-Dimensional Flow Magnetic Resonance Imaging (PC-VIPR).*

## Measurements

*Aims 1, 2, and 3*

Height and weight were measured to calculate body mass index (BMI, kg $m^{-2}$). Waist and hip circumference were measured as an indicator of regional adiposity. Venous blood samples were obtained for the determination of glucose and lipids (Aims 1 and 2, insulin and sex

hormones were also be measured).  During each visit, subjects were instrumented for continuous measurement of heart rate (3-lead ECG), pulse oximetry oxygen saturation ($S_PO_2$, pulse oximeter), and blood pressure (MABP, automated physiological monitor; GE Datex-Ohmeda, Madison, WI, USA).  Inspiratory and expiratory gases were measured with a gas analyzer (Aims 1 and 2; GEMINI, CWE, Inc., Ardmore, PA: Aim 3; Medrad Veris MR Vital Signs Patient Monitor, Bayer Healthcare, Whippany, NJ, USA) and respiratory flow was determined with a heated pneumotachometer (Aims 1 and 2; Hans Rudolph Inc., Shawnee, KS).

## Hypoxia

*Aims 1 and 2*

Isocapnic hypoxia trials were performed as previously described in our laboratory (48, 49, 81).  Briefly, subjects inspired through a two-way non-rebreathing valve (2700 Series, Hans Rudolph Inc., Kansas City, MO), connected to a gas mixer (PM5300; Precision Medical, Northampton, PA), supplied by medical grade pressurized oxygen ($O_2$), carbon dioxide ($CO_2$), and nitrogen ($N_2$, Airgas, Madison, WI).  After 5-minutes of baseline room air breathing, hypoxia was introduced by decreasing inspired $O_2$ to elicit and sustain 5-minutes of $S_PO_2$=90%, followed immediately by a transition to and 5-minutes of $S_PO_2$=80%.  Isocapnia was achieved through the addition of $CO_2$ to inspired gas.  End-tidal $CO_2$ ($ETCO_2$) has been shown to be a valid predictor of arterial blood $CO_2$ levels (92).

*Aim 3*

After 5-minutes of baseline room-air breathing and baseline scans, hypoxia was introduced.  Isocapnic hypoxia was performed by having subjects inspire through a two-way non-rebreathing valve (2700 Series, Hans Rudolph Inc., Kansas City, MO) supplied by a medical grade pressurized gas mixture containing 11% $O_2$ and the balance $N_2$ (Airgas, Madison, WI).  It

was expected that an $F_IO_2 = 11\%$ would elicit an $S_PO_2 \sim 80\%$. Isocapnia was achieved by titrating the hypoxic gas mixture accordingly with medical grade pressurized $CO_2$ (100% $CO_2$; Airgas, Madison, WI). Steady $S_PO_2$ and $ETCO_2$ were reached in <5 minutes (no change in $S_PO_2$ and $ETCO_2$ for > 1 min), after which 4D Flow MRI scan commenced.

## Hypercapnia

*Aims 1 and 2*

Hypercapnic trials were performed as previously described in our laboratory (48, 49). Briefly, subjects inspired through a three-way sliding rebreathing valve (Model 2870, Hans Rudolph Inc., Shawnee, KS) attached to a latex balloon containing a hyperoxic ($O_2$=35%-40%), hypercapnic ($CO_2$=3%) gas mixture with the balance $N_2$. The balloon was filled to a volume exceeding estimated vital capacity (as determined by age, sex, and height) by 1-liter. After 5-minutes of baseline room air breathing, hypercapnia commenced and was sustained (~2-minutes) until $ETCO_2$ values reach 10 mmHg above baseline values. A total of two hypercapnic trials were completed and separated by 10-minutes of quiet rest and room air breathing. Values for the two trials were averaged.

## Plasma Assays

*Aims 1 and 2*

Venous blood samples were drawn at baseline and after 90-minutes of placebo and indomethacin. Blood was centrifuged with plasma and serum drawn off and stored at -80$^o$C. Circulating estrogen and insulin were determined with radioimmunoassay (RIA), while EIA was utilized for determination of progesterone, testosterone, and dihydrotestosterone.

## Data Analysis

*Aims 1 and 2*

All cardiovascular and respiratory data were recorded, stored, and analyzed with PowerLab and LabChart (ADInsturments Inc., Dunedin, NZ).  The effect of drug administration in Aims 1 and 2 were examined over 90-minutes with the last 30-seconds of each 5-minute data recording interval used for analysis.  During hypoxia, the last 30-seconds of recording intervals for baseline, $S_PO_2=90\%$, and $S_PO_2=80\%$ were analyzed.  Analysis of hypercapnia data included the last 30-seconds of baseline and the last 10-seconds of hypercapnia, equating to a 10 mmHg increase in $ETCO_2$.  Automated blood pressure recordings were taken during the last 30-seconds of each recording interval to coincide with analysis intervals.  The main outcome variable was MCA blood flow velocity (MCAv), but to account differences in perfusion pressure, cerebrovascular conductance index was also be calculated and presented (CVCi = MCAv*100/MABP).

*Aim 3*

Data processing was completed using commercial software (Matlab, The Mathworks, Natick, MA, USA) which has proven to be reliable in the assessment of cerebral arteries (139). Vessel position was interactively selected with an underlying algorithm segmenting the vessel in a plane perpendicular to the vessel path, one voxel in width (0.69 mm), simultaneously providing velocity, cross sectional area (CSA), and flow measures.  CSA and flow were averaged over five consecutive cross sections (5 x 0.69 mm = 3.45 mm) producing a 3.45 mm long segment in each artery of interest, providing an accurate measurement of flow (120).  Eleven arteries of interest were assessed including: left and right vertebral arteries (VA-L, VA-R), basilar artery (BA), left and right posterior cerebral arteries (PCA-L, PCA-R), left and right internal carotid arteries

(ICA-L, ICA-R), left and right middle cerebral arteries (MCA-L, MCA-R), and left and right anterior cerebral arteries (ACA-L, ACA-R). Individual vessel segmentation occurred at precise locations which are as follows: VA was measured 4-5 mm from the junction with the BA, BA was measured near the junction with the VA, PCA was measured 4-5 mm from the junction with the BA, ICA was measured in the straight portion of the C4 segment (13), and ACA and MCA were be measured 4-5 mm from their junction with the ICA (A1 and M1, respectively; refer to blue cut planes Appendix A, *4-Dimensional Flow Magnetic Resonance Imaging (PC-VIPR)*).

The main outcome variables were blood flow and vessel cross sectional area (CSA), but to account for potential differences in perfusion pressure, cerebrovascular conductance was also calculated and presented (CVC = CBF*100/MABP). To account for potential differences in baseline flow associated with vessel and indomethacin, data was expressed as percent change from baseline which is considered to the most accurate representation of changes in vascular tone (17, 88).

## Statistical Considerations

Minintab 16 (State College, PA, USA) was used for all statistical analysis. Level of significance was set at $p < 0.05$. When ANOVA yielded significance, multiple comparisons on factor means were be performed with Tukey's post hoc analysis. Data was expressed as mean ± standard error of the mean.

*Aim 1*

Subject characteristics were compared using an unpaired student's t-test. Significance of sex (men vs. women) and time (0, 15, 30, 45, 60, 75, and 90 min) on basal MCAv, with and without indomethacin, were determined utilizing a general linear model to perform two-way analysis of variance (ANOVA). Unpaired student's t-tests were used to examine sex differences

in the change in MCAv following indomethacin. Significance of sex (men vs. women) and hypoxia (baseline, $S_PO_2$=90%, and $S_PO_2$=80%) on MCAv and change in MCAv from baseline, with and without indomethacin, were determined by two-way general linear model ANOVA. Significance of sex (men vs. women) and hypercapnia (baseline and hypercapnia) on MCAv, with and without indomethacin, were determined by two-way general linear model ANOVA. Unpaired student's t-tests were used to examine sex differences in the change in MCAv with hypercapnia following placebo and indomethacin. The contribution of COX to hypoxic or hypercapnic mediated increases in MCAv was determined with general linear model ANOVA.

Given no prior studies have examined sex-differences in hypoxic vasodilation we chose to power our study based upon sex-differences in the contribution of COX to hypercapnic reactivity and basal sex differences in MCAv. Based upon prior research (64) a sample size of n=28 (with an estimated standard deviation of 1.3) was necessary to provide 80% power to detect an expected 33% group difference in the contribution of COX to hypercapnic reactivity at $\alpha$= 0.05. This also provided >99% power to detect an expected 20% basal sex-difference in MCAv (138).

*Aim 2*

Significance of menstrual phase (early follicular versus late follicular) on circulating sex hormones (estrogen, progesterone, testosterone, and dihydrotestosterone) during early and late follicular phases was determined with t-tests. Significance of menstrual phase (early follicular or late follicular), time (0, 15, 30, 45, 60, 75, and 90 min), and drug (placebo or indomethacin) on basal CVCi and change in CVCi ($\Delta$MCAV) 90-minutes after the administration of drug were determined utilizing a general linear model to perform an analysis of variance (ANOVA). Significance of menstrual phase (early follicular or late follicular), hypoxia (baseline,

$S_PO_2$=90%, and 80%), and drug (placebo or indomethacin) on CVCi and ΔCVCi from baseline were determined by general linear model ANOVA. Significance of menstrual phase (early follicular or late follicular), hypercapnia (baseline or hypercapnia), and drug (placebo or indomethacin) on CVCi and ΔCVCi were be determined general linear model ANOVA.

Based upon research conducted in our laboratory and that of others (71) a sample size of n=10 (with an estimated standard deviation of 13) was necessary to provide 80% power to detect an expected 15% group difference in basal CVCi between menstrual phases at α= 0.05.

*Aim 3*

Significance of hypoxia (baseline, $S_PO_2$~80%), and drug (placebo or indomethacin) on cardiorespiratory variables were determined by general linear model ANOVA. Significance of drug (placebo or indomethacin) and vessel (R-VA, L-VA, BA, R-PCA, L-PCA, R-ICA, L-ICA, R-MCA, L-MCA, R-ACA, L-ACA) on hypoxia-mediated increases in CBF were determined by general linear model ANOVA.

Based upon prior research investigating greater relative hypoxic cerebrovascular reactivity in the VA (140), a sample size of n=9 (with an estimated standard deviation of 1.3) at an α= 0.05 was necessary to provide 80% power to detect an expected 50% greater VA relative reactivity compared to the ICA, MCA, and PCA. Additionally, a sample size of n=9 provided >80% power (with an estimated standard deviation of 1.0) to detect an expected 50% reduction in absolute hypoxic VA reactivity associated with COX inhibition (51).

# Experimental Design

## Aim 1

In Aim 1 determined the sex-specific contribution of COX to hypoxic vasodilation in the MCA of young, healthy men and women.  In a randomized, double-blind, and placebo-controlled design subjects (~ ½ men and ~ ½ women; women studied during the early follicular phase) completed two visits, under control (placebo) and experimental conditions (COX inhibition). Both placebo and the non-selective COX inhibitor indomethacin (100 mg) were administered orally.  After baseline data collection subjects received either placebo or indomethacin in addition to 20 ml of Maalox.  Maalox as provided to prevent gastrointestinal discomfort occasionally associated with oral indomethacin.  Subjects then rested quietly for 90-minutes while MCAv, respiratory, and cardiovascular variables were recorded for 5-minutes, spaced by 10-minute non-recording intervals.  After 90-minutes, hypoxic and hypercapnic trials were conducted in randomized order while recording MCAv, respiratory, and cardiovascular variables during each trial.  All trials were separated by 10-minutes of quiet rest while breathing room air. TCD placement was recorded and maintained during study visits and between study visits.  Refer to Appendix C, *Study Timeline for Addressing Aim 1 and Aim 2.*

## Aim 2

In Aim 2 we determined the menstrual phase specific contribution of COX to hypoxic vasodilation in the MCA in young, healthy women.  Aim 2 followed an identical experimental design as Aim 1 (Appendix C, *Study Timeline for Addressing Aim 1 and Aim 2*) but differed by subject enrollment and number of study visits.  Aim 2 required women not to be currently taking

any form of hormonal birth control and to be studied during the early follicular (days 1-5) and late follicular (days 12-16) phases of the menstrual cycle. We chose these two phases in an attempt to naturally isolate the effects of elevated estrogen without the confounding effects of changes in additional hormones.

## Aim 3:

In Aim 3 we determined the region-specific contribution of COX to hypoxic cerebral vasodilation in eleven separate cerebral arteries with 4D Flow MRI imaging techniques. We utilized an acquisition scheme developed at the University of Wisconsin Madison known as PC VIPR (phase contrast vastly under-sampled isotropic projection reconstruction). This MRI sequence is unique in its capability to acquire volumetric data sets with three-directional velocity encoding and high spatial resolution with short scan times and without the use of a contrast agent.

In a randomized, double-blind, and placebo-controlled design subjects completed two visits, under control (placebo) and experimental conditions (COX inhibition). Both placebo and the non-selective COX inhibitor indomethacin (100 mg) were administered orally, in addition to 20 ml of Maalox to prevent gastrointestinal discomfort occasionally associated with oral indomethacin. Subjects then rested quietly for 90-minutes outside of the MRI scanner.

After 90-minutes, subjects were placed in the MRI scanner for the hypoxia trial. Hypoxia trials consisted of a baseline scan (PC Viper and 2D radial scanning sequences ~ 5 minutes), initiation of hypoxia and transition to steady state (~5 min), and hypoxia scan upon reaching steady state (PC Viper and 2D radial scanning sequences, ~5 min). Cardiovascular variables and $ETCO_2$ were measured and recording during all trials. Refer to Appendix C, *Study Timeline for Addressing Aim 3*

# Project I

**Cerebrovascular regulation in men and women: stimulus-specific role of cyclooxygenase**

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## Abstract

Greater cerebral artery vasodilation mediated by cyclooxygenase (COX) in female animals is unexplored in humans. We hypothesized young, healthy women would exhibit greater basal cerebral blood flow (CBF) and greater vasodilation during hypoxia or hypercapnia compared to men, mediated by a larger contribution of COX. We measured middle cerebral artery velocity (MCAv, transcranial Doppler ultrasound) in 42 adults (24 women, 18 men; 24±1 yrs) during two visits, in a double-blind, placebo-controlled design (COX inhibition, 100 mg oral indomethacin). Women were studied early in the follicular phase of the menstrual cycle (days 1–5). Two levels of isocapnic hypoxia ($S_{P}O_2$= 90% and 80%) were induced for 5-minutes each. Separately, hypercapnia was induced by increasing end-tidal carbon dioxide ($PET_{CO2}$) 10 mmHg above baseline. A positive change in MCAv ($\Delta$MCAv) reflected vasodilation. Basal MCAv was greater in women compared to men (P<0.01) across all conditions. Indomethacin decreased baseline MCAv (P<0.01) similarly between sexes. Hypoxia increased MCAv (P<0.01), but $\Delta$MCAv was not different between sexes. Indomethacin did not alter hypoxic vasodilation in either sex. Hypercapnia increased MCAv (P<0.01), but $\Delta$MCAv was not different between sexes. Indomethacin elicited a large decrease in hypercapnic vasodilation (P<0.01) that was similar between sexes. During the early follicular phase, women exhibit greater basal CBF than men, but similar vasodilatory responses to hypoxia and hypercapnia. Moreover, COX is not obligatory for hypoxic vasodilation, but plays a vital and similar role in the regulation of basal CBF (~30%) and hypercapnic response (~55%) between sexes.

## Introduction

Cerebrovascular disease is a leading cause of death in the United States and accounts for over $38 billion dollars in annual medical costs (43). Stroke risk is highly sex-specific, as men

exhibit a 33% greater incidence, 41% greater prevalence, and earlier onset of first-ever stroke than women (4, 111). Cerebrovascular reactivity is also greater in women (65) and is associated with reduced stroke risk (146). Despite these epidemiologic observations, there is a lack of mechanistic human studies investigating potential sex differences in cerebral blood flow (CBF) regulation.

Animal data indicate sex differences in CBF regulation. Isolated cerebral arteries from female rats exhibit larger diameters than males (40). The reduced cerebrovascular tone in female animal models is due, in part, to cyclooxygenase (COX) mediated production of the vasodilatory prostanoid, prostacyclin (70, 102, 103). It should be noted that animal studies utilize supra-physiologic sex hormone supplementation after gonadectomy, which does not accurately represent the internal environment in young, healthy humans.

Several human studies are consistent with observations seen in animals. Women display greater resting global CBF (30, 115) and higher CBF velocities compared to men (75, 89, 101, 133, 138). Additionally, greater cerebrovascular reactivity in women has been suggested to be mediated by COX metabolites (64). Importantly, these studies did not control for menstrual phase or account for sex hormones that are known to acutely alter CBF and cerebrovascular responsiveness (14, 71-74, 95). Without accounting for menstrual phase, it is difficult to differentiate whether greater CBF responsiveness in women is due to a fundamental sex difference, or acute fluctuations in sex hormones during the menstrual cycle.

Hypercapnia and hypoxia are environmental stressors that both elicit robust increases in CBF. Women demonstrate a greater hypercapnic increase in CBF than men (62, 64, 65, 101) that may be mediated by COX (64). However, as previously noted, designs that do not control for menstrual cycle or account for sex hormones limit interpretation. From a clinical perspective

hypoxia may hold greater relevance than hypercapnia as it is characteristic of sleep disordered breathing, a condition noted for sex-specific pathophysiology (61). To our knowledge, no studies to date have examined sex differences in hypoxic vasodilation. Animal studies suggest COX contributes to hypoxic CBF regulation (23, 37), whereas in humans it appears that COX may (51) or may not play a substantial role in mediating hypoxic vasodilation (31, 49). Importantly, neither animal nor human studies have addressed potential sex differences in COX mediated regulation of hypoxic cerebral vasodilation. Human studies with physiologically relevant hormone levels and accounting for menstrual phase may provide direct translational insight into sex-specific regulation of CBF (60).

Exploration of the sex-specific mechanisms responsible for the regulation of CBF is warranted to develop strategies that will slow or prevent the onset of cerebrovascular disease in both men and women. The primary purpose of this study was to investigate potential sex differences in CBF regulation during two distinct environmental stressors, hypoxia and hypercapnia. The secondary purpose of this study was to determine if the contribution of COX to the regulation of basal CBF, hypoxia, or hypercapnia differs between the sexes. We hypothesized women would exhibit greater basal CBF, greater vasodilation to hypoxia and hypercapnia, and a greater contribution of COX. Our rationale focused on comparing women during the early follicular phase of the menstrual cycle (when sex hormones least different from men) to understand fundamental sex-differences in CBF regulation without the confounding effects of acute elevations in sex hormones. Results from this study will lay the framework for follow-up studies comparing across the menstrual cycle.

## Methods

*Subjects*

A total of 42 young healthy adults were recruited to participate (18 men, 26±1 yrs; 24 women, 23±1 yrs).  Subjects were free of disease, otherwise healthy, not currently taking medication with the exception of birth control, and sedentary (<120 minutes of moderate physical activity per week) as determined by health history and physical activity questionnaire. Women were not pregnant (urine pregnancy test) and studied during the early follicular phase of the menstrual cycle (days 1 – 5) or the low hormone phase of birth control (birth control; n=9). The experimental protocol conformed to the standards set forth by the Declaration of Helsinki and was approved by the University of Wisconsin-Madison Institutional Review Board.  The nature, purpose, and risks of the study were provided to each subject before written informed consent was obtained.  Data from a subset of subjects (men, n=6; women, n=6) were previously reported in a study comparing healthy controls to adults with metabolic syndrome (49).

*Measurements*

Height and weight were measured to calculate body mass index (BMI, kg m$^{-2}$).  Waist and hip circumferences were measured as indicators of regional adiposity.  Venous blood samples were obtained for the determination of glucose, lipids, insulin, and sex hormones. During each visit, subjects were studied in a semi-recumbent position and instrumented for continuous measurement of heart rate (3-lead ECG), pulse oximetry oxygen saturation (S$_P$O$_2$, pulse oximeter), and blood pressure (MABP, automated physiological monitor; GE Datex-Ohmeda, Madison, WI, USA).  A 2-MHz transcranial Doppler ultrasound probe (TCD, Neurovision model 500M, Multigon Industries, Inc.; Yonkers, NY, USA) was placed over the right or left temporal window and after obtaining an optimal signal secured by an adjustable headband for measurement of middle cerebral artery velocity (MCAv) (7, 48, 49, 128). Inspiratory and expiratory gases were measured with a gas analyzer (GEMINI, CWE, Inc.,

Ardmore, PA) and respiratory flow was determined with a heated pneumotachometer (Hans Rudolph Inc., Shawnee, KS).

*Protocol*

In a randomized, double-blind, and placebo-controlled design subjects completed two visits, under control (placebo) and experimental conditions (COX inhibition). Both placebo and the non-selective COX inhibitor indomethacin (100 mg, Heritage Parmaceuticals Inc., Edison, NJ) were administered orally. Subjects reported to the laboratory after completing a 10-hour fast and having abstained from exercise, alcohol, caffeine, and non-steroidal anti-inflammatory drugs for a minimum of 18-hours. After baseline data collection subjects received either placebo or indomethacin in addition to 20 ml of Maalox. Maalox was provided to prevent gastrointestinal discomfort occasionally associated with oral indomethacin. Subjects then rested quietly for 90-minutes while MCAv, respiratory, and cardiovascular variables were recorded for 5-minutes, spaced by 10-minute non-recording intervals. After 90-minutes, hypoxic and hypercapnic trials were conducted in randomized order while recording MCAv, respiratory, and cardiovascular variables during each trial. All trials were separated by 10-minutes of quiet rest while breathing room air (Figure 1). TCD placement was maintained during study visits and was similar between study visits.

*Hypoxia*

Isocapnic hypoxia trials were performed as previously described in our laboratory (48, 49, 81). Briefly, subjects inspired through a two-way non-rebreathing valve (2700 Series, Hans Rudolph Inc., Kansas City, MO), connected to a gas mixer (PM5300; Precision Medical, Northampton, PA), supplied by medical grade pressurized oxygen ($O_2$), carbon dioxide ($CO_2$), and nitrogen ($N_2$, Airgas, Madison, WI). After 5-minutes of baseline room air breathing,

hypoxia was introduced by decreasing inspired $O_2$ to elicit and sustain 5-minutes of $S_PO_2=90\%$, followed immediately by a transition to and 5-minutes of $S_PO_2=80\%$. Isocapnia was achieved through the addition of $CO_2$ to inspired gas. $PET_{CO2}$ has been shown to be a valid predictor of arterial blood $CO_2$ levels (92). Two women were unable to complete the $S_PO_2=80\%$ hypoxia trial due to hypoxia intolerance and were omitted from $S_PO_2=80\%$ hypoxia data analysis.

*Hypercapnia*

Hypercapnic trials were performed as previously described in our laboratory (48, 49). Briefly, subjects inspired through a three-way sliding rebreathing valve (Model 2870, Hans Rudolph Inc., Shawnee, KS) attached to a latex balloon containing a hyperoxic ($O_2=40\%$), hypercapnic ($CO_2=3\%$) gas mixture with the balance $N_2$. The balloon was filled to a volume exceeding estimated vital capacity (as determined by age, sex, and height) by 1-liter. After 5-minutes of baseline room air breathing, hypercapnia commenced and was sustained (~2-minutes) until $PET_{CO2}$ values reached 10 mmHg above baseline values. A total of two hypercapnic trials were completed and separated by 10-minutes of quiet rest and room air breathing. Values for the two trials were averaged.

*Plasma Assays*

Venous blood samples were drawn at baseline and after 90-minutes of placebo and indomethacin. Blood was centrifuged and plasma was drawn off and stored at $-80^oC$. Circulating estrogen and insulin were determined with radioimmunoassay (RIA), while enzyme immunoassay (EIA) was utilized for the determination of progesterone, testosterone, and dihydrotestosterone.

*Data Analysis*

All cardiovascular and respiratory data were recorded, stored, and analyzed with PowerLab and LabChart (ADInsturments Inc., Dunedin, NZ). The effect of drug administration was examined over 90-minutes with the last 30-seconds of each 5-minute data recording interval used for analysis. During hypoxia, the last 30-seconds of the 5-minute recording intervals for baseline, $S_PO_2$=90%, and $S_PO_2$=80% were analyzed. Analysis of hypercapnia data included the last 30-seconds of baseline and the last 10-seconds of hypercapnia, equating to a 10 mmHg increase in $PET_{CO2}$. Automated blood pressure recordings were taken during the last 30-seconds of each recording interval to coincide with analysis intervals. The main outcome variable was MCAv, but to account for potential differences in perfusion pressure, cerebrovascular conductance index was also calculated and is presented in tables (CVCi = MCAv*100/MABP).

*Statistical Analysis*

Minintab 16 (State College, PA, USA) was used for statistical analysis. Subject characteristics were compared using an unpaired student's t-test. Significance of sex (men vs. women) and time (0, 15, 30, 45, 60, 75, and 90 min) on basal MCAv, with and without indomethacin, were determined utilizing a general linear model to perform two-way analysis of variance (ANOVA). Unpaired student's t-tests were used to examine sex differences in the change in MCAv following indomethacin. Significance of sex (men vs. women) and hypoxia (baseline, $S_PO_2$=90%, and $S_PO_2$=80%) on MCAv and change in MCAv from baseline, with and without indomethacin, were determined by two-way general linear model ANOVA. Significance of sex (men vs. women) and hypercapnia (baseline and hypercapnia) on MCAv, with and without indomethacin, were determined by two-way general linear model ANOVA. Unpaired student's t-tests were used to examine sex differences in the change in MCAv with hypercapnia following placebo and indomethacin. In a secondary analysis, the contribution of

COX to hypoxic or hypercapnic mediated increases in MCAv was determined with general linear model ANOVA.  Level of significance was set *a priori* at P<0.05.  When ANOVA yielded significance, multiple comparisons on factor means were performed with Tukey's post hoc analysis.  Data are expressed as mean ± standard error of the mean.

## Results

*Subjects*

Subject characteristics are summarized in Table 1.  Men and women were similar in age and matched for activity level.  Men had significantly greater height, weight, and BMI.  Women had significantly greater total cholesterol and HDL.  Values in both groups were considered healthy.  Due to a lower body mass, women received a larger relative dose of indomethacin (P<0.01).  Circulating levels of sex hormones were determined in all 18 men and 20 of 24 women.  Testosterone and DHT were greater in men (P<0.01). By design, estrogen and progesterone were similar between sexes, confirming our menstrual phase selection criteria and allowing us to focus on sex differences when female sex hormones differences are minimized.

*Basal Cerebral Blood Flow: Sex Comparison*

Cardiorespiratory variables collected prior to and serially for 90-minutes after the administration of placebo while breathing normal room air are presented in Table 2.  Men had greater MABP and $PET_{CO2}$ than women (P<0.05).  Basal MCAv was greater in women (Figure 2A; P<0.01).  During placebo, MCAv did not change over the course of 90-minutes.

*Basal Cerebral Blood Flow: Contribution of COX*

Cardiorespiratory variables collected prior to and serially for 90-minutes after the administration of indomethacin, while breathing normal room air are presented in Table 2.  Men

had greater MABP and PET$_{CO2}$ than women (P<0.01).  Indomethacin increased MABP over the course of indomethacin wash-in (P<0.01).  MCAv decreased by the 30-minute time point (P<0.01) and remained suppressed for the reminder of the 90-minutes following indomethacin (Figure 2B).  Women displayed higher MCAv than men during 90-minutes of indomethacin wash-in (Figure 2B; P<0.01).  The absolute change in MCA (ΔMCAv) with indomethacin was not different between sexes (Figure 2C).

*Hypoxia-Mediated Cerebral Vasodilation: Sex Comparison*

Hemodynamic and gas exchange variables collected during hypoxia are presented in Table 3.  Hypoxia reduced S$_P$O$_2$ (P<0.01), but was not different between sexes. Hypoxia increased heart rate and MABP (P<0.01).  PET$_{CO2}$ did not change during hypoxia, but was greater in men (P<0.01).  MCAv was not significantly different from baseline at S$_P$O$_2$=90%, but was increased at S$_P$O$_2$=80% (Figure 3A; P<0.01).  Accordingly, the ΔMCAv from baseline was greater at SpO$_2$=80% than SpO$_2$=90%, (Figure 3B; P<0.01).  MCAv was greater in women during hypoxia, but ΔMCAv was not different between sexes (Figure 3B).

*Hypoxia-Mediated Cerebral Vasodilation: Contribution of COX*

Hemodynamic and gas exchange variables collected during hypoxia with indomethacin are presented in Table 3.  Hypoxia reduced S$_P$O$_2$ (P<0.01), but SpO$_2$ was not different between sexes. MCAv increased significantly at S$_P$O$_2$=80%, but not S$_P$O$_2$=90% (Figure 3C; P<0.01).  Accordingly, ΔMCAv from baseline was greater at SpO$_2$=80% than SpO$_2$=90% (Figure 3D; P<0.01).  MCAv was greater (Figure 3C; main effect, P<0.05) and MABP was lower in women during hypoxia with indomethacin.  The ΔMCAv during hypoxia with indomethacin was not different between sexes (Figure 3D) and was not different from the placebo condition.  PET$_{CO2}$ did not change during hypoxia (Table 3) and was not different between sexes.

*Hypercapnia-Mediated Cerebral Vasodilation: Sex Comparison*

Hemodynamic and gas exchange variables collected during hypercapnia are presented in Table 4. By design, hypercapnia increased $PET_{CO2}$ (P<0.01). MCAv increased during hypercapnia (Figure 4A; P<0.01) and was greater in women (Figure 4A; P<0.05). However, ΔMCAv from baseline was not different between sexes (Figure 4B). $PET_{CO2}$ was greater in men, but the change in $PET_{CO2}$ was not different between sexes.

*Hypercapnia-Mediated Cerebral Vasodilation: Contribution of COX*

Hemodynamic and gas exchange variables collected during hypercapnia with indomethacin are presented in Table 4. By design, hypercapnia increased $PET_{CO2}$ (P<0.01). MCAv increased during hypercapnia (Figure 4C; P<0.01) and was greater in women (Figure 4C; P<0.01). The ΔMCAv with indomethacin was lower than placebo hypercapnia (~55% reduction, P<0.01), but ΔMCAv was not different between sexes (Figure 4D). Change in $PET_{CO2}$ was not different between sexes.

*Alternative Data Expression*

CBF data are expressed as MCAv or ΔMCAv. Normalizing MCAv for perfusion pressure (MABP) and expressed as CVCi yielded the same results. Additionally, data expressed as relative changes in MCAv from baseline did not alter conclusions. One exception was the %ΔMCAv and %ΔCVCi during hypoxia with indomethacin. Despite similar ΔMCAv during hypoxia with placebo or indomethacin, decreased baseline MCAv with indomethacin resulted in significantly greater relative ΔMCAv compared to placebo. Since indomethacin substantially reduced basal MCAv, but did not change hypoxic absolute ΔMCAv, expressing data as a relative change from baseline is inappropriate and does not provide additional insight into our findings.

There were 24 women who participated in the current study, of which 9 were on birth control and 15 were not. Comparing the hypoxic and hypercapnic responses between women on birth control and not on birth control revealed no differences in placebo or indomethacin trials. When women on birth control were excluded from analysis between sexes (men, n=18; women, n=15), similar conclusions were reached, and so they were included in analysis (men, n=18; women, n=24).

## Discussion

The purpose of this study was to determine the fundamental sex-difference in CBF regulation between men and women without the confounding factor of acute elevations in sex hormones associated with the female menstrual cycle. Our aim was to determine the sex-specific contribution of COX to cerebrovascular control. The findings of our investigations indicate: 1) there is a similar and substantial contribution (30%) of COX to basal CBF in both sexes, 2) the increase in CBF to hypoxia or hypercapnia is not different between sexes, 3) the contribution of COX to the hypoxic increase in CBF is minimal and similar between sexes, and 4) the contribution of COX to hypercapnic vasodilation is substantial (55%) and similar between sexes. Taken together, data suggest women exhibit greater basal CBF, but similar hypoxic and hypercapnic mediated vasodilation when sex hormone profiles are most comparable to men.

*Sex Differences in Basal CBF Regulation*

Previous TCD studies indicate pre-menopausal women exhibit higher basal MCAv than age-matched men (15, 75, 86, 89, 133, 138) and our data support this concept. The average basal MCAv measured for men and women in our study over the course of 90-minutes was found to be approximately 70 cm s$^{-1}$ and 80 cm s$^{-1}$, respectively. These are nearly identical to previously published TCD values (75, 138), lending confidence in our ability to accurately measure MCAv

with TCD and compare sexes.  In the context of comparing our present findings to studies

lacking control of menstrual phase, we provide clear evidence that when estrogen and

progesterone (Table 1) are comparable to that of men, women have fundamentally higher basal

CBF.

Estrogen increases cerebral artery vasodilation partially through stimulating production

of the COX metabolite prostacyclin (102, 103, 129).  With this in mind, we hypothesized COX

would have a greater contribution to basal CBF in women, potentially related to a greater chronic

estrogen exposure than men.  Contrary to our hypothesis, COX contributes significantly (~30%)

and equally to the maintenance of basal CBF in men and women (Figure 2).  This reduction in

resting CBF with COX inhibition is in accord with the previous work (8, 31, 49-51, 54, 79) and

is supported when comparing absolute and relative reductions in basal MCAv.  Interestingly after

COX inhibition, there still remains an unidentified vasodilator mechanism maintaining a slightly

greater resting CBF in women (Figure 2B).

*Hypoxia*

Hypoxia is clinically relevant.  Sleep disordered breathing is characterized by hypoxia

and is noted for sex-specific pathophysiology (61).  This is the first study to demonstrate hypoxic

vasodilation is not different between men and women (Figure 3).  Furthermore, we provide clear

evidence COX does not contribute to hypoxic vasodilation in either sex (Figure 3).  This

supports previous studies (not examining sex differences) indicating COX does not play an

obligatory role in regulating hypoxic vasodilation in humans, at least when lowering $S_PO_2$ to

80% (31, 49).

*Hypercapnia*

Our new data indicate women exhibit similar $CO_2$ reactivity compared to men, which contrasts with prior studies suggesting greater $CO_2$ reactivity in women (62, 64, 65, 101). The higher $CO_2$ reactivity reported in prior studies might be explained by the lack of control for menstrual phase and fluctuations in female sex hormones, which were not reported previously (62, 64, 65, 101). Along these lines, cerebrovascular reactivity to breath holding ($CO_2$ accumulation) is greater in women during the luteal phase compared to the follicular phase of the menstrual cycle (25). Furthermore, sex hormone oscillations in younger women and hormone replacement therapy in older women are known to alter cerebrovascular reactivity to hypercapnia (9, 63, 73). In the current study, women were studied during the early follicular phase of the menstrual cycle when circulating sex-hormones are least different from men (Table 1). This likely explains the absence of a sex difference in $CO_2$ reactivity (Figure 4). Taken in context with previous studies, fundamental sex differences in $CO_2$ reactivity do not exist between healthy young men and women in the early follicular phase. Rather, greater $CO_2$ reactivity in women during late follicular or luteal phases of menstrual cycle is likely explained by cyclic increases in circulating female sex hormones.

COX inhibition in the current study reduced hypercapnic vasodilation by ~55% which is similar to that seen in prior studies not focused on sex comparisons (7, 49). Though there was a robust decrease in hypercapnic vasodilation with COX inhibition, the contribution of COX was not different between sexes (Figure 4). Contrary to our findings, Kastrup et al. demonstrated women had greater $CO_2$ reactivity that was abolished with COX inhibition when menstrual phase was not controlled (64). By controlling for menstrual cycle phase and quantifying circulating sex hormone levels, our new findings strongly suggest men and women demonstrate similar

vasodilation to hypercapnia, as well as a similar mechanistic contribution of COX eliciting this response.

*Limitations*

This study included a large number of well characterized subjects, controlled for menstrual cycle, quantified sex hormone levels, tightly controlled experimental conditions, and was a double-blind, randomized research design with multiple physiologic stressors. Given the strengths, there are limitations that need consideration. First, measuring MCAv with TCD is an estimation of CBF, as it is assumed that middle cerebral artery (MCA) diameter does not change. Based upon the levels of hypoxia and hypercapnia used in the current study, MCA diameter likely remains constant (112, 122, 144). MCA diameter has been shown to change during hypoxic challenges more extreme than those used in this study (144) and during hypercapnia (21). There is no evidence to indicate that these diameter changes are sex-specific, therefore if MCA diameter were to change in response to our stressors we would be underestimating CBF (21) equally in both sexes. However, sex-specific MCA dilation cannot be completely ruled out and could contribute to our lack of sex difference. Secondly, we only measured CBF through one MCA but previous studies demonstrate inter-hemispheric asymmetry is lowest in MCA resulting in insignificant differences in MCAv between left and right hemispheres (89, 138). Third, though we did not measure plasma metabolites of COX to test efficacy of COX inhibition, we previously reported a large and similar decrease in circulating COX metabolites, between two groups receiving different relative doses, coupled with large decreases in basal CBF and hypercapnic responses (49). Furthermore, the robust decrease in basal CBF 30-minutes after indomethacin administration and the ~55% decrease in hypercapnic responses in both sexes indicate COX was inhibited. Fourth, women in our group received a larger relative dose of

indomethacin due to a smaller body size.  Despite the greater relative dose,  there was an ~30% decrease in basal CBF in both sexes which is similar to that seen during indomethacin administration relative to body weight (51).  A larger relative dose of indomethacin in women suggests we did not underestimate the hypothesized greater vasodilation in women and further supports our conclusions.  Fifth, our findings are limited by the comparison of men to women during the early follicular phase of the menstrual cycle, which we sought to explore the basic sex difference in CBF regulation when sex-hormones are most similar.  An intriguing follow-up investigation should focus on whether acute elevations in female sex hormones in varying phases of the menstrual cycle and these hormonal influence CBF regulation and the contribution of COX.

*Summary and Conclusion*

We systematically tested the hypothesis that women would exhibit greater basal CBF, greater cerebral vasodilation to hypercapnia and hypoxia, and greater contribution of COX.  Our findings establish women in the early follicular phase of the menstrual cycle exhibit greater basal CBF compared to men.  New findings demonstrate hypoxia and hypercapnia mediated increases in CBF are remarkably similar between sexes.  Additionally, COX does not appear to contribute to hypoxic vasodilation in either men or women.  Finally, our results indicate COX contributes substantially to both basal CBF and hypercapnic vasodilation, and this contribution is similar between sexes.  Taken together, these data establish fundamental insight into CBF regulation that can be used to design mechanistic studies to unravel the complex sex-age-hormone interactions seen in overt clinical cerebrovascular disease developing during middle to old age.

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## Disclosures

The authors declare no conflict of interest.

Tables

**Table 1.1.** Subject characteristics

| | Men | | | Women | | | |
|---|---|---|---|---|---|---|---|
| | n=18 | | | n=24 | | | |
| Age (yrs) | 26 | ± | 1 | 23 | ± | 1 | |
| Height (cm) | 178 | ± | 2 | 165 | ± | 1 | * |
| Weight (kg) | 74 | ± | 2 | 58 | ± | 2 | * |
| BMI (kg m$^{-2}$) | 23 | ± | 1 | 21 | ± | 0 | * |
| Waist (cm) | 83 | ± | 1 | 77 | ± | 3 | |
| Hip (cm) | 101 | ± | 1 | 93 | ± | 2 | * |
| PAQ (kcal/wk) | 1654 | ± | 294 | 1976 | ± | 311 | |
| Glucose (mg dL$^{-1}$) | 75 | ± | 2 | 75 | ± | 1 | |
| Insulin (μU ml$^{-1}$) | 11 | ± | 1 | 10 | ± | 1 | |
| Total Cholesterol (mg dL$^{-1}$) | 141 | ± | 7 | 162 | ± | 5 | * |
| HDL (mg dL$^{-1}$) | 52 | ± | 3 | 71 | ± | 4 | * |
| LDL (mg dL$^{-1}$) | 75 | ± | 8 | 76 | ± | 4 | |
| Triglycerides (mg dL$^{-1}$) | 73 | ± | 8 | 73 | ± | 3 | |
| Systolic BP (mmHg) | 118 | ± | 2 | 113 | ± | 2 | |
| Diastolic BP (mmHg) | 74 | ± | 1 | 74 | ± | 1 | |
| MABP (mmHg) | 89 | ± | 1 | 87 | ± | 1 | |
| Estradiol (pg mL$^{-1}$) | 58 | ± | 3 | 84 | ± | 25 | |
| Progesterone (pg mL$^{-1}$) | 409 | ± | 59 | 352 | ± | 46 | |
| Testosterone (pg mL$^{-1}$) | 4120 | ± | 251 | 315 | ± | 29 | * |
| DHT (pg mL$^{-1}$) | 552 | ± | 34 | 206 | ± | 15 | * |
| Indo Dose (mg kg$^{-1}$) | 1.4 | ± | 0 | 1.7 | ± | 0 | * |

Values are presented as mean ± SE. Sex hormones (men, n=18; women, n=20). BMI, body mass index; PAQ, physical activity questionnaire; HDL, high density lipoprotein; LDL, low density lipoprotein; BP, blood pressure; MABP, mean arterial blood pressure; DHT, dihydrotestosterone; Indo, indomethacin. * Men vs. women; P<0.05.

**Table 1.2.** Cerebrovascular and cardiorespiratory variables prior to and for 90-minutes following placebo or indomethacin administration.

| | Placebo | | | | Indomethacin | | | |
|---|---|---|---|---|---|---|---|---|
| | Men | | Women | | Men | | Women | |
| MABP (mmHg)*† | | | | | | | | |
| Baseline | 88 ± 1 | | 86 ± 1 | | 90 ± 2 | | 86 ± 2 | |
| 30 min | 90 ± 2 | | 87 ± 1 | | 94 ± 2 | | 90 ± 1 | |
| 60 min | 89 ± 2 | | 87 ± 2 | | 92 ± 2 | | 93 ± 1 | |
| 90 min | 89 ± 1 | | 86 ± 1 | | 94 ± 2 | | 91 ± 1 | |
| $PET_{CO2}$ (mmHg)*† | | | | | | | | |
| Baseline | 38 ± 1 | | 36 ± 1 | | 38 ± 1 | | 37 ± 1 | |
| 30 min | 39 ± 0 | | 37 ± 1 | | 38 ± 0 | | 37 ± 0 | |
| 60 min | 39 ± 0 | | 38 ± 1 | | 38 ± 0 | | 37 ± 1 | |
| 90 min | 38 ± 1 | | 38 ± 1 | | 38 ± 1 | | 37 ± 1 | |
| $SpO_2$ (%) | | | | | | | | |
| Baseline | 99 ± 0 | | 99 ± 0 | | 99 ± 0 | | 99 ± 0 | |
| 30 min | 99 ± 0 | | 99 ± 0 | | 96 ± 3 | | 99 ± 0 | |
| 60 min | 99 ± 0 | | 99 ± 0 | | 99 ± 0 | | 99 ± 0 | |
| 90 min | 99 ± 0 | | 99 ± 0 | | 99 ± 0 | | 99 ± 0 | |
| MCAv (cm s$^{-1}$)*†‡ | | | | | | | | |
| Baseline | 69 ± 3 | | 75 ± 3 | | 70 ± 3 | | 74 ± 3 | |
| 30 min | 71 ± 3 | | 78 ± 3 | | 57 ± 3 | | 60 ± 3 | |
| 60 min | 72 ± 3 | | 78 ± 4 | | 50 ± 3 | | 52 ± 2 | |
| 90 min | 71 ± 3 | | 79 ± 3 | | 47 ± 3 | | 52 ± 2 | |
| ΔMCAv | | | | | | | | |
| 90 min | 1 ± 1 | | 4 ± 2 | | 22 ± 2 | | 23 ± 2 | |
| % ΔMCAv | | | | | | | | |
| 90 min | 2 ± 2 | | 6 ± 3 | | 32 ± 3 | | 30 ± 2 | |
| CVCi (cm s$^{-1}$ mmHg$^{-1}$)*†‡ | | | | | | | | |
| Baseline | 79 ± 3 | | 88 ± 4 | | 78 ± 4 | | 87 ± 3 | |
| 30 min | 79 ± 3 | | 90 ± 4 | | 61 ± 4 | | 67 ± 3 | |
| 60 min | 81 ± 3 | | 91 ± 4 | | 54 ± 3 | | 57 ± 3 | |
| 90 min | 80 ± 3 | | 92 ± 4 | | 50 ± 3 | | 57 ± 2 | |
| Δ CVCi | | | | | | | | |
| 90 min | 1 ± 2 | | 4 ± 3 | | 28 ± 3 | | 30 ± 3 | |
| % Δ CVCi | | | | | | | | |
| 90 min | 1 ± 2 | | 6 ± 4 | | 35 ± 3 | | 34 ± 3 | |

Values are presented as mean ± SE. MABP, mean arterial blood pressure; $PET_{CO2}$, end-tidal carbon dioxide; $SpO_2$, pulse oximetry oxygen saturation; MCAv, middle cerebral artery velocity; ΔMCAv, absolute change in MCAv; %ΔMCAv, relative change in MCAv; CVCi, cerebrovascular conductance index; ΔCVCi, absolute change in CVCi; %ΔCVCi, relative change in CVCi. * Placebo main effect of group, † indomethacin main effect of group, ‡ indomethacin main effect of time; P<0.05.

**Table 1.3.** Cerebrovascular and cardiorespiratory variables during graded systemic hypoxia.

| | Placebo | | Indomethacin | |
| --- | --- | --- | --- | --- |
| | Men | Women | Men | Women |
| MABP (mmHg) $\unlhd$† | | | | |
| Baseline | 90 ± 1 | 89 ± 1 | 95 ± 2 | 94 ± 1 |
| 90% | 94 ± 1 | 93 ± 2 | 97 ± 2 | 94 ± 1 |
| 80% | 94 ± 1 | 91 ± 2 | 98 ± 2 | 95 ± 1 |
| HR (beats min$^{-1}$)*$\unlhd$‡ | | | | |
| Baseline | 65 ± 5 | 70 ± 2 | 67 ± 6 | 62 ± 2 |
| 90% | 75 ± 4 | 82 ± 2 | 70 ± 4 | 71 ± 2 |
| 80% | 79 ± 3 | 89 ± 2 | 73 ± 2 | 76 ± 3 |
| PET$_{co2}$ (mmHg) * | | | | |
| Baseline | 40 ± 1 | 38 ± 0 | 38 ± 1 | 37 ± 1 |
| 90% | 40 ± 1 | 38 ± 0 | 38 ± 1 | 38 ± 0 |
| 80% | 39 ± 1 | 38 ± 0 | 38 ± 1 | 38 ± 1 |
| SpO$_2$ (%) $\unlhd$‡ | | | | |
| Baseline | 99 ± 0 | 99 ± 0 | 99 ± 0 | 99 ± 0 |
| 90% | 90 ± 0 | 90 ± 0 | 90 ± 0 | 90 ± 0 |
| 80% | 80 ± 0 | 81 ± 0 | 80 ± 0 | 81 ± 0 |
| MCAv (cm s$^{-1}$) *$\unlhd$†‡ | | | | |
| Baseline | 73 ± 3 | 78 ± 3 | 49 ± 2 | 54 ± 2 |
| 90% | 78 ± 3 | 86 ± 4 | 53 ± 3 | 59 ± 3 |
| 80% | 86 ± 4 | 93 ± 4 | 62 ± 3 | 65 ± 3 |
| ΔMCAv $\unlhd$‡ | | | | |
| 90% | 5 ± 1 | 8 ± 1 | 4 ± 1 | 6 ± 1 |
| 80% | 13 ± 2 | 14 ± 1 | 12 ± 1 | 11 ± 1 |
| % ΔMCAv $\unlhd$‡ | | | | |
| 90% | 7 ± 2 | 10 ± 2 | 9 ± 2 | 11 ± 2 |
| 80% | 17 ± 2 | 18 ± 2 | 25 ± 3 | 20 ± 2 |
| CVCi (cm s$^{-1}$ mmHg$^{-1}$) *$\unlhd$†‡ | | | | |
| Baseline | 82 ± 4 | 88 ± 4 | 52 ± 3 | 57 ± 2 |
| 90% | 84 ± 4 | 93 ± 4 | 55 ± 3 | 63 ± 3 |
| 80% | 92 ± 4 | 102 ± 4 | 63 ± 3 | 68 ± 4 |
| Δ CVCi $\unlhd$‡ | | | | |
| 90% | 2 ± 2 | 5 ± 1 | 3 ± 1 | 6 ± 1 |
| 80% | 10 ± 2 | 13 ± 1 | 11 ± 2 | 11 ± 2 |
| % Δ CVCi $\unlhd$‡ | | | | |
| 90% | 2 ± 2 | 6 ± 2 | 7 ± 2 | 11 ± 2 |
| 80% | 13 ± 2 | 16 ± 2 | 22 ± 3 | 18 ± 2 |

Values are means ± SE. MABP, mean arterial blood pressure; HR, heart rate; PET$_{CO2}$, end-tidal carbon dioxide; S$_P$O$_2$, pulse oximetry oxygen saturation; MCAv, middle cerebral artery velocity; ΔMCAV, change in MCAv from baseline; %ΔMCAV, percent change in MCAv from baseline; CVCi, cerebrovascular conductance index; ΔCVCi, change in CVCi from baseline; %ΔCVCi, percent change in CVCi from baseline. * Placebo, main effect of group; $\unlhd$ Placebo, main effect of hypoxia; † indomethacin, main effect of group; ‡ indomethacin, main effect of hypoxia; P<0.05.

**Table 1.4.** Cerebrovascular and cardiorespiratory variables during hypercapnia.

| | Placebo | | Placebo | | Indomethacin | | Indomethacin | |
|---|---|---|---|---|---|---|---|---|
| | Men | | Women | | Men | | Women | |
| MABP (mmHg) | | | | | | | | |
|    Baseline | 90 | ± 1 | 87 | ± 1 | 95 | ± 2 | 91 | ± 2 |
|    Hypercapnia | 92 | ± 1 | 89 | ± 2 | 96 | ± 2 | 94 | ± 2 |
| HR (beats min$^{-1}$) | | | | | | | | |
|    Baseline | 65 | ± 6 | 69 | ± 2 | 62 | ± 6 | 63 | ± 2 |
|    Hypercapnia | 65 | ± 5 | 64 | ± 3 | 61 | ± 6 | 62 | ± 2 |
| $PET_{CO2}$ (mmHg)*$\mathfrak{s}$‡ | | | | | | | | |
|    Baseline | 39 | ± 1 | 38 | ± 0 | 38 | ± 1 | 37 | ± 0 |
|    Hypercapnia | 49 | ± 1 | 47 | ± 0 | 48 | ± 1 | 47 | ± 0 |
| $S_PO_2$ (%)$\mathfrak{s}$‡ | | | | | | | | |
|    Baseline | 99 | ± 0 | 99 | ± 0 | 99 | ± 0 | 99 | ± 0 |
|    Hypercapnia | 100 | ± 0 | 100 | ± 0 | 100 | ± 0 | 100 | ± 0 |
| MCAv (cm s$^{-1}$)*$\mathfrak{s}$†‡ | | | | | | | | |
|    Baseline | 72 | ± 3 | 79 | ± 3 | 48 | ± 2 | 55 | ± 2 |
|    Hypercapnia | 91 | ± 4 | 101 | ± 4 | 55 | ± 3 | 64 | ± 3 |
| ΔMCAv | | | | | | | | |
|    Hypercapnia | 19 | ± 1 | 23 | ± 2 | 8 | ± 1 | 9 | ± 1 |
| %ΔMCAv | | | | | | | | |
|    Hypercapnia | 27 | ± 2 | 30 | ± 2 | 16 | ± 2 | 17 | ± 2 |
| CVCi (cm s$^{-1}$ mmHg$^{-1}$)*$\mathfrak{s}$†‡ | | | | | | | | |
|    Baseline | 80 | ± 3 | 90 | ± 4 | 50 | ± 2 | 60 | ± 2 |
|    Hypercapnia | 100 | ± 4 | 114 | ± 4 | 58 | ± 3 | 69 | ± 3 |
| Δ CVCi | | | | | | | | |
|    Hypercapnia | 20 | ± 2 | 23 | ± 2 | 8 | ± 1 | 9 | ± 1 |
| %Δ CVCi | | | | | | | | |
|    Hypercapnia | 25 | ± 2 | 27 | ± 2 | 15 | 2 | 14 | ± 2 |

Values are means ± SE. MABP, mean arterial blood pressure; HR, heart rate; $PET_{CO2}$, end-tidal carbon dioxide; $S_PO_2$, pulse oximetry oxygen saturation; MCAv, middle cerebral artery velocity; ΔMCAV, change in MCAv from baseline; %ΔMCAV, percent change in MCAv from baseline; CVCi, cerebrovascular conductance index; ΔCVCi, change in CVCi from baseline; %ΔCVCi, percent change in CVCi from baseline. * Placebo, main effect of group; $\mathfrak{s}$ Placebo, main effect of hypercapnia; † indomethacin, main effect of group; ‡ indomethacin, main effect of hypercapnia; $P<0.05$.
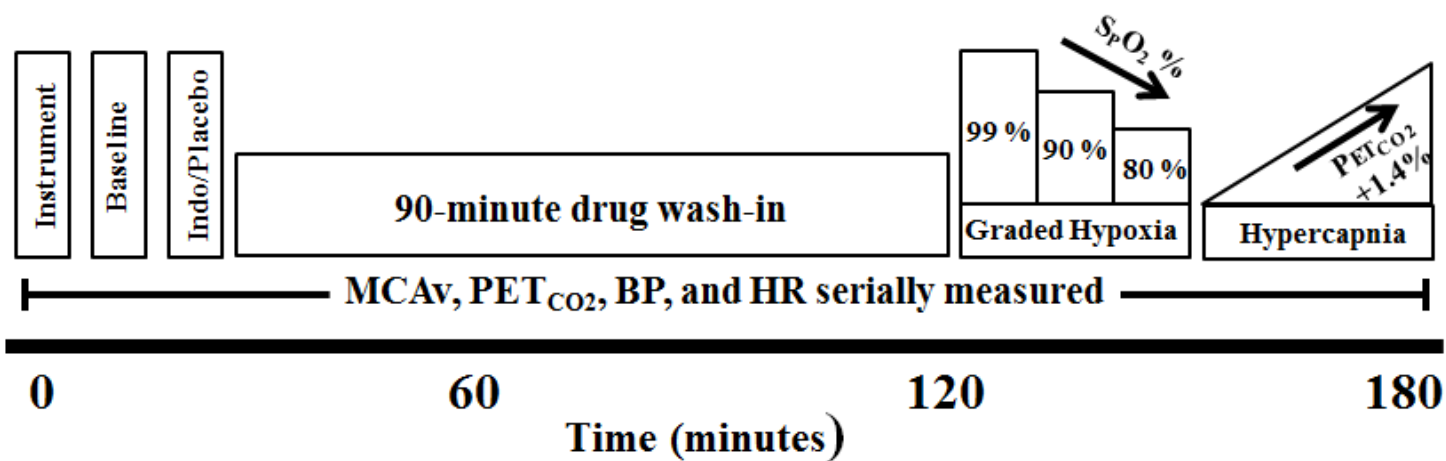
Figures

**Figure 1.1.** Timeline of study visits. Placebo and indomethacin were administered in a randomized, double-blind order. Order of graded hypoxia and hypercapnia were randomized for each study visit. Indo, indomethacin; $S_PO_2$, pulse oximetry oxygen saturation; $PET_{CO2}$, end-tidal carbon dioxide; MCAv, middle cerebral artery velocity; BP, blood pressure; HR, heart rate.
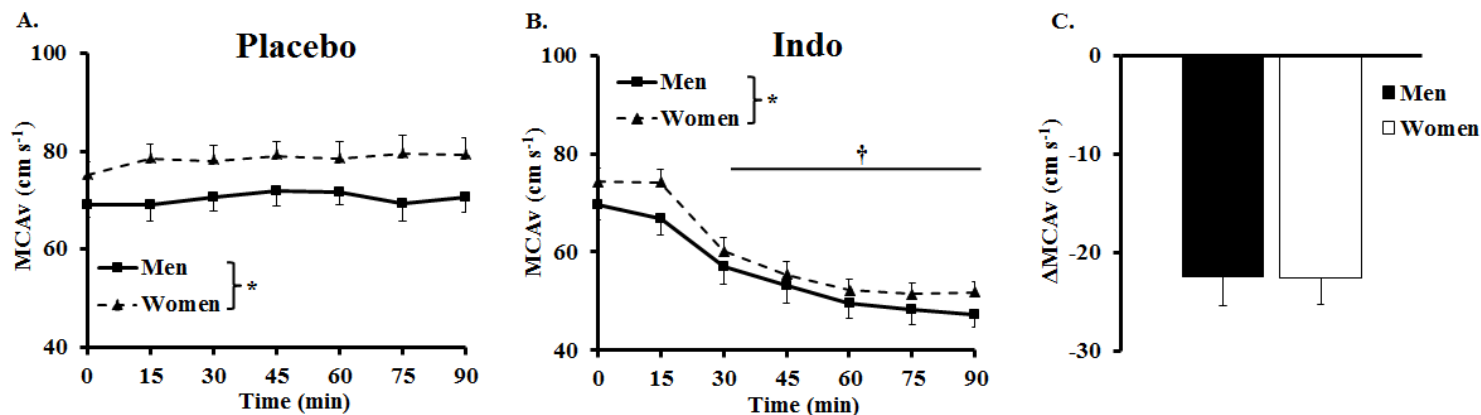
**Figure 1.2.** Basal middle cerebral artery velocity (MCAv) following placebo or indomethacin (Indo) administration. **A.** MCAv was greater in women over the 90-minutes of placebo wash-in. **B.** MCAv was decreased by 30-minutes of indomethacin wash-in, but remained greater in women. **C.** The absolute change in MCAv ($\Delta$MCAv) 90-minutes following indomethacin was similar in both sexes.

* Main effect of sex; † main effect of indomethacin; $P<0.01$.
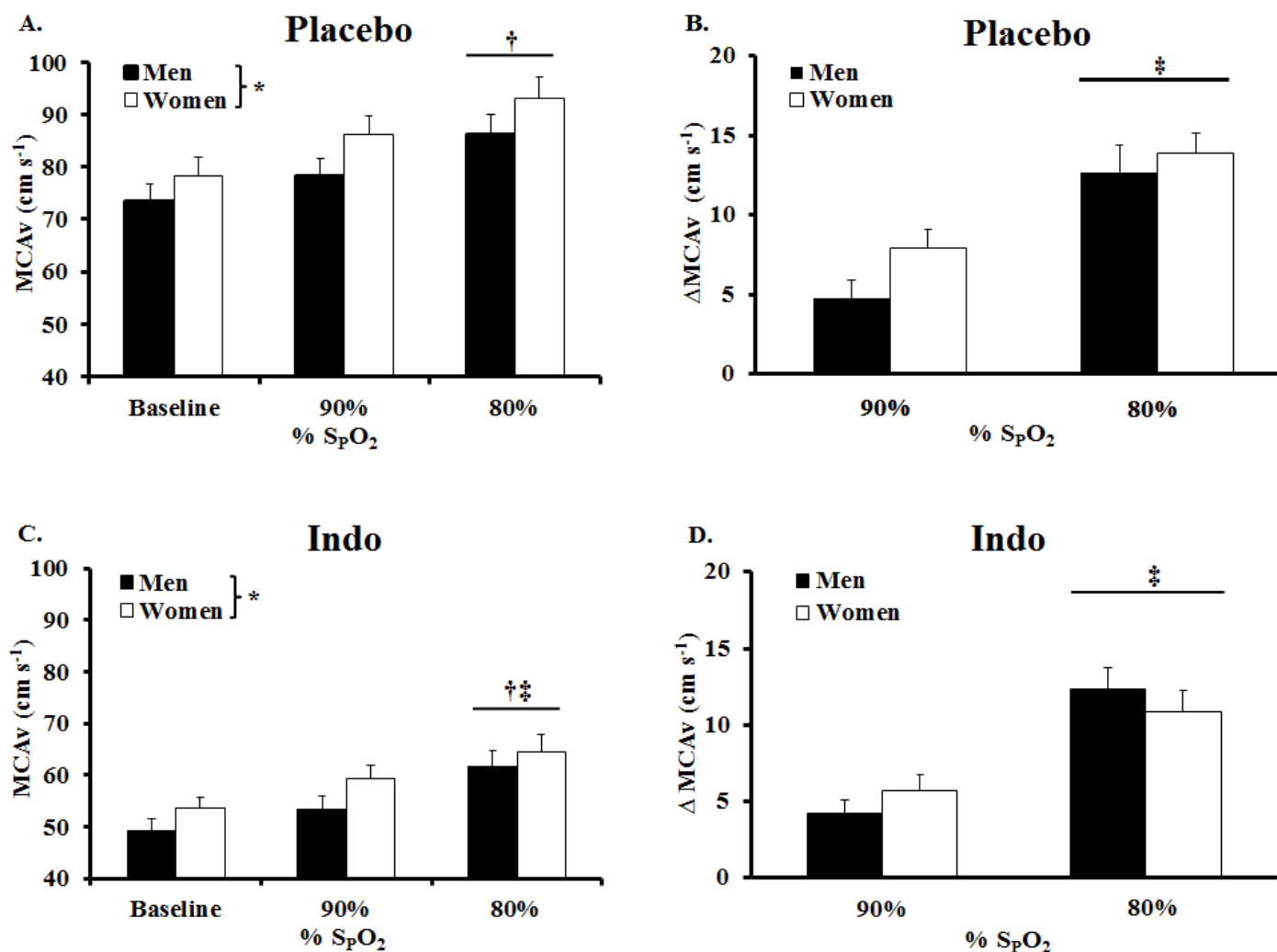
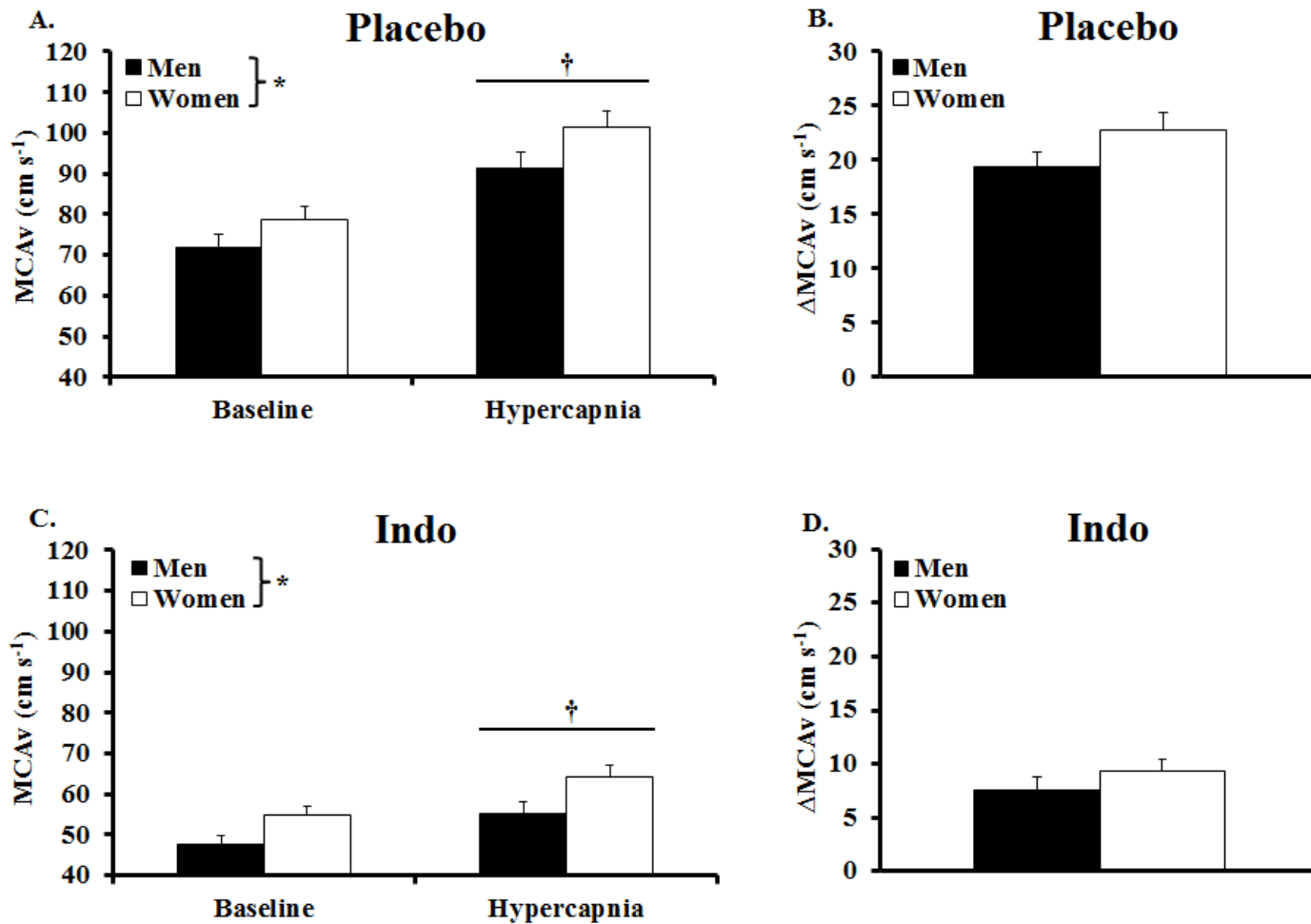**Figure 1.3.** Middle cerebral artery velocity (MCAv) during hypoxia with placebo and indomethacin (Indo). **A.** Hypoxia increased MCAv at $S_PO_2$ 80% and MCAv was greater in women with placebo. **B.** The change in MCAv ($\Delta$MCAv) was greater at $S_PO_2$ 80% than 90% with placebo, but $\Delta$MCAv was not different between groups. **C.** Hypoxia increased MCAv at $S_PO_2$ 80% with indomethacin and MCAv was greater in women. **D.** $\Delta$MCAv was not different between sexes during hypoxia with indomethacin, but $\Delta$MCAv was greater at $S_PO_2$ 80% than 90%. * Main effect of sex, $P<0.05$; † 80% vs. baseline, $P<0.05$; ‡ 80% vs. 90%, $P<0.01$

**Figure 1.4.** Middle cerebral artery velocity (MCAv) during hypercapnia with placebo and indomethacin (Indo). **A.** Hypercapnia increased MCAv and MCAv was greater in women with placebo. **B.** The change in MCAv ($\Delta$MCAv) with hypercapnia was not different between groups. **C.** Hypercapnia increased MCAv with indomethacin and MCAv was greater in women. **D.** $\Delta$MCAv was not different between sexes during hypercapnia with indomethacin. * Main effect of sex, $P<0.05$; † hypercapnia vs. baseline, $P<0.01$.

# Project II

**Cerebral blood flow regulation in women across menstrual phase: differential contribution of cyclooxygenase to basal, hypoxic, and hypercapnic vascular tone.**

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## Abstract

In healthy young women, basal cerebral blood flow (CBF) and cerebrovascular reactivity may change across the menstrual cycle, but mechanisms responsible remain untested. Compared to the early follicular phase of the menstrual cycle, we hypothesized women in late follicular phase would exhibit: 1) greater basal CBF, 2) greater hypercapnic increases in CBF, 3) greater hypoxic increases in CBF, and 4) increased cyclooxygenase (COX) signaling. We measured middle cerebral artery velocity (MCAv, transcranial Doppler ultrasound) in 11 healthy women ($23\pm1$ yrs) during rest, hypoxia, and hypercapnia. Subjects completed four visits; two during the early follicular (~day 3) and two during the late follicular (~day 14) phase of the menstrual cycle, with and without COX inhibition (oral indomethacin). Isocapnic hypoxia elicited an $S_PO_2$=90% and $S_PO_2$=80% for 5-minutes each. Separately, hypercapnia increased end-tidal carbon dioxide 10mmHg above baseline. Cerebral vascular conductance index (CVCi=MCAv/MABP *100) was calculated and a positive change reflected vasodilation ($\Delta$CVCi). Basal CVCi was greater in the late follicular phase (P<0.001). Indomethacin decreased basal CVCi (~37%) and abolished the phase difference (P<0.001). Hypoxic $\Delta$CVCi was similar between phases and was unaffected by indomethacin. Hypercapnic $\Delta$CVCi was similar between phases, and indomethacin decreased hypercapnic $\Delta$CVCi (~68%; P<0.001) similarly between phases. In summary, while neither hypercapnic nor hypoxic vasodilation is altered by menstrual phase, increased basal CBF in the late follicular phase is fully explained by a greater contribution of COX. These data provide new mechanistic insight into CBF regulation across menstrual phases and contribute to our understanding of CBF regulation in women.

## Introduction

Circulating estrogen is associated with reduced stroke risk and favorable stroke outcomes (84, 90). Increasing levels of estrogen lowers cerebrovascular impedance (72) and resistance (74), and is positively associated with cerebral blood flow (CBF) velocity (71). Accordingly, the highest CBF velocities occur during the late follicular phase of the menstrual cycle, when estrogen is at its highest (71). Conditions where estrogen is elevated such as pregnancy (9), ovarian stimulation (95, 126), and post-menopausal hormone replacement therapy (100, 126) are also associated with increased basal CBF. Despite these notable associations between estrogen and CBF, human research studies have not rigorously tested mechanisms responsible for increased CBF across menstrual phase in healthy young women.

While resting CBF is related to cardiovascular risk (94, 107) the ability to increase CBF during physiological challenges (i.e. hypoxia and hypercapnia) also holds important implications in health and disease. Hypoxic vasodilation studies are clinically relevant as hypoxia is a characteristic of sleep-disordered breathing and is experienced during sojourns to high altitude environments. To our knowledge, the influence of menstrual phase on hypoxic vasodilation has not been examined. Hypercapnia is also clinically relevant as poor hypercapnic cerebrovascular reactivity is predictive of cerebrovascular disease risk (87, 146). Cyclic oscillations in estrogen across the menstrual cycle are positively associated with the ability of the cerebrovasculature to vasodilate (reactivity) when exposed to the physiologic stress of hypercapnia, but the mechanisms remain unknown (73).

Cyclooxygenase (COX) is an important CBF control mechanism that may be influenced by menstrual phase. COX is known to regulate basal CBF and hypercapnic cerebral vasodilation (7, 49, 64, 110), while its role during hypoxic vasodilation is equivocal (31, 49, 51, 110).

Evidence suggests that female sex hormones may modulate COX-mediated CBF regulation. *In vitro* animal data show estrogen enhances the production of the vasodilatory cyclooxygenase (COX) metabolite prostacyclin (PGI₂) by upregulating endothelial COX and PGI₂ synthase (102, 103). Likewise, estrogen increases PGI₂ production in isolated human umbilical vein endothelial cells (129). To date, we are unaware of any human studies that have examined potential mechanisms responsible for greater resting CBF and greater hypercapnic reactivity associated with cyclic oscillations of endogenous estrogen across the menstrual cycle.

With this in mind, the overall aim of this study was to determine the active cerebrovascular responses to hypoxia and hypercapnia during the early and late follicular phases of the menstrual cycle. Secondly, we aimed to determine whether there is a menstrual phase difference in the contribution of COX to basal, hypoxic, and hypercapnic CBF regulation. We hypothesized that women in the late follicular phase of the menstrual cycle would exhibit greater basal CBF and greater vasodilation to hypoxia and hypercapnia. Further, we hypothesized the contribution of COX will be greater during the late follicular phase of the menstrual cycle. To test these hypotheses we compared the natural low versus high estrogen phases of the menstrual cycle, providing novel insight into CBF control during cyclic hormonal fluctuations associated with the menstrual cycle.

## Methods

*Subjects*

A total of 11 young, healthy women were recruited to participate in the study ($23 \pm 1$ yrs). All subjects were free of overt disease, non-medicated, and sedentary (<120 minutes of moderate physical activity per week) as determined by a medical history and physical activity questionnaire. All women had regular menstrual cycles, were not using any form of birth-

control, and were not pregnant as determined by a urine pregnancy test.  Subjects were studied

twice each during the early follicular (cycle days 1-5) and late follicular (cycle days 12-16)

phases of the menstrual cycle.  Cycle phase was confirmed by measuring the circulating sex

hormones estrogen and progesterone.  The nature, purpose, and risks of the study were provided

to each subject before written informed consent was obtained.  Protocols were approved by the

University of Wisconsin-Madison Institutional Review Board and conformed to the standards set

forth by the Declaration of Helsinki.

*Measurements*

Height and weight were measured to calculate body mass index (BMI; kg m$^{-2}$) and

regional adiposity was determined by measurement of hip and waist circumference.  Venous

blood samples were obtained to determine fasting plasma glucose, lipids, and sex-hormones.

Subjects were studied in a semi-recumbent position and instrumented with 3-lead

electrocardiogram (heart rate), pulse oximeter (pulse oximetry oxygen saturation; $S_PO_2$), and

blood pressure cuff (blood pressure; MABP; automated physiological monitor; GE Datex-

Ohmeda, Madison, WI, USA).  Hemodynamic parameters were continuously measured with

finger plethysmography (Nexfin HD; BMEYE, Amsterdam, NL).  Middle cerebral artery

velocity (MCAv) was measured with a 2-MHz transcranial Doppler ultrasound probe that was

held in place over the temporal window with an adjustable headband (TCD, Neurovision model

500M, Multigon Industries, Inc.; Yonkers, NY, USA).  Placement of TCD was recorded and

maintained for subsequent visits.  Respiratory flow and inspiratory and expiratory gases were

measured with a heated pneumotachometer (Hans Rudolph Inc., Shawnee, KS) and gas analyzer

(GEMINI, CWE, Inc., Ardmore, PA).

*Protocol:*

Subjects were studied twice during each the early follicular and late follicular phases of the menstrual cycle in a randomized, placebo-controlled, double blind study design. Prior to all four visits, subjects completed a 10-hour fast; abstained from exercise, alcohol, caffeine, and non-steroidal anti-inflammatory drugs for a minimum of 18-hours. After instrumentation, venous blood draw, and resting baseline measures, subjects received either placebo or oral indomethacin (100 mg, Heritage Pharmaceuticals Inc., Edison, NJ). Subjects were also given ~20 mL of Maalox for the prophylactic treatment of occasional gastrointestinal distress associated with indomethacin. Following drug administration, subjects rested quietly for 90-minutes while MCAv and cardiorespiratory variables were measured during the last 5-minutes of each 15-minute time interval. After 90-minutes, hypoxia and hypercapnia trials were conducted in a randomized order with MCAv and cardiorespiratory variables recorded. All trials were separated by 10-minutes of quiet rest. From instrumentation to completion, each study visit lasted ~3 hours (Figure 1).

*Hypoxia*

Isocapnic hypoxia trials were conducted as previously described in our laboratory (48, 49, 81, 110). After 5-minutes of baseline room-air breathing, inspired oxygen ($O_2$) was titrated with nitrogen ($N_2$) via medical grade gas-mixer to achieve and sustain $S_PO_2 = 90\%$ for 5-minutes. Subjects were then transitioned to achieve and sustain $S_PO_2 = 80\%$ for an additional 5-minutes. Isocapnia, relative to baseline room-air breathing, was maintained by the addition of carbon dioxide ($CO_2$) to inspired air. After baseline recording, the duration of hypoxia exposure was ~20 minutes. All inspired gases were medical grade quality (Airgas, Madison, WI).

*Hypercapnia:*

Hypercapnia trials were conducted as previously described in our laboratory (48, 49, 110). Briefly, after 5-minutes of baseline room-air breathing, subjects rebreathed a hyperoxic ($O_2$=40%) hypercapnic ($CO_2$=3%) gas mixture until end-tidal $CO_2$ ($P_{ET}CO_2$) increased 10 mmHg above baseline values (~2-minutes). Two hypercapnic trials were performed and separated by 10-minutes of quiet rest with both trials being averaged.

*Venous Blood Sample and Plasma Assays*

Venous blood samples were obtained prior to drug administration and 90-minutes after drug administration. Whole blood was used to determine fasting glucose and lipids (PTS Cardio Chek P-A; Indianapolis, IN). Blood was stored on ice, centrifuged (5500 rpm at 4ºC), and plasma was drawn off and stored at -80ºC for later analysis. Circulating estrogen and insulin were determined from plasma via radioimmunoassay (RIA). Progesterone, testosterone, and dihydrotestosterone were determined from plasma via enzyme immunoassay (EIA). Insulin and sex hormone assays were conducted by the University of Wisconsin-Madison National Primate Research Center.

*Data Analysis*

All MCAv and cardiorespiratory data were recorded, stored, and analyzed with PowerLab and LabChart (ADInsturments Inc., Dunedin, NZ). The effect of drug (placebo or indomethacin) was examined serially over 90-minutes following baseline measures. Data was recorded for the last 5-minutes every 15 minutes, with the last 30 seconds of each 5-minute recording interval analyzed. During hypoxia the last 30 seconds of 5-minute recording intervals during baseline, sustained $S_PO_2$=90%, and $S_PO_2$=80% were analyzed. For hypercapnia, the final 30-seconds of 5-minute baseline recording and the last 10-seconds of hypercapnia, equating to a 10-mmHg increase in $P_{ET}CO_2$ beyond basal values, were analyzed. Blood pressure recordings

were taken during the last 30-seconds of each recording interval to coincide with analysis

intervals. To account for small differences in perfusion pressure due to menstrual phase,

hypoxia/hypercapnia, and/or indomethacin administration, cerebrovascular conductance index

was calculated (CVCi = MCAv*100/MABP). A positive change in CVCi ($\Delta$CVCi) indicated

vasodilation.

*Statistical Analysis*

Minitab 16 (State College, PA, USA) was used for statistical analysis. Significance of

menstrual phase (early follicular versus late follicular) on circulating sex-hormones (estrogen,

progesterone, testosterone, and dihydrotestosterone) in 6 subjects during early and late follicular

phases, plus 2 additional subjects during the late follicular phase of the menstrual cycle were

determined with 2 sample t-tests. Significance of menstrual phase (early follicular or late

follicular), time (0, 15, 30, 45, 60, 75, and 90 min), and drug (placebo or indomethacin) on basal

CVCi and change in CVCi ($\Delta$MCAV) 90-minutes after the administration of drug were

determined utilizing a general linear model to perform an analysis of variance (ANOVA).

Significance of menstrual phase (early follicular or late follicular), hypoxia (baseline,

$S_PO_2$=90%, and 80%), and drug (placebo or indomethacin) on CVCi and $\Delta$CVCi from baseline

were determined by general linear model ANOVA. Significance of menstrual phase (early

follicular or late follicular), hypercapnia (baseline or hypercapnia), and drug (placebo or

indomethacin) on CVCi and $\Delta$CVCi were determined general linear model ANOVA. Level of

significance was set *a priori* at P<0.05. When ANOVA yielded significance, multiple

comparisons of factor means were performed with Tukey's post hoc analysis. Data are

expressed as mean $\pm$ standard error of the mean.

## Results

*Subjects*

Subject characteristics are presented in Table 1 and menstrual phase specific hormone concentrations are presented in Table 2. By design, subjects were studied during the early follicular (day 3±0) and late follicular (day 14±0) phases of the menstrual cycle. Circulating estradiol was greater during the late follicular phase of the menstrual cycle compared to the early follicular phase (P=0.01) as was testosterone (P=0.03). Progesterone and dihydrotestosterone were not different between phases (P=0.19 and P=0.94).

*Basal Cerebral Blood Flow: Influence of Menstrual Phase and the Contribution of COX*

Cardiorespiratory and cerebrovascular variables collected prior to and serially for 90-minutes after the administration of placebo and indomethacin while breathing room air are presented in Table 3 and Figure 2. Compared to the early follicular phase of the menstrual cycle, $S_PO_2$ was greater during the late follicular phase (main effect of phase; P=0.001). $P_{ET}CO_2$ was not different between phase, drug, or time point. During placebo, CVCi was greater in the late follicular phase compared to the early follicular phase of the menstrual cycle (Figure 2A; phase x drug interaction, P<0.001). Indomethacin administration increased MABP (main effect of drug, P<0.001), decreased HR (main effect of drug, P<0.001), and decreased CVCi by the 45-minute time point (Figure 2A; time x drug interaction, P<0.001). Indomethacin also abolished the phase difference in basal CVCi (Figure 2A; phase x drug interaction, P<0.001), yielding a ~37% decrease in basal CVCi (main effect of drug; P<0.001). Expressing data as MCAv yielded similar conclusions. Indomethacin abolished the phase difference in basal MCAv (Figure 2B; phase x drug interaction, P=0.001).

*Hypoxic Cerebral Vasodilation: Influence of Menstrual Phase and the Contribution of COX*

Cardiorespiratory and cerebrovascular variables collected prior to and during hypoxia after the administration of either placebo or indomethacin are presented in Table 4. Hypoxia decreased $S_PO_2$ (Figure 3A, 3C; main effect of condition, P<0.001), increased HR (main effect of condition, P<0.001), and increased CVCi at $S_PO_2$=80% but not at $S_PO_2$=90% (Figure 3A; main effect of condition, P=0.01). Accordingly, the absolute and relative $\Delta$CVCi were greater at $S_PO_2$=80% compared to $S_PO_2$=90% (Figure 3B; main effect of condition, P<0.001). Expressing data as MCAv yielded similar conclusions (Figure 3D).

Indomethacin increased MABP (main effect of drug, P=0.001), decreased heart rate (main effect of drug, P<0.001), decreased $P_{ET}CO_2$ (~1mmHg, main effect of drug, P<0.001), and decreased basal CVCi (Figure 3A; phase x drug interaction, P=0.014). Absolute and relative $\Delta$CVCi during hypoxia were not different between placebo and indomethacin (Figure 3B; main effect drug, absolute, P>0.131). Interestingly, expressing data as MCAv, the absolute $\Delta$MCAv during hypoxia was lower with indomethacin compared to placebo (Figure 2D; main effect drug, P=0.012), but relative $\Delta$MCAv was not different (main effect drug, P=0.885).

During the late follicular phase of the menstrual cycle, MABP was lower (main effect of phase, P=0.036) and CVCi was greater compared to the early follicular phase (Figure 3A; phase x drug interaction, P=0.014). Indomethacin decreased CVCi in both phases, abolishing the menstrual phase difference in CVCi (Figure 3A; phase x drug interaction, P=0.014), however the absolute and relative $\Delta$CVCi during hypoxia with placebo or indomethacin were not different between menstrual phases (Figure 3B; phase x drug interaction, P=0.435, P=0.791).

MCAv was not different between menstrual phases with placebo. Indomethacin decreased MCAv but was not different between menstrual phases (Figure 3C; phase x drug interaction, P=0.023). Absolute and relative $\Delta$MCAv during hypoxia with and without

indomethacin were also not different between menstrual phases (Figure 3D; phase x drug interaction, P=0.924, P=0.272)

*Hypercapnic Cerebral Vasodilation: Influence of Menstrual Phase and the Contribution of COX*

Cardiorespiratory variables collected prior to and during hypoxia after the administration of either placebo or indomethacin are presented in Table 5.  Hypercapnia increased $P_{ET}CO_2$ (+10mmHg from baseline), $S_PO_2$, CVCi, and MCAv (Table 5, Figures 4A and 4C; main effect of drug, P<0.01).  Indomethacin increased MABP and decreased heart rate, $P_{ET}CO_2$, CVCi, and MCAv (Table 5, Figures 4A and 4C; main effect of drug, P<0.01).

During hypercapnia with placebo, CVCi was greater in the late follicular compared to early follicular phase of the menstrual cycle (Figure 4A, phase x drug interaction, P=0.005).  The absolute and relative hypercapnia-mediated ΔCVCi were not different between phases (Figure 4B, phase x pill interaction, P>0.490).  Indomethacin decreased CVCi in both menstrual phases, abolishing the phase difference in CVCi (phase x drug interaction, P=0.005).  The absolute and relative hypercapnic-mediated ΔCVCi was reduced with indomethacin (Figure 4B, main effect of drug, P<0.001) but was not different between phases (Figure 4B, main effect of phase, P=0.118)

During hypercapnia with placebo, MCAv was not different between menstrual phases (Figure 4C, phase x drug interaction, P=0.010).  The absolute and relative hypercapnia-mediated increases in MCAv (ΔMCAv) during placebo also were not different between menstrual phases (Figure 4D, phase x drug interaction, P>0.358).  Indomethacin decreased MCAv but there was no menstrual phase difference (Figure 4C, phase x drug interaction, P=0.010).  The absolute and relative hypercapnia-mediated increases in MCAv (ΔMCAv) were reduced with indomethacin but were not different between phases (Figure 4D, phase x drug interaction, P>0.358).  The relative ΔMCAv during placebo and indomethacin was lower during the late follicular phase

(main effect of phase, P=0.021), which is likely due to greater baseline flow during the late follicular phase of the menstrual cycle.

## Discussion

The purpose of the study was to test the hypothesis that women in the late follicular phase of the menstrual cycle would exhibit greater basal CBF and greater vasodilation to hypoxia and hypercapnia. Further, we hypothesized the contribution of COX would be greater during the late follicular compared to early follicular phase of the menstrual cycle. Findings from the current study are: 1) greater basal CVCi and MCAv during the late follicular phase of the menstrual cycle are abolished COX inhibition, 2) hypoxia-mediated cerebral vasodilation is similar across early and late follicular phases and is unaffected by COX inhibition, and 3) hypercapnia-mediated cerebral vasodilation is similar across early and late follicular phase, as is the large reduction in vasodilation seen with COX inhibition. Our findings suggest COX contributes substantially to regulation of basal CBF and hypercapnic vasodilation. Interestingly, the contribution of COX to basal CBF differs between early and late follicular phases menstrual cycle. This is the first extensive CBF study focused solely on women. These novel findings provide insight into cerebrovascular physiology and have implications for understanding cerebrovascular disease risk in women.

*Basal Cerebral Blood Flow: Influence of Menstrual Phase and the Contribution of COX*

We provide the first *in vivo* human data that demonstrate COX mediates the late follicular phase increase in basal CBF (Figure 2). These data clarify conflicting reports that CBF velocity in the internal carotid artery is greater (71) or not different (73) during the late follicular versus early follicular phase of the menstrual cycle. Compared to the early follicular phase, circulating levels of estrogen are higher during the late follicular phase (Table 2). Elevated estrogen is

positively associated with CBF (71) and is known to have beneficial effects on the cerebral

vasculature, such as improving stroke outcomes (84, 90).  These beneficial effects may be due to

an estrogen-mediated reduction in cerebral vascular impedance (72), reduction in  resistance

(74), and an increase in CBF (71), which are consistent with current results.  Our findings are

also in agreement with prior *in vitro* studies showing that estrogen upregulates endothelial COX

and $PGI_2$ synthase (102, 103, 129).  Considered collectively, the most likely explanation for

increased basal CBF during the late follicular phase is an estrogen-mediated increase in the

contribution of COX products.

*Hypoxic Cerebral Vasodilation: Influence of Menstrual Phase and the Contribution of COX*

Hypoxia is a potent vasodilatory stimulus as the cerebrovasculature attempts to maintain

oxygen delivery despite a reduction in arterial oxygen content.  We provide the first evidence in

humans that hypoxic cerebral vasodilation is not increased during the late follicular compared to

early follicular phase of the menstrual cycle.  Recently, our lab provided evidence that hypoxic

vasodilation was not different between men and women, when women were studied only during

the early follicular phase and plasma estrogen was similar between sexes (110).  With this in

mind, we hypothesized women during the late follicular phase, when estrogen is higher, would

exhibit increased hypoxic vasodilation due to the vasoactive effects of estrogen.  Contrary to our

hypothesis, we found no differences in hypoxic vasodilation between early and late follicular

phases of the menstrual cycle despite higher levels of estrogen (Figure 3, Table 2).

These novel findings are in contrast to the reduced vascular tone observed in animal

models (102, 103), but may be explained by the following observations.  First, animal studies

often utilize ovarectomized rats with supra-physiologic estrogen supplementation.  This model

may not directly translate to cerebrovascular control in humans where physiologic levels of sex

hormones would provide a less potent vascular stimulus. Second, our lack of menstrual phase difference in hypoxic cerebral vasodilation could be due to chronic pulsatile exposure to estrogen. Although estrogen oscillates throughout the menstrual cycle in an acute fashion, these acute oscillations result in chronic pulsatile estrogen exposure. Chronic estrogen exposure may be sufficient to elicit the beneficial vascular effects of estrogen and therefore acute elevations may not alter vascular responsiveness to hypoxia. However, this appears to be unlikely given the lack of sex-difference in our recent human hypoxic vasodilation study (110). Lastly, women in the present study demonstrated elevated levels of testosterone during the late follicular phase, as testosterone is converted to estrogen by aromatase. During the late follicular phase of the menstrual cycle it is plausible that vasodilatory effects of estrogen (102, 103) are negated by the influence of elevated testosterone. Testosterone increases production of thromboxane and therefore, may increase cerebrovascular tone (41, 44, 45). Collectively, it appears that normally fluctuating, physiologic levels of estrogen do not enhance hypoxic vasodilation.

This is the first study to directly examine the contribution of COX during hypoxic vasodilation across menstrual phases. Previous studies have suggested that COX does (51) and does not (31, 49, 109) mediate hypoxic cerebral vasodilation. In the current investigation, COX inhibition (indomethacin) significantly reduced $\Delta$MCAv, but not $\Delta$CVCi during hypoxia. We believe $\Delta$CVCi is the most appropriate way to express data for several reasons. Indomethacin significantly increased blood pressure during hypoxia and there was a menstrual phase difference in blood pressure. Additionally, indomethacin substantially decreased baseline flow, and although absolute $\Delta$MCAv during hypoxia was lower with indomethacin, relative $\Delta$MCAv was not. Considered collectively, present findings suggest COX does not mediate hypoxic cerebral vasodilation.

*Hypercapnic Cerebral Vasodilation: Influence of Menstrual Phase and the Contribution of COX*

We provide clear evidence hypercapnic cerebral vasodilation is similar between the early and late follicular phases of the menstrual cycle, and that the substantial contribution of COX to hypercapnic vasodilation is similar between phases. Accordingly, others have shown that hypercapnic reactivity in the left common carotid artery, and left and right internal carotid arteries is not different between early and late follicular phases (73). Hypercapnic reactivity in right common carotid artery however, appears to be menstrual phase specific (73). Considered collectively, it seems that hypercapnic reactivity between early follicular and late follicular phases of the menstrual cycle is similar in carotid and middle cerebral arteries, although responses might be hemispheric specific. We would not expect there to be differences between either right or left MCA, as inter-hemispheric asymmetry is lowest in MCA compared to other cerebral arteries (89, 138).

To our knowledge, we are first to demonstrate that the contribution of COX to hypercapnic reactivity is similar between the early and late follicular phases of the menstrual cycle. Our data indicate COX contributes to ~68% of hypercapnia-mediated vasodilation, which is in agreement with prior studies from our lab and others (7, 49). Although *in vitro* animal data suggest increased estrogen may enhance COX-mediated cerebrovascular regulation (102, 103), our results suggest otherwise. A likely explanation for this discrepancy is that physiologic levels of estrogen are much lower in women in the current study compared to rats with supra-physiologic hormone supplementation (102, 103).

*Limitations*

The present study controlled for menstrual cycle, quantified sex hormone levels, tightly controlled experimental conditions, and employed a double-blind, randomized research design

with multiple physiologic stressors. Despite these strengths, there are limitations to be considered. First, measuring MCAv is only an estimation of MCA blood flow with the primary assumption that MCA diameter does not change. Given the levels of hypoxia and hypercapnia used in the current study, there may (21, 22, 144) or may not (112, 122) be a change in MCA diameter. Though we cannot rule out changes in MCA diameter, there is no evidence to indicate menstrual phase alters MCA diameter. If diameter were to increase during hypoxia and hypercapnia, we would be underestimating CBF equally in both phases (21). Second, we did not measure the concentration of plasma COX metabolites to demonstrate adequate COX inhibition. In a prior study in our lab, an absolute dose of indomethacin (100 mg) resulted in a large decrease in circulating COX metabolites (49). Combined with our observed ~37% reduction in basal CVCi and ~68% reduction in hypercapnic response, we are confident we effectively inhibited COX. Third, we relied upon the natural sex hormone oscillations associated with the menstrual cycle to attempt to isolate the effects of estrogen in a healthy physiologic context. Aside from measuring plasma sex-hormones on a daily basis, which does not seem practical from a subject standpoint, there are few methods to accurately predict sex-hormone concentrations on a given day of the menstrual cycle. Therefore, there is inherent variability in sex-hormone levels. We were unable to completely isolate the effects of estrogen from testosterone and therefore cannot conclude that our lack of phase differences is due to a small estrogen effect or testosterone counteracting estrogen.

*Summary and Conclusions*

We tested the hypothesis that women in the late follicular phase of the menstrual cycle would exhibit greater basal CBF and greater vasodilation to hypoxia and hypercapnia. Further, we hypothesized the contribution of COX would be greater during the late follicular compared to

early follicular phase of the menstrual cycle. In the same subjects, we compared the natural low versus high estrogen phases of the menstrual cycle. A key new finding indicates basal CBF is greater in the late follicular phase due to an increased contribution of COX. Second, hypoxic and hypercapnic vasodilation is remarkably similar between menstrual phases. Third, COX contributes to hypercapnic vasodilation similarly in both menstrual phases, but COX does not significantly contribute to hypoxic vasodilation in either phase. This study provides novel insight into CBF control during cyclic hormonal fluctuations associated with the menstrual cycle and contribute new understanding to cerebrovascular regulation in women.

## Acknowledgements

## Funding

## Disclosure

The authors declare no conflicts of interest.

Tables

**Table 2.1.** Subject Characteristics

| | | | |
|---|---|---|---|
| Age (yrs) | 23 | ± | 1 |
| Height (cm) | 161 | ± | 2 |
| Weight (kg) | 55 | ± | 2 |
| BMI (kg m$^{-2}$) | 21 | ± | 0 |
| Waist (cm) | 71 | ± | 2 |
| Hip (cm) | 94 | ± | 2 |
| PAQ (kcal/wk) | 2141 | ± | 511 |
| Glucose (mg dL$^{-1}$) | 74 | ± | 2 |
| Insulin (μU ml$^{-1}$) | 9 | ± | 1 |
| Total Cholesterol (mg dL$^{-1}$) | 172 | ± | 8 |
| HDL (mg dL$^{-1}$) | 71 | ± | 6 |
| LDL (mg dL$^{-1}$) | 80 | ± | 6 |
| Triglycerides (mg dL$^{-1}$) | 79 | ± | 5 |
| Systolic BP (mmHg) | 111 | ± | 3 |
| Diastolic BP (mmHg) | 73 | ± | 2 |
| MABP (mmHg) | 85 | ± | 2 |
| Indo Dose (mg kg$^{-1}$) | 1.8 | ± | 0.1 |

Values are presented as mean ± SE. BMI, body mass index; PAQ, physical activity questionnaire; HDL, high density lipoprotein; LDL, low density lipoprotein; BP, blood pressure; MABP, mean arterial blood pressure; Indo, indomethacin.

**Table 2.2.** Early follicular and late follicular phase sex-hormone concentrations

| | Early Follicular | | | Late Follicular | | | |
|---|---|---|---|---|---|---|---|
| Cycle Day | 3 | ± | 0 | 14 | ± | 0 | * |
| Estradiol (pg mL$^{-1}$) | 67 | ± | 8 | 145 | ± | 25 | * |
| Progesterone (pg mL$^{-1}$) | 435 | ± | 78 | 705 | ± | 174 | |
| Testosterone (pg mL$^{-1}$) | 295 | ± | 37 | 411 | ± | 44 | * |
| DHT (pg mL$^{-1}$) | 216 | ± | 25 | 213 | ± | 33 | |

Values are presented as mean ± SE. DHT, dihydrotestosterone. * early follicular vs. late follicular phase of menstrual cycle; P<0.05.

**Table 2.3.** Cardiorespiratory and cerebrovascular variables prior to and for 90-minutes following placebo or indomethacin administration.

| | Placebo | | | | Indomethacin | | | |
|---|---|---|---|---|---|---|---|---|
| | Early Follicular | | Late Follicular | | Early Follicular | | Late Follicular | |
| **HR (beats min$^{-1}$) †‡** | | | | | | | | |
| Baseline | 67 ± 2 | | 69 ± 2 | | 68 ± 2 | | 69 ± 2 | |
| 30 min | 66 ± 3 | | 66 ± 2 | | 65 ± 3 | | 65 ± 2 | |
| 60 min | 67 ± 2 | | 65 ± 2 | | 61 ± 3 | | 60 ± 3 | |
| 90 min | 68 ± 2 | | 68 ± 2 | | 59 ± 2 | | 60 ± 2 | |
| **MABP (mmHg) †** | | | | | | | | |
| Baseline | 84 ± 3 | | 82 ± 2 | | 85 ± 3 | | 81 ± 2 | |
| 30 min | 85 ± 2 | | 84 ± 2 | | 87 ± 2 | | 89 ± 2 | |
| 60 min | 85 ± 2 | | 83 ± 1 | | 91 ± 2 | | 88 ± 2 | |
| 90 min | 84 ± 1 | | 85 ± 2 | | 88 ± 1 | | 91 ± 2 | |
| **S$_P$O$_2$ (%)*** | | | | | | | | |
| Baseline | 98 ± 0 | | 99 ± 0 | | 98 ± 0 | | 99 ± 0 | |
| 30 min | 98 ± 0 | | 99 ± 0 | | 98 ± 0 | | 99 ± 0 | |
| 60 min | 98 ± 0 | | 99 ± 0 | | 99 ± 0 | | 99 ± 0 | |
| 90 min | 98 ± 0 | | 99 ± 0 | | 98 ± 0 | | 99 ± 0 | |
| **P$_{ET}$CO$_2$ (mmHg)** | | | | | | | | |
| Baseline | 38 ± 1 | | 38 ± 0 | | 38 ± 0 | | 39 ± 1 | |
| 30 min | 39 ± 0 | | 39 ± 1 | | 38 ± 1 | | 38 ± 1 | |
| 60 min | 39 ± 1 | | 39 ± 1 | | 38 ± 1 | | 39 ± 1 | |
| 90 min | 39 ± 0 | | 39 ± 1 | | 38 ± 1 | | 38 ± 1 | |
| **CVCi (cm s$^{-1}$ mmHg$^{-1}$) *†‡ṧ⊹** | | | | | | | | |
| Baseline | 82 ± 5 | | 93 ± 5 | | 84 ± 5 | | 88 ± 5 | |
| 30 min | 85 ± 6 | | 94 ± 6 | | 72 ± 6 | | 67 ± 6 | |
| 60 min | 83 ± 5 | | 95 ± 5 | | 59 ± 5 | | 57 ± 4 | |
| 90 min | 84 ± 4 | | 96 ± 6 | | 57 ± 5 | | 52 ± 3 | |
| **Δ CVCi†** | | | | | | | | |
| 90 min | 2 ± 3 | | 3 ± 4 | | -27 ± 4 | | -37 ± 5 | |
| **% Δ CVCi†** | | | | | | | | |
| 90 min | 3 ± 4 | | 4 ± 4 | | -32 ± 4 | | -40 ± 4 | |
| **MCAv (cm s$^{-1}$) †‡ṧ⊹** | | | | | | | | |
| Baseline | 68 ± 3 | | 76 ± 5 | | 71 ± 3 | | 71 ± 4 | |
| 30 min | 71 ± 4 | | 78 ± 4 | | 62 ± 4 | | 59 ± 5 | |
| 60 min | 70 ± 4 | | 79 ± 4 | | 53 ± 4 | | 50 ± 3 | |
| 90 min | 71 ± 4 | | 81 ± 5 | | 50 ± 4 | | 47 ± 2 | |
| **ΔMCAv†** | | | | | | | | |
| 90 min | 2 ± 2 | | 5 ± 2 | | -21 ± 3 | | -24 ± 4 | |
| **% ΔMCAv†** | | | | | | | | |
| 90 min | 3 ± 2 | | 8 ± 4 | | -30 ± 4 | | -33 ± 4 | |

Values are presented as mean ± SE. HR, heart rate; MABP, mean arterial blood pressure; S$_P$O$_2$, pulse oximetry oxygen saturation; P$_{ET}$CO$_2$, end-tidal carbon dioxide; CVCi, cerebrovascular conductance index; ΔCVCi, absolute change in CVCi; %ΔCVCi, relative change in CVCi; MCAv, middle cerebral artery velocity; ΔMCAv, absolute change in MCAv; %ΔMCAv, relative change in MCAv. * main effect of phase, † main effect of drug, ‡ main effect of time, ṧ phase x drug interaction, ⊹ time x drug interaction; $P<0.05$.

**Table 2.4.** Cardiorespiratory and cerebrovascular variables during graded systemic hypoxia.

| | Placebo | | Indomethacin | |
|---|---|---|---|---|
| | **Early Follicular** | **Late Follicular** | **Early Follicular** | **Late Follicular** |
| **HR (beats min$^{-1}$)†‡** | | | | |
| Baseline | 72 ± 2 | 69 ± 3 | 62 ± 2 | 66 ± 4 |
| 90% | 80 ± 3 | 81 ± 4 | 72 ± 2 | 68 ± 3 |
| 80% | 89 ± 3 | 88 ± 4 | 76 ± 4 | 74 ± 3 |
| **MABP (mmHg)*†** | | | | |
| Baseline | 88 ± 2 | 86 ± 2 | 93 ± 2 | 91 ± 2 |
| 90% | 90 ± 2 | 87 ± 2 | 91 ± 2 | 91 ± 2 |
| 80% | 90 ± 2 | 86 ± 2 | 93 ± 2 | 91 ± 2 |
| **S$_P$O$_2$ (%)‡** | | | | |
| Baseline | 99 ± 0 | 99 ± 0 | 99 ± 0 | 98 ± 0 |
| 90% | 90 ± 0 | 90 ± 1 | 90 ± 1 | 90 ± 1 |
| 80% | 81 ± 1 | 81 ± 1 | 81 ± 1 | 80 ± 1 |
| **P$_{ET}$CO$_2$ (mmHg)†** | | | | |
| Baseline | 39 ± 0 | 40 ± 1 | 39 ± 0 | 39 ± 1 |
| 90% | 40 ± 0 | 40 ± 1 | 39 ± 0 | 38 ± 1 |
| 80% | 40 ± 0 | 40 ± 1 | 39 ± 0 | 39 ± 1 |
| **CVCi (cm s$^{-1}$ mmHg$^{-1}$)†‡ꜱ** | | | | |
| Baseline | 82 ± 6 | 93 ± 6 | 54 ± 4 | 51 ± 2 |
| 90% | 87 ± 6 | 101 ± 8 | 60 ± 5 | 55 ± 3 |
| 80% | 96 ± 7 | 109 ± 8 | 64 ± 7 | 62 ± 4 |
| **ΔCVCi‡** | | | | |
| 90% | 5 ± 2 | 8 ± 3 | 6 ± 2 | 4 ± 1 |
| 80% | 14 ± 2 | 16 ± 3 | 10 ± 3 | 11 ± 3 |
| **%ΔCVCi‡** | | | | |
| 90% | 7 ± 2 | 8 ± 3 | 11 ± 4 | 9 ± 3 |
| 80% | 18 ± 3 | 17 ± 3 | 18 ± 4 | 23 ± 5 |
| **MCAv (cm s$^{-1}$)†‡ꜱ** | | | | |
| Baseline | 71 ± 5 | 80 ± 5 | 50 ± 4 | 46 ± 2 |
| 90% | 78 ± 6 | 87 ± 6 | 54 ± 4 | 50 ± 2 |
| 80% | 85 ± 6 | 94 ± 6 | 59 ± 5 | 56 ± 3 |
| **ΔMCAv†‡** | | | | |
| 90% | 7 ± 2 | 7 ± 3 | 4 ± 1 | 4 ± 1 |
| 80% | 14 ± 1 | 14 ± 3 | 9 ± 2 | 10 ± 2 |
| **%ΔMCAv‡** | | | | |
| 90% | 9 ± 2 | 8 ± 3 | 8 ± 2 | 9 ± 2 |
| 80% | 20 ± 2 | 17 ± 3 | 18 ± 3 | 22 ± 4 |

Values are presented as mean ± SE. HR, heart rate; MABP, mean arterial blood pressure; S$_P$O$_2$, pulse oximetry oxygen saturation; P$_{ET}$CO$_2$, end-tidal carbon dioxide; CVCi, cerebrovascular conductance index; ΔCVCi, absolute change in CVCi; %ΔCVCi, relative change in CVCi; MCAv, middle cerebral artery velocity; ΔMCAv, absolute change in MCAv; %ΔMCAv, relative change in MCAv. * main effect of phase, † main effect of drug, ‡ main effect of condition, ꜱ phase x drug interaction; $P<0.05$.

**Table 2.5.** Cardiorespiratory and cerebrovascular variables during hypercapnia.

| | Placebo | | | | Indomethacin | | | |
|---|---|---|---|---|---|---|---|---|
| | **Early Follicular** | | | **Late Follicular** | | | **Early Follicular** | | | **Late Follicular** | |
| **HR (beats min$^{-1}$) †** | | | | | | | | |
| Baseline | 68 | ± | 2 | 69 | ± | 3 | 62 | ± | 2 | 64 | ± | 3 |
| Hypercapnia | 69 | ± | 2 | 69 | ± | 3 | 63 | ± | 2 | 64 | ± | 2 |
| **MABP (mmHg)†** | | | | | | | | |
| Baseline | 87 | ± | 2 | 84 | ± | 2 | 91 | ± | 1 | 91 | ± | 2 |
| Hypercapnia | 89 | ± | 2 | 85 | ± | 2 | 94 | ± | 2 | 93 | ± | 2 |
| **S$_P$O$_2$ (%)‡** | | | | | | | | |
| Baseline | 99 | ± | 0 | 99 | ± | 0 | 99 | ± | 0 | 99 | ± | 0 |
| Hypercapnia | 99 | ± | 0 | 99 | ± | 0 | 99 | ± | 0 | 99 | ± | 0 |
| **P$_{ET}$CO$_2$ (mmHg)†‡** | | | | | | | | |
| Baseline | 39 | ± | 0 | 39 | ± | 1 | 38 | ± | 0 | 38 | ± | 1 |
| Hypercapnia | 49 | ± | 0 | 49 | ± | 1 | 48 | ± | 0 | 47 | ± | 1 |
| **CVCi (cm s$^{-1}$ mmHg$^{-1}$)†‡ṡ** | | | | | | | | |
| Baseline | 80 | ± | 6 | 97 | ± | 6 | 57 | ± | 5 | 51 | ± | 3 |
| Hypercapnia | 101 | ± | 7 | 117 | ± | 8 | 64 | ± | 6 | 56 | ± | 4 |
| **Δ CVCi†** | | | | | | | | |
| Hypercapnia | 21 | ± | 3 | 20 | ± | 3 | 8 | ± | 2 | 5 | ± | 2 |
| **%Δ CVCi†** | | | | | | | | |
| Hypercapnia | 27 | ± | 3 | 21 | ± | 3 | 13 | ± | 2 | 10 | ± | 3 |
| **MCAv (cm s$^{-1}$)†‡ṡ** | | | | | | | | |
| Baseline | 69 | ± | 4 | 82 | ± | 5 | 52 | ± | 4 | 46 | ± | 3 |
| Hypercapnia | 90 | ± | 6 | 99 | ± | 6 | 60 | ± | 5 | 52 | ± | 3 |
| **ΔMCAv†** | | | | | | | | |
| Hypercapnia | 21 | ± | 2 | 18 | ± | 3 | 9 | ± | 2 | 6 | ± | 1 |
| **%ΔMCAv*†** | | | | | | | | |
| Hypercapnia | 31 | ± | 3 | 22 | ± | 3 | 16 | ± | 2 | 12 | ± | 2 |

Values are presented as mean ± SE. HR, heart rate; MABP, mean arterial blood pressure; S$_P$O$_2$, pulse oximetry oxygen saturation; P$_{ET}$CO$_2$, end-tidal carbon dioxide; CVCi, cerebrovascular conductance index; ΔCVCi, absolute change in CVCi; %ΔCVCi, relative change in CVCi; MCAv, middle cerebral artery velocity; ΔMCAv, absolute change in MCAv; %ΔMCAv, relative change in MCAv. * main effect of phase, † main effect of drug, ‡ main effect of condition, ṡ phase x drug; P<0.05.

Figures
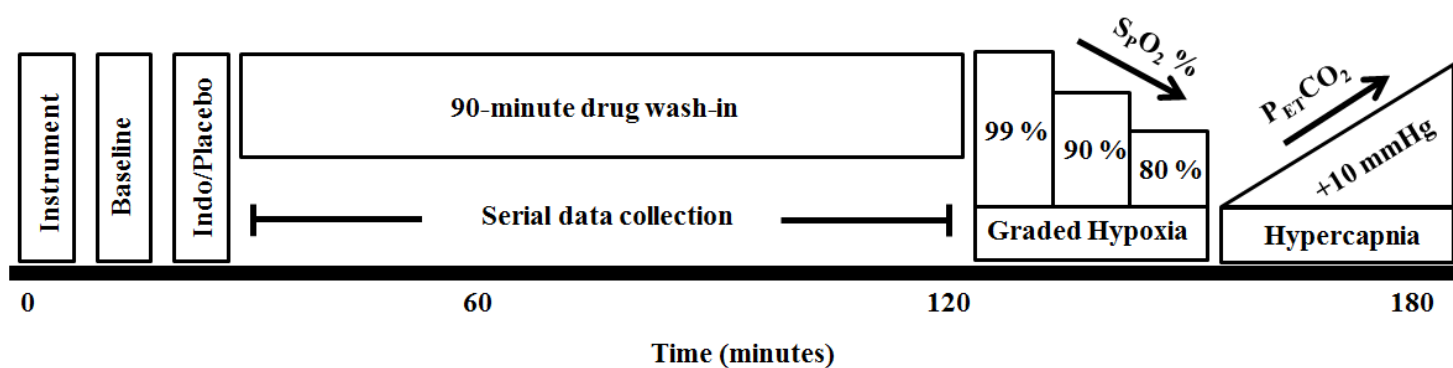
**Figure 2.1**. Timeline of study visits. Placebo and indomethacin were administered in a randomized, double-blind order. Order of graded hypoxia and hypercapnia were randomized for each study visit. Indo, indomethacin; $S_PO_2$, pulse oximetry oxygen saturation; $PET_{CO2}$, end-tidal carbon dioxide; MCAv, middle cerebral artery velocity; BP, blood pressure; HR, heart rate.
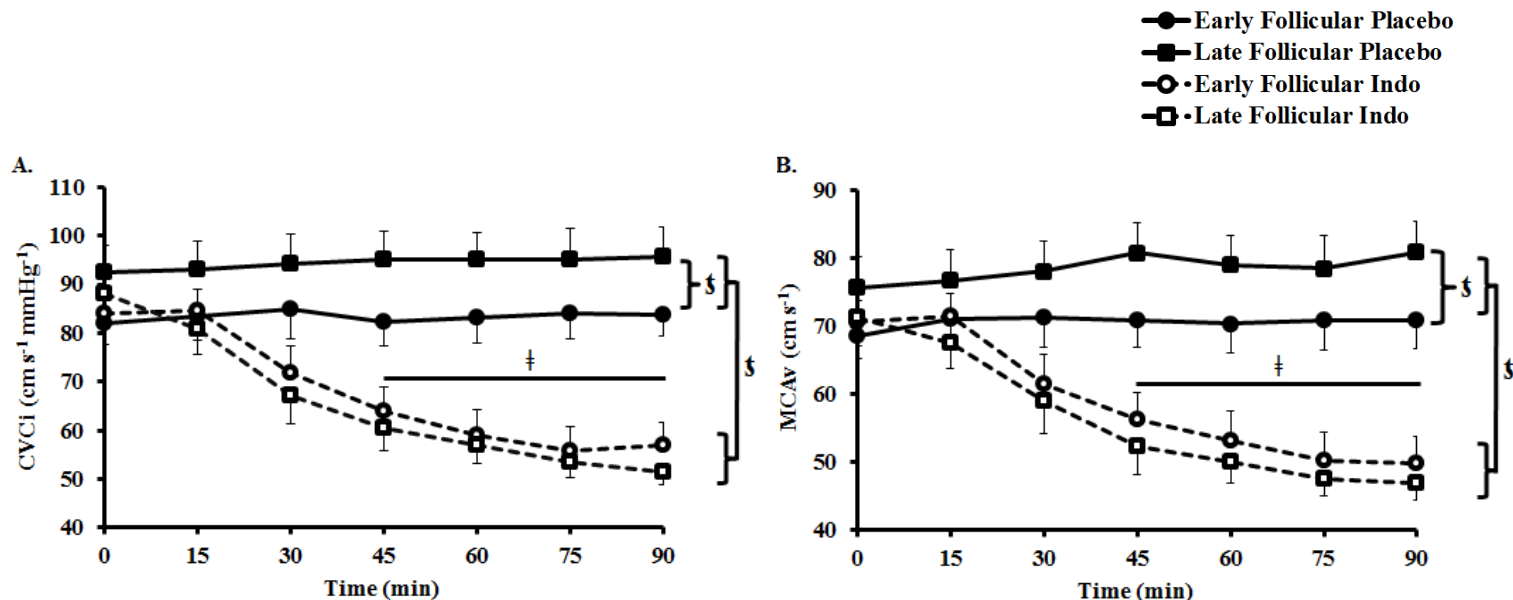
**Figure 2.2.** Basal middle cerebral artery vascular conductance index (CVCi) and velocity (MCAv) during early and late follicular phases of the menstrual cycle with placebo or indomethacin. **A.** During placebo CVCi was greater in the late follicular phase compared to the early follicular phase of the menstrual cycle (phase x drug, P<0.001). Administration of indomethacin decreased CVCi by the 45-minute time point (drug x time, P<0.001) and abolished the phase difference in basal CVCi (phase x drug, P<0.001). **B.** During placebo MCAv was greater in the late follicular phase compared to the early follicular phase of the menstrual cycle (phase x drug, P=0.001). Administration of indomethacin decreased MCAv by the 45-minute time point (drug x time, P<0.001) and abolished the phase difference in basal MCAv (phase x drug, P=0.001). $\int$ phase x drug interaction; ‡ drug x time interaction, vs time-point 0; P≤0.001.
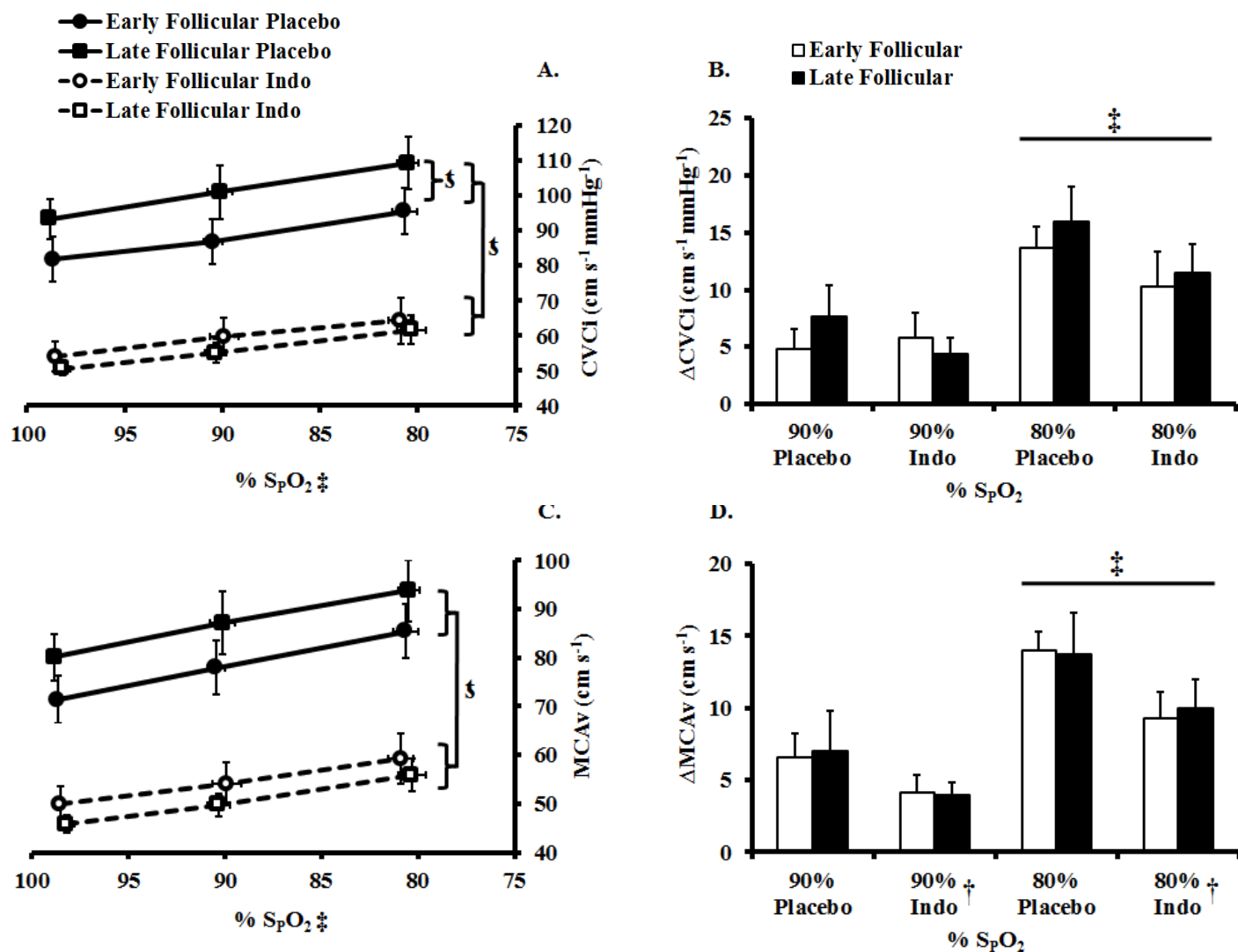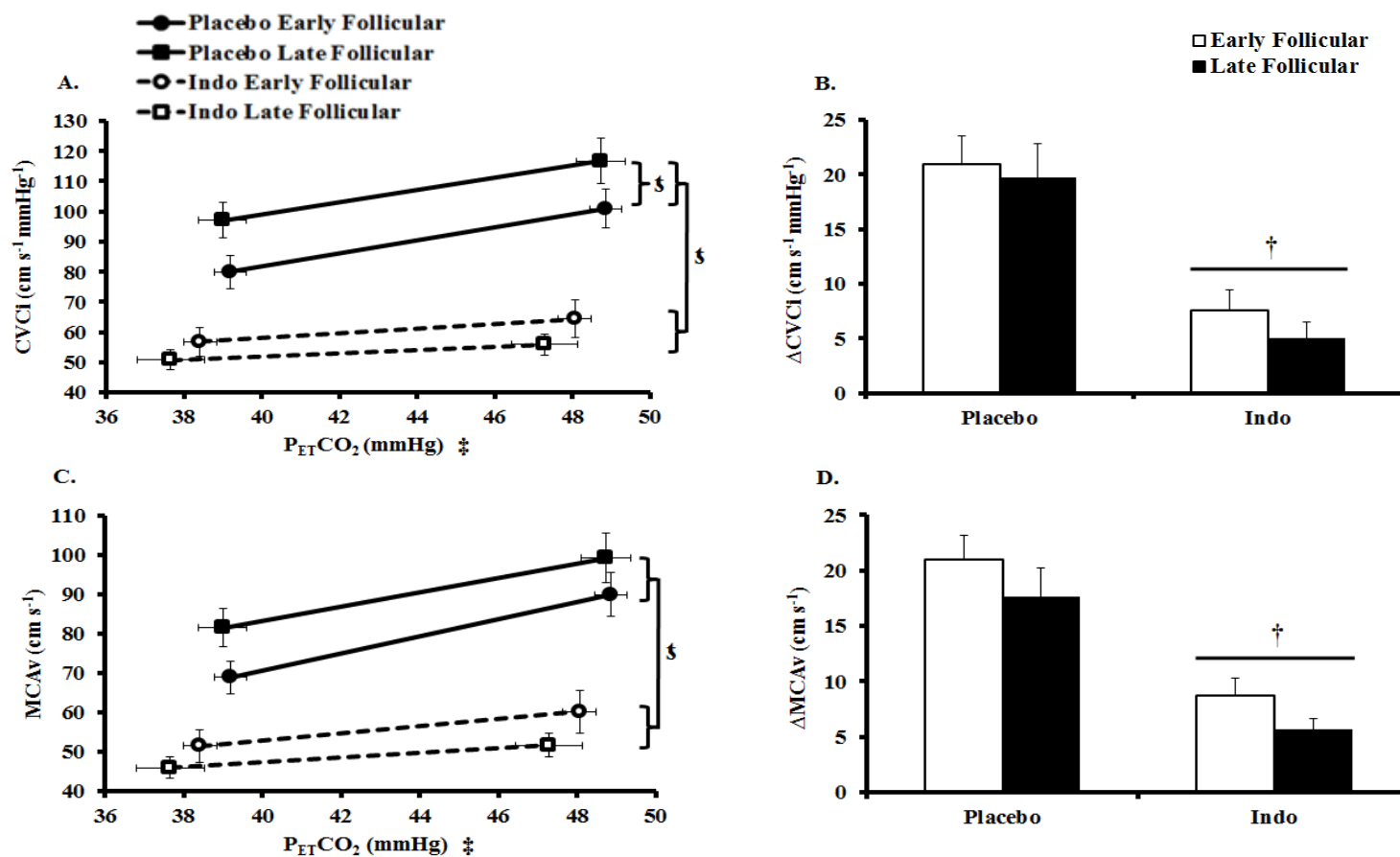
**Figure 2.3.** Hypoxia mediated middle cerebral artery vascular conductance index (CVCi) and velocity (MCAv) during early and late follicular phases of the menstrual cycle with placebo or indomethacin. **A.** CVCi during hypoxia was greater in the late follicular phase of the menstrual cycle, indomethacin decreased CVCi in both menstrual phases, abolishing the phase difference in CVCi (phase x drug interaction, P=0.014). **B.** The hypoxic mediated increase in CVCi (ΔCVCi) was not different between phases with placebo or indomethacin (phase x drug interaction, P=0.435). **C.** MCAv was not different between menstrual phases with placebo. Indomethacin decreased MCAv but was not different between menstrual phases (phase x drug interaction,

P=0.023).  **D.** The hypoxic mediated increase in MCAv (ΔMCAv) was not different between phases with placebo (phase x drug interaction, P=0.924).  Indomethacin decreased hypoxic ΔMCAv (main effect of drug, P=0.012), but was not different between phases (phase x drug interaction, P=0.924).  † main effect of drug;  ‡ main effect of condition;  ꙅ phase x drug interaction; P<0.05.

**Figure 2.4.** Hypercapnia mediated middle cerebral artery vascular conductance index (CVCi) and velocity (MCAv) during early and late follicular phases of the menstrual cycle with placebo or indomethacin. **A.** CVCi during hypercapnia was greater in the late follicular phase of the menstrual cycle. Indomethacin decreased CVCi in both menstrual phases, abolishing the phase difference in CVCi (phase x drug interaction, P=0.005). **B.** The hypercapnic mediated increase in CVCi ($\Delta$CVCi) was reduced with indomethacin (main effect of pill, P<0.001) but was not different between menstrual phases (main effect of phase, P=0.427). **C.** The hypercapnic mediated increase in MCAv was reduced with indomethacin but was not different between menstrual phases (phase x drug interaction, P=0.010). **D.** The hypercapnic mediated increase in MCAv ($\Delta$MCAv) was reduced with indomethacin (main effect of drug, P<0.001) but was not

different between phases (main effect of phase, P=0.122).  † main effect of drug; ‡ main effect of

condition; $ phase x drug interaction; P≤0.01.

# Project III

**Regional Contribution of Cyclooxygenase to Hypoxic Cerebral Vasodilation in Healthy Adults**

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The following data are in preparation for publication.

## Abstract

The precise mechanisms mediating hypoxic increases in cerebral blood flow (CBF) have not been well characterized. Data suggest potential region-specific increases in CBF during hypoxia, and region-specific contributions of COX. With this in mind, we hypothesized that hypoxic vasodilation would be greater in the posterior circulation compared to the anterior circulation, due in part to a greater contribution of COX. With four-dimensional flow magnetic resonance imaging (4D Flow MRI) we quantified CBF, cross-sectional area (CSA), and CBF velocity concurrently in 11 cerebral arteries (left and right: internal carotid, middle cerebral, anterior cerebral, vertebral, and posterior cerebral arteries and basilar artery). In a randomized, double-blinded, placebo-controlled design we studied 9 healthy, young adults ($28\pm1$ yrs; $23\pm1$ kg/m$^2$) on two separate occasions with and without COX inhibition (oral indomethacin). During each visit subjects were studied at baseline and during steady-state hypoxia ($F_IO_2 = 11\%$) while heart rate, blood pressure, pulse oximetry oxygen saturation ($S_PO_2$), respiratory rate, and end-tidal $CO_2$ ($ETCO_2$) were measured. Cerebrovascular conductance was calculated (CVC = CBF*100/MABP) and was expressed as percent change from baseline. Basal CVC was reduced from $663 \pm 64$ ml/min to $367 \pm 28$ ml/min following indomethacin ($p < 0.001$), a $44 \pm 2\%$ reduction in basal CVC ($p < 0.001$). Hypoxia elicited an $S_PO_2 \sim 81 \pm 1\%$ and was not different between conditions. Contrary to the hypothesis, hypoxia increased CVC in all arteries to a similar relative extent. Further, the hypoxia-mediated increase in relative CVC was augmented with indomethacin (main effect; $p < 0.001$) and this was similar between vessels. Hypoxia mediated changes in relative vessel CSA were greater with indomethacin compared to placebo (main effect, $p = 0.006$) and similar between vessels. The hypoxia-mediated increase in relative mean velocity was not affected by indomethacin and similar between vessels. We conclude that

hypoxia increases CVC uniformly across the cerebral circulation, and COX inhibition with indomethacin augments hypoxic cerebral vasodilation homogeneously across the cerebral circulation.

## Introduction

The brain comprises ~2% of body mass but accounts for ~20% of total body oxygen ($O_2$) consumption and cardiac output at rest (42). This extraordinary oxygen demand, driven by a high metabolic rate and almost total reliance on oxidative metabolism, necessitates tight regulation of cerebral blood flow (CBF) across a wide range of environmental and metabolic perturbations. One such perturbation is hypoxia, which elicits a robust increase in global CBF (11, 16, 80) to maintain cerebral oxygen delivery ($CDO_2$) in the face of reduced arterial oxygen content. Despite the importance of maintaining adequate blood flow to the brain, relatively little is known about region-specific CBF regulation.

Data in humans suggest heterogeneous increases in macrovascular blood flow during hypoxia (80, 99, 140). There appears to be greater relative increases in vertebral artery flow and diameter when compared to the internal carotid artery during hypoxia (80, 99). These regional differences can be substantial as the vertebral artery demonstrates an ~50% greater increase in flow when compared to the internal carotid, middle cerebral, and posterior cerebral arteries at an $S_PO_2 = 70\%$ (140).

Studies exploring hypoxia-induced regional increases in microvascular perfusion also support the idea of heterogeneity. Microvascular perfusion during hypoxia has been shown to increase in phylogenically older regions of the brain associated with autonomic function, including the  brain stem, when compared to newer regions of the brain like the cerebral cortex (11). Similarly, hypoxia has been shown to induce a disproportionate increase in hypothalamic

and cerebellar flow when compared to cortical brain regions (16). One thought is that the more primitive posterior brain regions, housing the brainstem and cardiorespiratory centers, supplied by the vertebral and basilar arteries are more sensitive to hypoxia than anterior brain regions supplied by the internal carotid, middle cerebral, and anterior cerebral arteries (80, 140). This is in line with the observation that neuropsychological function associated with anterior brain regions (cerebral cortex) is impaired during hypoxic exposure (137, 145), while posterior brain regions (i.e. brain stem) housing the respiratory and cardiovascular control centers become more active to increase ventilation and cardiac output to maintain homeostasis (80).

However, it is important to point out that there are data suggesting greater hypoxia-mediated increases in anterior macrovascular flow (58), anterior cross sectional area (67), and anterior microvascular perfusion compared to the posterior circulation (77). Existing animal data also support the idea of greater anterior hypoxic sensitivity as chronic hypoxia in fetal sheep results in greater vasodilation of the anterior cerebral artery, compared to the middle cerebral and posterior cerebral arteries (26). Further complicating matters, others suggest there are no regional differences in macrovascular flow (51, 53, 67, 99) and microvascular perfusion (29, 104, 105).

It is well known that hypoxia increases CBF, and evidence suggests potential of region-specific hypoxic vasodilation. Regardless, the precise mechanisms mediating hypoxic increases in CBF are relatively unknown. One potential mechanism mediating hypoxic cerebral vasodilation is cyclooxygenase (COX). Animal data clearly indicate that COX plays a role (23, 37), whereas human data remains equivocal (31, 49, 51). There is only one study to date in humans that has investigated the potential region-specific contribution to COX to hypoxic cerebral vasodilation (51). In this study, COX was shown to contribute to hypoxic vasodilation

in both the middle cerebral and vertebral arteries but not the internal carotid and posterior cerebral arteries (51), suggesting that COX signaling may be region-specific and contribute to potential region-specific increases in CBF during hypoxia.

Although data suggest possible region-specific increases in CBF and a region-specific contribution of COX during hypoxia, there has yet to be a comprehensive study examining the CBF responses to hypoxia in multiple anterior and posterior arteries, while sufficiently clamping end-tidal $CO_2$ ($ETCO_2$) to isolate the effects of hypoxia from hyperventilation-induced hypocapnia.

With regional cerebrovascular specificity in mind, we aimed to investigate region-specific increases in CBF and the region-specific contributions of COX to hypoxic cerebral vasodilation. Utilizing four-dimensional flow magnetic resonance imaging (4D Flow MRI), we directly quantified CBF, during hypoxia while clamping $ETCO_2$, concurrently in eleven major cerebral arteries that comprise the anterior and posterior macrovascular circulations. We hypothesized that hypoxic vasodilation would be greater in the posterior circulation (vertebral, basilar, and posterior cerebral arteries) compared to the anterior circulation (internal carotid, middle cerebral, and anterior cerebral arteries), due in part to a greater contribution of COX.

## Methods

*Subjects*

A total of nine young, healthy volunteers participated in the study (28±1 yrs). A portion of this data (hypoxia placebo) has been previously published (66). All subjects were lean (23±1 kg/m$^2$), non-smoking, free of disease, and not currently taking medication with the exception of birth control, as determined by a health history questionnaire. Women were not pregnant and studied either during the early follicular phase of the menstrual cycle (cycle days 1-5) or low

hormone phase of birth control (self-report).  Subjects were instructed to report to the laboratory on all study days having abstained from exercise, alcohol, caffeine, supplements, over the counter medications, and non-steroidal anti-inflammatory drugs for $\geq 24$ hours, and to have fasted for $\geq 4$-hours.  The nature, purpose, and risks of the study were provided to each subject before written informed consent was obtained.  All protocols were approved by the University of Wisconsin-Madison Institutional Review Board and conformed to the standards set forth by the Declaration of Helsinki.

*Screening Visit*

Subjects performed an initial screening visit to determine eligibility that included a healthy history and physical activity questionnaire.  Height and weight were measured to calculate body mass index (BMI, kg m$^{-2}$), waist and hip circumference were measured as an indicator of regional adiposity, brachial artery blood pressure was measured, and venous blood samples were obtained for the determination of glucose and lipids (Table 1).

*Protocol*

Following informed consent and screening procedures, subjects completed two study visits in a randomized, double-blind, and placebo-controlled design under control (placebo) and experimental conditions (COX inhibition). Both placebo and the non-selective COX inhibitor indomethacin (100 mg) were administered orally, in addition to 20 ml of Maalox to prevent gastrointestinal discomfort occasionally associated with oral indomethacin.  Subjects then rested quietly for 90-minutes outside of the MRI scanner.

After 90-minutes, subjects entered the MRI scanner and were instrumented for continuous measurement of pulse oximetry oxygen saturation ($S_PO_2$, pulse oximeter), heart rate (via pulse oximeter), blood pressure (MABP, automated sphygmomanometery, Medrad Veris

MR Vital Signs Patient Monitor, Bayer Healthcare, Whippany, NJ, USA) and ETCO$_2$ (Medrad Veris MR Vital Signs Patient Monitor, Bayer Healthcare, Whippany, NJ, USA). Hypoxia consisted of a baseline scan (PC Viper scanning sequence, ~ 5 minutes), transition to steady state hypoxia ( <5 min), and hypoxia scan once steady state hypoxia was reached (PC Viper scanning sequence, ~5 minutes). Figure 3.1

*Hypoxia*

Following instrumentation and 5-minutes of baseline room-air breathing and baseline scans, hypoxia was introduced. Isocapnic hypoxia was introduced by having subjects inspire through a two-way non-rebreathing valve (2630 Series, Hans Rudolph Inc., Shawnee, KS) supplied by a medical grade pressurized gas mixture containing 11% O$_2$ and the balance N$_2$ (Airgas, Madison, WI). This gas mixture was expected elicited an S$_P$O$_2$ ~ 80%. Isocapnia was achieved by titrating the hypoxic gas mixture accordingly with medical grade pressurized CO$_2$ (100% CO$_2$; Airgas, Madison, WI), with ETCO$_2$ serving as a valid predictor of arterial blood CO$_2$ levels (92). Steady S$_P$O$_2$ and ETCO$_2$ were reached in <5 minutes (no change in S$_P$O$_2$ and ETCO$_2$ for > 1 min), after which 4D Flow MRI scan commenced.

*4-Dimensional Flow Magnetic Resonance Imaging (PC-VIPR)*

We utilized 4D Flow MRI or more specifically Phase Contrast-Vastly Under Sampled Isotropic Projection Reconstruction (PC-VIPR) to determine the region-specific contribution of COX to hypoxic cerebral vasodilation concurrently in eleven separate cerebral arteries. PC-VIPR is an acquisition scheme developed at the University of Wisconsin-Madison that measures three-dimensional flow across time, without the use of a contrast agent. PC-VIPR has been validated and is known to provide accurate blood flow measures within cerebral arteries (47, 139). All study imaging was completed on a 3T MRI (Discovery MR750, GE Healthcare,

Waukesha, WI, USA) with an eight-channel head coil. The following scan parameters were

utilized: imaging volume = 22 x 22 x 22 cm$^3$, (0.69 mm)$^3$ acquired isotropic resolution, scan time

= 5 min 30 seconds, velocity encoding (Venc) = 100 cm/s, flip angle = 20$^0$, TR/TE = 6.7/2.8 ms,

20 reconstructed cardiac time frames using retrospective cardiac gating and temporal view

sharing  (83).

*Data Processing*

Data processing was completed using commercial software (Matlab, The Mathworks,

Natick, MA, USA) that has proven to be reliable in the assessment of cerebral artery blood flow

parameters (139).  Vessel position was selected with a centerline-processing scheme that utilizes

an algorithm to segment individual vessels in a plane perpendicular to the vessel path, one voxel

in width (0.69 mm), simultaneously providing velocity, cross sectional area (CSA), and flow

measures.  Velocity, CSA and blood flow were averaged over five consecutive cross sections (5

x 0.69 mm = 3.45 mm) producing a 3.45 mm long segment in each artery of interest, providing

an accurate measurement of CBF (120).  Eleven arteries of interest were assessed including: left

and right vertebral arteries (VA-L, VA-R), basilar artery (BA), left and right posterior cerebral

arteries (PCA-L, PCA-R), left and right internal carotid arteries (ICA-L, ICA-R), left and right

middle cerebral arteries (MCA-L, MCA-R), and left and right anterior cerebral arteries (ACA-L,

ACA-R).  Individual vessel segmentation occurred at precise locations which are as follows:  VA

was measured 4-5 mm from the junction with the BA, BA was measured near the junction with

the VA, PCA was measured 4-5 mm from the junction with the BA, ICA was measured in the

straight portion of the C4 segment (13), and ACA and MCA were be measured 4-5 mm from

their junction with the ICA (A1 and M1, respectively (Figure 3.2).  Total CBF was calculated as

the sum of the ICA-L, ICA-R, and BA. Anterior CBF was calculated at the sum of the ICA-L and ICA-R, while the BA was used to represent posterior CBF.

*Statistics*

The main outcome variable was CBF. To account for potential differences in perfusion pressure, cerebrovascular conductance was calculated and presented (CVC = CBF*100/MABP). To account for potential differences in baseline flow associated with vessel and indomethacin, data was expressed as percent change in CVC from baseline, with a positive change reflecting vasodilation and a negative change reflecting vasoconstriction. Expressing data as percent change in CVC is considered the most accurate representation of changes in vascular tone between conditions that vary widely in flow (17, 76, 88).

Minitab 16 (State College, PA) was used for statistical analysis. Significance of systemic hemodynamics variables in response to condition (baseline or hypoxia) and drug (placebo or indomethacin) were determined with a general linear model ANOVA.

Significance of the basal effect of indomethacin on total and regional CVC was determined with two-sample *t*-tests, one sample *t*-tests were used to determine if basal changes in CVC with indomethacin were less than zero.

Significance of hypoxia on vessel and region-specific increases in CVC was determined with general linear model ANOVA and two-sample *t*-tests, respectively.

Significance of drug (placebo or indomethacin) on hypoxia-mediated increases on total CVC was determined with two-sample *t*-tests. Significance of drug (placebo or indomethacin) and region (anterior or posterior) on hypoxia-mediated increases in CVC were determined by general linear model ANOVA.

Significance of drug (placebo or indomethacin) and vessel (R-VA, L-VA, BA, R-PCA, L-PCA, R-ICA, L-ICA, R-MCA, L-MCA, R-ACA, L-ACA) on hypoxia-mediated increases in CVC were determined by general linear model ANOVA.

Based upon prior research investigating greater relative hypoxic cerebrovascular reactivity in the VA (140), a sample size of n=9 (with an estimated standard deviation of 1.3) at an α= 0.05 was necessary to provide 80% power to detect an expected 50% greater VA relative reactivity compared to the ICA, MCA, and PCA. Additionally, a sample size of n=9 provided >80% power (with an estimated standard deviation of 1.0) to detect an expected 50% reduction in absolute hypoxic VA reactivity associated with COX inhibition (51).

## Results

*Subjects*

Subjects were young, lean, healthy, and free of disease (Table 3.1). All subjects completed both study visits. In 4 of 9 subjects all eleven cerebral vessels of interest were visible with and without indomethacin, during baseline and hypoxia conditions. For the remaining five subjects at least one or more of the eleven cerebral vessels were not visible for the following reasons. One subject had a fetal type PCA-R (originating from the ICA-R) and anatomically did not have a VA-R, while a second subject was anatomically missing a VA-L. Methodologically, indomethacin substantially decreased basal CBF and in some instances, the resultant flow was below the detectable limit of the current methodology. Thus, in a third subject during indomethacin baseline the ACA-L VA-L, PCA-L, and PCA-R were not detectable, in a fourth subject during indomethacin baseline the PCA-R was not detectible, and in a fifth subject during indomethacin baseline the ACA-L was not detectable and in this same subject indomethacin baseline and indomethacin hypoxia the PCA-L was also not detectable (Table 3.2). Considering

that hypoxia, in all cases but one, increased flow with indomethacin above detectable limits, these values were included in the flow analysis.  However, given that indomethacin baseline data was missing in these cases, we were unable to calculate changes in CBF or reactivities in these specific instances.

*Systemic Hemodynamic Responses*

By design, $S_PO_2$ was lower during hypoxia compared to normoxia (main effect, p < 0.001), but not different between indomethacin and placebo.  Heart rate was higher during hypoxia (main effect, $p < 0.001$), but was lower with indomethacin compared to placebo (main effect, $p = 0.02$).  We sufficiently clamped $ETCO_2$, as there was no difference between hypoxia and normoxia, nor was there any difference between indomethacin and placebo.  Systolic blood pressure (SBP), diastolic blood pressure (DBP), MABP, and respiratory rate did not differ by condition or pill.  Table 3.3.

*Basal Effects of Indomethacin*

Total basal CVC was reduced from $663 \pm 64$ ml/min to $367 \pm 28$ ml/min following indomethacin ($p < 0.001$), equating to a $44 \pm 2\%$ reduction in global CVC (Figure 3.3, Table 3.4).  Anterior CVC was reduced from $518 \pm 49$ ml/min to $290 \pm 24$ ml/min ($p < 0.001$) and posterior CVC was reduced from $145 \pm 18$ ml/min to $77 \pm 7$ ml/min ($p < 0.001$), yielding similar relative reductions in CVC of $44 \pm 2\%$ and $45 \pm 3\%$, respectively (Figure 3.3, Table 3.4).  On a vessel-by-vessel basis, the relative decrease in CVC was similar between all vessels (Figure 3.4, Table 3.5).  Expressing data as flow yielded similar conclusions (Table 3.5).

Vessel CSA was reduced following indomethacin compared to placebo (Table 3.5; main effect, $p < 0.001$).  The relative decrease in CSA of both ICAs following indomethacin was

greater than that of the PCA-L and VA-R (Table 3.5; $p = 0.004$), but similar across all other vessels. Expressing data as diameter yielded similar conclusions (Table 3.5).

Mean blood flow velocity was reduced following indomethacin compared to placebo (Table 3.5; main effect, $p < 0.001$). The relative decrease in vessel mean velocity following indomethacin was greater in the VA-R compared to ICA-R (Table 3.5; $p = 0.027$), but was similar across all other vessels.

*Hypoxic Cerebral Vasodilation*

Hypoxia increased CVC ($p < 0.01$) and the relative increase in CVC was similar between vessels and regions. Expressing data as flow yielded similar conclusions. This is in agreement with data previously collected in our lab (66). Hypoxia-mediated changes in relative vessel CSA and relative mean velocity were also similar between vessels.

*Total and Regional Contribution of COX to Hypoxic Cerebral Vasodilation*

To explore the role of COX in a region-specific manner, we calculated total CBF (ICAs + BA), anterior CBF (ICAs) and posterior CBF (BA). Total CVC was greater during hypoxia (main effect, $p = 0.044$) and was lower with indomethacin (Figure 3.5, Table 3.4; main effect, $p < 0.001$). The relative increase in total CVC during hypoxia was greater with indomethacin (Figure 3.7, Table 3.6; $15 \pm 3\%$ vs $28 \pm 4\%$; $p = 0.028$). Similarly, relative total CVC reactivity was greater following indomethacin (Figure 3.7, Table 3.6; $0.8 \pm 0.2$ vs $2.0 \pm 0.3$; $p = 0.005$). Expressing data as flow yielded similar conclusions with the exception that the greater relative increase in total CBF with indomethacin trended towards significance (Figures 3.5 and 3.6, Table 3.6; $19 \pm 3\%$ vs $29 \pm 4\%$; $p = 0.069$).

Comparing anterior and posterior circulations, CVC was greater during hypoxia (main effect, $p < 0.05$) and was lower with indomethacin (Figure 3.5, Table 3.6; main effect, $p < 0.001$). The hypoxia-mediated change in relative CVC was greater with indomethacin (main effect, $p = 0.002$) and not different between regions ($p = 0.974$). Likewise, relative hypoxic CVC reactivity was greater with indomethacin (main effect, $p < 0.001$) and was not different between regions (Figure 3.7, Table 3.6). Expressing data as flow yielded similar conclusions (Figures 3.5 and 3.6, Table 3.6).

*Vessel Specific Contribution of COX to Hypoxic Cerebral Vasodilation*

CVC was greater during hypoxia compared to normoxia (main effect, $p < 0.001$) and was lower with indomethacin compared to placebo (Figure 3.8, Table 3.7; main effect, $p < 0.001$). The hypoxia-mediated increase in relative CVC was greater with indomethacin (main effect; $p < 0.001$) and was similar between all vessels (Figure 3.10, Table 3.7). Similarly, relative CVC reactivity was greater with indomethacin (main effect, $p < 0.001$), but was lower in the PCA-L compared to the MCA-L and ACA-R (Figure 3.10, Table 3.7; main effect, $p = 0.023$). Expressing data as flow yielded similar conclusions with the exception that the hypoxia-mediated increase in relative flow was greater in the ACA-R compared to the PCA-L (Figure 3.9, Table 3.7; main effect, $p = 0.030$)

Vessel CSA was greater during hypoxia compared to baseline (main effect, $p = 0.014$) and was reduced following indomethacin compared to placebo (Table 3.7; main effect, $p < 0.001$). The hypoxia-mediated changes in relative vessel CSA were greater with indomethacin compared to placebo (main effect, $p = 0.006$) but not different between vessels (Figure 3.11, Table 3.7). Expressing data as diameter yielded similar conclusions (Table 3.5).

Mean velocity was greater during hypoxia compared to baseline (main effect, $p < 0.001$) and lower following indomethacin compared to placebo (Table 3.7; main effect, $p < 0.001$).  The hypoxia-mediated increase in relative mean velocity was not different between indomethacin and placebo and not different between vessels (Figure 3.11, Table 3.7).

## Discussion

The aim of this investigation was to examine the region-specific increases in CVC and the region-specific contribution of COX to hypoxic cerebral vasodilation.  We directly quantified CBF during hypoxia, while clamping ETCO$_2$, concurrently in eleven major cerebral arteries that comprise the anterior and poster macrovascular circulations.  This is the first study to directly quantify the region-specific contribution of COX to the maintenance of basal CVC and the region-specific contribution of COX to hypoxia-mediated increases in CVC.

The novel findings of this study are as follows:  1) indomethacin decreased basal CVC uniformly across the cerebral circulation (anterior vs posterior) by $44 \pm 2\%$, 2) hypoxia-mediated increases in relative CVC are not region-specific and are augmented with indomethacin, 3) hypoxia-mediated increases in relative CSA are augmented with indomethacin, 4) Hypoxia-mediated increases in relative mean velocity are not altered with indomethacin, 5) and cerebral macrovascular responses to indomethacin are not region-specific.  Thus, we conclude that COX inhibition uniformly reduces resting CVC by $44 \pm 2\%$ and contrary to our hypothesis, COX inhibition augments hypoxic cerebral vasodilation, which is not different across the cerebral circulation

*Basal Effects of Indomethacin*

We demonstrate that indomethacin decreases basal CVC largely uniformly across the cerebral circulation by $44 \pm 2\%$. This decrease in basal CVC with indomethacin is substantially greater than the $31 \pm 2\%$ and $32 \pm 4\%$ reduction in middle cerebral artery velocity (MCAv) shown in the sex-specific and menstrual phase-specific investigations of Aim 1 and Aim 2. Collectively, previous investigations report a range of reductions from 25% - 41%, with an overall average MCAv reduction of ~30% with indomethacin (8, 31, 49-51, 54, 79). In the current investigation, MCAv was reduced by $35 \pm 2\%$, which is in agreement with these prior velocity measures. Given the limitations of using MCAv as an index of CBF, specifically the assumption of constant vessel diameter (our data indicate indomethacin decreases vessel diameter), these prior investigations may have under-estimated the contribution of COX to the maintenance of resting CBF.

There is one study to date (51), utilizing duplex ultrasound, that quantified blood flow through the ICA and VA prior to and following indomethacin administration. In this study, indomethacin decreased blood flow in both the ICA and VA by ~32%, which is less than the $44 \pm 2\%$ and $38 \pm 5\%$ shown in the ICA and VA of the current study. The most likely explanations for this discrepancy includes greater variability in duplex ultrasound measures of blood flow ($\pm 13\% - 18\%$) compared to our 4D Flow MRI measures ($\pm 2\% - 5\%$). Additionally, a longer hypoxia protocol was utilized (10-minutes post steady-state vs 5 minutes post-steady state), $S_PO_2$ values were not reported (hypoxic stimulus may have been different), and there was a slight, non-significant increase in perfusion pressure with indomethacin. All of which may contribute to the differential findings.

Our data demonstrate that indomethacin reduces both CBF velocity and vessel CSA. By not accounting indomethacin-mediated decreases in CSA, the indomethacin-mediated reductions

in CBF velocity of prior investigations may underestimate the contribution of COX to the maintenance of basal CBF.

*Hypoxic Cerebral Vasodilation*

To date, investigations exploring region-specific, hypoxia-mediated increases in CBF are equivocal. Some studies suggest that the posterior circulation demonstrates greater hypoxia induced increases in CBF compared to the anterior circulation (11, 16, 80, 99, 140). It has been postulated that this heterogeneous CBF distribution during hypoxia may serve to preserve vital autonomic function associated with older areas of the brain at the expense of cognitive function (11). In contrast, others demonstrate that there are no regional differences in hypoxic cerebral vasodilation (29, 51, 53, 99, 104, 105), or that the anterior circulation may even have greater hypoxic sensitivity than the poster circulation (58, 77). The uncertainty of region-specific increases in CBF is highlighted by findings from a single research group, utilizing a similar protocol across studies that have yielded conflicting results. For example, during sequential 15-minute isocapnic hypoxia bouts eliciting $S_PO_2$ of ~90%, ~80%, and ~70 % respectively, the relative increase in VA blood flow was found to be ~50% greater than that of the ICA, MCA, or PCA at $S_PO_2$ ~70 % (140). However, this same research group also reports that 10-minutes of isocapnic hypoxia eliciting an $S_PO_2$~80%, showed no difference in relative cerebrovascular reactivity between the MCA and PCA, as well as no difference in reactivity when comparing the ICA and VA (51). Similarly, they demonstrate that sequential 5-minute stages of isocapnic hypoxia eliciting an $S_PO_2$ ~90%, ~80%, and ~70% results in no difference in relative cerebrovascular reactivity between the MCA and PCA, and no difference in reactivity between the ICA and VA (53).

Considering these equivocal findings it is important to note that many hypoxia studies utilize varying degrees of hypoxic severity and often do not clamp arterial $CO_2$, making it difficult to isolate the effects of hypoxia from hyperventilation-induced hypocapnia. Considering that CBF responses during hypoxia are dependent upon the balance between hypoxic vasodilation and hyperventilation induced hypocapnic vasoconstriction, variable hypoxic severities and $CO_2$ responses limit interpretation. Data indicate that differences in hypoxic severity may alter region-specific hypoxic cerebral vasodilation (140), while clamping arterial $CO_2$ is especially important given regional specificity in hypercapnic vasodilation (56, 119, 125). Additional limitations of hypoxia studies include utilizing CBF velocity as an index of CBF and quantifying hypoxic CBF responses in a limited number of cerebral arteries.

In the current investigation, we examined CBF responses to hypoxia in multiple anterior and posterior arteries, while clamping $ETCO_2$ in order to isolate the effects of hypoxia from hyperventilation-induced hypocapnia. We demonstrate that there are no regional differences in hypoxic cerebral vasodilation at a hypoxic severity eliciting an $S_PO_2 = 81 \pm 1\%$. These results are in agreement with prior findings from our lab (67) and others (29, 51, 53, 99, 104, 105). Thus, if there are regional differences in hypoxic cerebral vasodilation it may only be evident during more severe levels of hypoxia (i.e. $S_PO_2 = 70\%$) (140) or during poikilocapnic hypoxia (16).

*Contribution of COX to Hypoxic Cerebral Vasodilation*

The role of COX in mediating hypoxic cerebral vasodilation is uncertain. *In vivo* animal data indicate no contribution of COX during severe hypoxia (23, 78, 118), while *in vitro* data suggest a substantial contribution of COX (37). Likewise, human data are equivocal as investigations in young, healthy adults suggest that COX may mediate hypoxic cerebral

vasodilation (51), while others suggest no role of COX in mediating hypoxic cerebral vasodilation (31, 49). These human studies are limited in that they only measured CBF velocity in a single cerebral vessel, the MCA. Therefore, any potential region-specific contribution of COX was not investigated.

There is only one study in humans that has proposed vessel-specific differences in the contribution to COX to hypoxic cerebral vasodilation (51). In this study, COX was shown to mediate hypoxic vasodilation in both the MCA and VA, but not in the ICA or PCA (51), suggesting that COX signaling may be region-specific. With this as rationale, we hypothesized that COX-mediated hypoxic cerebral vasodilation would be region specific. Contrary to our hypothesis, we demonstrate that the contribution of COX is not region-specific and that COX does not contribute to hypoxic vasodilation. As alluded to earlier, most hypoxia studies investigating COX-mediated hypoxic vasodilation have utilized transcranial Doppler ultrasound (TCD) to quantify MCAv. Although TCD is a useful tool it is limited in the assumption of constant vessel diameter, which has been shown to change during hypoxia (66, 144). Given that flow is proportional to radius raised to the fourth power, small changes in diameter can have profound impacts on CBF and ultimately interpretation of data. Despite this limitation, most of the prior TCD studies (31, 49), including our findings from Aim 1 and Aim 2 are in agreement that COX does *not* mediate hypoxic cerebral vasodilation. Interestingly, not only does COX not appear to facilitate hypoxic vasodilation, based upon our current findings COX appears to be restraining vasodilation as indicated by the greater relative increase in CVC with indomethacin.

The one study to date suggesting a region-specific contribution of COX in the MCA and VA utilizing a combination of TCD and duplex ultrasound (allowing the quantification of vessel diameter) has several limitations (51). First, the contribution of COX in MCA was determined

by using MCAv, which does not account for potential hypoxia induced increases in CSA or

indomethacin mediated decreases in CSA. Second, although a decrease in absolute

cerebrovascular reactivities to hypoxia was found in both the MCA and VA following

indomethacin, there was no reduction in relative reactivities. Given the widely different blood

flows between vessels and large reduction in CBF associated with indomethacin, relative

reactivity seems to be the most appropriate form of data expression (17). Additionally,

variability is greater in duplex ultrasound measures of CBF when compared to CBF measures

provided in the current study. This study also utilized a longer hypoxia protocol, did not report

$S_PO_2$ values, and indomethacin caused a slight, albeit insignificant increase in perfusion pressure,

all of which have the potential to explain the discord between studies.

*Differential Contribution of Cyclooxygenase*

COX-mediated CBF regulation appears to be stimulus specific. It has been well

characterized that COX contributes to the maintenance of basal CBF, as indomethacin facilitates

an overall ~30% reduction in MCAv (8, 31, 49-51, 54, 79). This is in agreement with both Aims

1 and 2 of this dissertation, which also showed $31 \pm 2\%$ and $32 \pm 4\%$ reductions in MCAv with

indomethacin. Likewise, the current investigation demonstrated a significant contribution of

COX to the maintenance of basal CBF, albeit a larger contribution as indomethacin reduced

resting CVC by $44 \pm 2\%$. We provide evidence that indomethacin decreases vessel CSA,

suggesting prior studies quantifying blood flow velocity may underestimate the contribution of

COX to resting cerebral perfusion.

Unlike basal CBF regulation, most investigations have found little evidence that COX

mediates hypoxic cerebral vasodilation. To date there have been two studies suggesting COX

does not mediate hypoxic cerebral vasodilation (31, 49), and one study suggesting vessel-specific

contributions of COX during hypoxia (51). Both Aim 1 and Aim 2 of this dissertation support

the notion that COX is not obligatory for hypoxic cerebral vasodilation. Unlike most previous

studies that almost exclusively measured blood flow velocity to quantify the contribution of

COX to hypoxia-mediated vasodilation, in Aim 3 we directly quantified CBF. Findings from

Aim 3 provide evidence that not only does COX not mediate hypoxic cerebral vasodilation, but

appears to be restraining hypoxic mediated increases in CVC.

Considered collectively, COX appears to contribute to the maintenance of basal CBF but

paradoxically restrains hypoxia-mediated increases in CBF. The simplest explanation for these

opposing roles of COX is that at rest COX mediates the production of the vasodilatory

prostaglandin $PGI_2$ or prostacyclin, while during hypoxia COX signaling shifts to support greater

production of the vasoconstrictor prostaglandin $TXA_2$ or thromboxane (108). A second

possibility is that COX normally contributes to the maintenance of basal CBF and does not

contribute to hypoxic cerebral vasodilation, but restrains an additional vasodilator mechanism

such as nitric oxide. In humans, nitric oxide has been shown to potentially mediate hypoxic

cerebral vasodilation (136). Interestingly, COX inhibition increases the synthesis of nitric oxide

in isolated human saphenous veins (6) and in the peripheral circulation of humans COX restrains

the nitric oxide portion of β-adrenergic receptor mediated vasodilation (82). Previous data from

our lab also suggests that COX restrains hypoxic vasodilation, albeit in individuals with

metabolic syndrome (49), while data in the human forearm also suggest that COX restrains

acetylcholine-mediated vasodilation (131). A third possibility is that the reduction in basal CBF

with COX inhibition causes a significant reduction in oxygen delivery. This reduced oxygen

delivery when combined with hypoxia and increased oxygen extraction may further activate

existing or additional compensatory vasodilator mechanisms to abrogate the further reduction in

oxygen delivery. For example, it is known that release of the vasodilator adenosine tri-phosphate exhibits a strong relationship with deoxygenated hemoglobin (46, 57) which increases as oxygen extraction increases. Although the current study design did not enable us to investigate these possibilities, future investigations exploring these possibilities may provide additional insight.

*Cerebral Oxygen Delivery*

The brain comprises ~2% of body mass but accounts for ~20% of total body oxygen ($O_2$) consumption at rest (42). Given the high metabolic rate and reliance on oxidative metabolism, regulation of CBF and maintenance of oxygen delivery is of utmost importance. During hypoxia CBF increases in proportion to the severity of the hypoxic stimulus, ensuring adequate oxygen delivery (52) as cerebral metabolic rate ($CMRO_2$) remains unchanged, at least in humans (68, 141). For the current investigation, we calculated cerebral oxygen delivery ($CDO_2$) with the following equations:

$$\textbf{CDO}_\textbf{2}\textbf{ (ml/min)} = \text{total CBF (ml/min)} \cdot CaO_2 \text{ (ml/dl)} / 100$$

$$\textbf{CaO}_\textbf{2}\textbf{ (ml/dl)} = [Hb] \cdot 1.36 \cdot [\% \ SpO_2 / 100] + [0.003 \cdot PaO_2]$$

Where

$CaO_2$ is the arterial oxygen content, $PaO_2$ is the arterial dissolved $O_2$, Hb is the arterial hemoglobin concentration, $SaO_2$ is the arterial hemoglobin saturation, 1.36 is the affinity for $O_2$ to hemoglobin, and 0.003 is the solubility of $O_2$ in blood. For the purposes of this calculation, average hemoglobin was assumed to be 14 g/dL, and arterial blood oxygen during normoxia was assumed to be 100 mmHg while during hypoxia it was assumed to be 45 mmHg (123).

During placebo normoxia $CDO_2$ = 119 ml/min, while during placebo hypoxia $CDO_2$ = 115 ml/min, equating to minimal 3% reduction in $CDO_2$ during hypoxia. CBF was significantly reduced with indomethacin, as was $CDO_2$. During indomethacin normoxia $CDO_2$ = 65 ml/min,

while during indomethacin hypoxia $CDO_2 = 71$ ml/min, equating to 9% increase in $CDO_2$ during hypoxia with indomethacin. During placebo conditions, the increase in CBF during hypoxia resulted in maintenance of $CDO_2$ comparable to that of normoxia.

With indomethacin, there was a significant reduction in $CDO_2$ during normoxic conditions compared to placebo (~45%). However, indomethacin induced a greater relative increase in CBF during hypoxia compared to placebo that resulted in an increase in $CDO_2$ of 9% from baseline. Although indomethacin resulted in a greater relative increase in CBF and $CDO_2$ during hypoxia, $CDO_2$ remained much lower than that during hypoxia placebo conditions (~38% less). Thus, indomethacin must be associated with a greater oxygen extraction (OEF) or a decrease in $CMRO_2$. Available evidence in humans suggests that indomethacin increases in OEF while $CMRO_2$ remains unchanged (5, 18, 39). Unfortunately, we were unable to measure cortical oxygen saturation and therefore were unable to determine if an increase in OEF compensated for the reduction in $CDO_2$ with indomethacin.

Available evidence suggests that at rest, OEF is in the range of 30 - 35% (36, 114, 134), thus during placebo normoxia assuming a $CDO_2 = 119$ ml/min and an $OEF = 35\%$, cerebral oxygen consumption $(CMRO_2) = 42$ ml/min. This is an agreement with previously published values (20). Given the large reduction in $CDO_2$ with indomethacin and no change in $CMRO_2$ (5, 18, 39), OEF can be expected in increase from 35% during placebo to ~65% with indomethacin. It has been shown that OEF can increase up to ~78% in humans prior to the onset of cerebral ischemia (91), thus making it unlikely that indomethacin during control or hypoxia resulted in ischemia.

*Alternative Data Expression*

To account for potential differences in perfusion pressure, cerebrovascular conductance was calculated and presented (CVC = CBF*100/MABP). To account for potential differences in baseline blood flow associated with vessel and indomethacin, data was expressed as percent change in CVC from baseline, which is considered the most accurate representation of changes in vascular tone (17, 76, 88). However, it is important to note that expressing data as absolute changes in CBF and CVC results in differing conclusions. Specifically, expressing data in absolute terms demonstrate that COX does not mediate hypoxic cerebral vasodilation, whereas expressing data in relative terms indicate that COX inhibition augments hypoxic cerebral vasodilation. It is our belief and others (17, 76, 88) that relative data expression is most appropriate. Regardless of data expression, COX does not appear to mediate hypoxic cerebral vasodilation.

*Limitations*

This is the first study to directly quantify the region-specific contribution of COX to the maintenance of basal CVC and hypoxia-mediated increases in CVC. We quantified CBF concurrently in eleven cerebral macrovascular arteries while tightly controlling experimental conditions. In light of these strengths, there are some limitations worthy of consideration. First, in five subjects at least one or more of the eleven cerebral vessels were not visible. One subject was anatomically missing a VA-R, while a second subject was anatomically missing a VA-L. Additionally, the large decrease in resting CBF with indomethacin in some vessels was substantial enough that it fell below the detectable limit of the current methodology. Thus, during the indomethacin baseline trial in a third subject the ACA-L VA-L, PCA-L, and PCA-R were not detectable in a third subject, in a fourth subject the PCA-R was not detectable, and in a fifth subject the ACA-L and PCA-L was not detectable. In the fifth subject, the PCA-L was also

not detectable during the indomethacin hypoxia trial (Table 3.2). Despite these limitations we were able to collect a complete data set (n = 9) on five cerebral arteries including ICAs, MCAs, and BA which is a more comprehensive investigation than has been conducted to date. In the remaining vessels, we analyzed a minimum of seven subjects for each vessel which has provided valuable information pertaining to the region-specific role if COX in the maintenance of basal CBF as well as hypoxia-mediated increases in CBF.

Second, our study was powered to detect an expected 50% greater VA relative reactivity compared to the ICA, MCA, and PCA. This power analysis was based upon prior research eliciting and $S_PO_2 = 70\%$ (140). Given the less severe hypoxia in the current investigation ($S_PO_2 = 81 \pm 1\%$) and inability to quantify CBF in all vessels, for all subjects, under all conditions, we may have been underpowered to detect regional differences in hypoxic cerebral vasodilation, as well as region-specific contributions of COX. Based upon the current data a sample size of 7 provides 80% power to detect an expected 17% increase in relative CVC during hypoxia. A sample size of 29 would provide 80% power to detect a 50% greater increase in relative CVC during hypoxia between vessels, as well as 85% power to detect a 50% greater relative increase CVC between vessels during hypoxia with indomethacin. Despite this limitation, we provide novel evidence that indomethacin restrains hypoxic cerebral vasodilation which is not different across the cerebral circulation.

Third, we did not measure cortical oxygen saturation and therefore were unable to determine if an increase in OEF compensated for the reduction in $CDO_2$ with indomethacin. Available evidence points towards an indomethacin induced increase in OEF however, we are unable to confirm this in the current investigation.

Fourth, although indomethacin augmented the relative increase in CVC as well as the relative increase in CSA, we were unable to determine if the augmented change in vessel CSA was due to the direct effects of indomethacin on the macrovasculature or elevations in shear stress from downstream vasodilation (due to the low temporal resolution of MRI technology).

Lastly, we utilized an absolute dose of indomethacin and did not measure the concentration of plasma COX metabolites to demonstrate adequate COX inhibition. Our absolute (100 mg) and relative dose (1.4 mg/kg) of indomethacin is greater than (54, 127) or similar (31, 51) to that previously published. In a prior study in our lab, we used an identical absolute dose of indomethacin that was shown to result in a large decrease in circulating COX metabolites (49). Considered collectively, and with our observed $44 \pm 2\%$ reduction in basal CVC and $35 \pm 2\%$ reduction in MCAv, we are confident we effectively inhibited COX. It is important to note that plasma concentrations of circulating COX metabolites are only reduced and not abolished (49). With this in mind, we may still be underestimating the contribution of COX to CBF regulation.

*Summary and Conclusion*

The novel findings of the study are as follows: 1) indomethacin decreased basal CVC similarly across the cerebral circulation by $44 \pm 2\%$, 2) hypoxia-mediated increases in relative CVC are not region-specific and are augmented with indomethacin, 3) hypoxia-mediated increases in relative CSA are augmented with indomethacin, 4) hypoxia-mediated increases in relative mean velocity are not altered with indomethacin, and 5) cerebral macrovascular responses to indomethacin are not region-specific. Thus, contrary to our hypothesis we conclude that COX inhibition augments hypoxic cerebral vasodilation uniformly across the cerebral circulation.

## Acknowledgements

## Disclosure

The authors declare no conflicts of interest.

Tables

**Table 3.1.** Subject Characteristics

| | |
|---|---|
| n | 9 (5 male, 4 female) |
| Age (yrs) | 28 ± 1 |
| Height (cm) | 174 ± 2 |
| Weight (kg) | 70 ± 3 |
| BMI (kg/m$^2$) | 23 ± 1 |
| Waist (cm) | 81 ± 2 |
| Hip (cm) | 98 ± 1 |
| Glucose (mg/dL) | 72 ± 2 |
| Total Cholesterol (mg/dL) | 155 ± 11 |
| HDL (mg/dL) | 57 ± 5 |
| LDL (mg/dL) | 89 ± 17 |
| Triglycerides (mg/dL) | 88 ± 18 |
| SBP (mmHg) | 119 ± 2 |
| DBP (mmHg) | 74 ± 2 |
| MAP (mmHg) | 89 ± 2 |
| Physical Activity (Kcal/wk) | 2287 ± 619 |
| Indo Dose (mg/kg) | 1.4 ± 0.1 |

Values are means ± SE; BMI, body mass index; HDL, high density lipoprotien; LDL, Low density lipoprotein; SBP, systolic blood pressure, DBP, diastolic blood pressure; MAP, mean arterial blood pressure; Indo, indomethacin.

**Table 3.2.** Vessels missing from analysis

| Subject | Pill | Condition | Vessel | Notes |
|---------|------|-----------|--------|-------|
| 1 | Placebo | Baseline | VA-R | |
| | Placebo | Hypoxia | VA-R | Anatomically missing VA-R and |
| | Indo | Baseline | VA-R | PCA-R of fetal origin |
| | Indo | Hypoxia | VA-R | |
| | | | | |
| 2 | Placebo | Baseline | VA-L | |
| | Placebo | Hypoxia | VA-L | Anatomically missing VA-L |
| | Indo | Baseline | VA-L | |
| | Indo | Hypoxia | VA-L | |
| | | | | |
| 3 | Indo | Baseline | ACA-L | |
| | Indo | Baseline | VA-L | |
| | Indo | Baseline | PCA-L | |
| | Indo | Baseline | PCA-R | |
| | | | | |
| 4 | Indo | Baseline | PCA-R | |
| | | | | |
| 5 | Indo | Baseline | ACA-L | |
| | Indo | Baseline | PCA-L | |
| | Indo | HX | PCA-L | |

Indo, indomethacin; L, left; R, right; ACA, anterior cerebral artery; VA, vertebral artery; PCA, posterior cerebral artery.

**Table 3.3**. Systemic hemodynamic responses

| | Placebo | | | | Indo | | | |
|---|---|---|---|---|---|---|---|---|
| | Baseline | | Hypoxia | | Baseline | | Hypoxia | |
| Heart Rate (beats/min) * † | 66 ± 3 | | 82 ± 3 | | 58 ± 3 | | 75 ± 4 | |
| SBP (mmHg) | 130 ± 4 | | 131 ± 3 | | 128 ± 3 | | 129 ± 3 | |
| DBP (mmHg) | 80 ± 3 | | 84 ± 3 | | 80 ± 3 | | 80 ± 3 | |
| MAP (mmHg) | 97 ± 3 | | 100 ± 3 | | 96 ± 3 | | 96 ± 3 | |
| $S_PO_2$ (%) * | 98 ± 0 | | 80 ± 1 | | 97 ± 0 | | 83 ± 1 | |
| $ETCO_2$ (mmHg) | 41 ± 1 | | 39 ± 1 | | 40 ± 1 | | 39 ± 1 | |
| Respiratory Rate (bpm) | 13 ± 1 | | 15 ± 1 | | 14 ± 1 | | 15 ± 1 | |

Values are presented as mean ± SE.  SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; $S_PO_2$, pulse oximetry oxygen saturation; $ETCO_2$, end-tidal carbon dioxide. * main effect of condition, † main effect of pill; $p < 0.05$.

**Table 3.4.** Regional cerebrovascular responses to hypoxia with and without indomethacin

| | Anterior | | | Posterior | | | Total | | |
|---|---|---|---|---|---|---|---|---|---|
| **Flow (ml/min) †Ω‡** | | | | | | | | | |
| Placebo | 489 | ± 32 | A | 137 | ± 14 | C | 626 | ± 43 | |
| Indo | 275 | ± 19 | B | 73 | ± 6 | C | 348 | ± 22 | |
| | | | | | | | | | |
| **Δ Flow (ml/min) †Ω** | | | | | | | | | |
| Indo | 214 | ± 17 | | 64 | ± 9 | | 278 | ± 25 | |
| | | | | | | | | | |
| **% Δ Flow Ω** | | | | | | | | | |
| Indo | 44 | ± 2 | | 45 | ± 2 | | 44 | ± 2 | |
| | | | | | | | | | |
| **CVC (100 ml/min/mmHg) †Ω‡** | | | | | | | | | |
| Placebo | 518 | ± 49 | A | 145 | ± 18 | C | 663 | ± 64 | |
| Indo | 290 | ± 24 | B | 77 | ± 7 | C | 367 | ± 28 | |
| | | | | | | | | | |
| **Δ CVC (ml/min) †Ω** | | | | | | | | | |
| Indo | 228 | ± 27 | | 68 | ± 12 | | 297 | ± 38 | |
| | | | | | | | | | |
| **% Δ CVC Ω** | | | | | | | | | |
| Indo | 44 | ± 2 | | 45 | ± 3 | | 44 | ± 2 | |

Values are presented as mean ± SE. Regional cerebrovascular responses (anterior vs posterior) were analyzed separately from the total response but are presented together. CVC, cerebral vascular conductance; Indo, indomethacin. † main effect of region (anterior vs posterior); Ω main effect of pill (different from zero); ‡ region by pill interaction, means that have but do not share a letter are significantly different; $p < 0.01$.

**Table 3.5.** Vessel specific cerebrovascular responses with and without indomethacin

| | ICA Left | ICA Right | MCA Left | MCA Right | ACA Left | ACA Right | VA Left | VA Right | BA - | PCA Left | PCA Right |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Flow (ml/min)†Ω‡** | | | | | | | | | | | |
| Placebo | 236 ± 21 A | 254 ± 16 A | 147 ± 9 B | 145 ± 10 B | 69 ± 13 EF | 92 ± 8 CDE | 89 ± 15 CDE | 83 ± 7 DEF | 137 ± 14 BC | 53 ± 4 EF | 51 ± 4 EF |
| Indo | 129 ± 13 BCD | 146 ± 9 B | 81 ± 5 DEF | 84 ± 6 DEF | 46 ± 9 EF | 51 ± 5 EF | 58 ± 7 EF | 48 ± 5 EF | 73 ± 6 EF | 34 ± 2 F | 32 ± 2 F |
| Δ Flow † | -107 ± 11 D | -108 ± 9 D | -66 ± 5 C | -60 ± 6 BC | -31 ± 8 AB | -41 ± 4 ABC | -38 ± 9 ABC | -35 ± 3 ABC | -64 ± 9 BC | -21 ± 4 A | -21 ± 3 A |
| % Δ Flow | -45 ± 2 | -42 ± 2 | -45 ± 2 | -41 ± 2 | -38 ± 7 | -45 ± 2 | -36 ± 7 | -42 ± 2 | -45 ± 2 | -37 ± 5 | -39 ± 3 |
| **CVC (100 ml/min/mmHg) †Ω‡** | | | | | | | | | | | |
| Placebo | 249 ± 26 A | 270 ± 27 A | 155 ± 13 B | 153 ± 14 BC | 72 ± 14 EF | 97 ± 11 BCDEF | 95 ± 18 BCDEF | 85 ± 7 CDEF | 145 ± 18 BCD | 56 ± 5 F | 53 ± 5 F |
| Indo | 135 ± 14 BCDE | 155 ± 14 B | 85 ± 5 DEF | 89 ± 7 CDEF | 47 ± 9 F | 54 ± 6 F | 61 ± 8 F | 50 ± 5 F | 77 ± 7 EF | 35 ± 3 F | 33 ± 2 F |
| Δ CVC † | -114 ± 14 CD | -115 ± 14 D | -70 ± 8 BC | -64 ± 8 AB | -33 ± 9 AB | -43 ± 6 AB | -42 ± 12 AB | -35 ± 4 AB | -68 ± 12 AB | -23 ± 5 A | -22 ± 5 A |
| % Δ CVC | -45 ± 2 | -42 ± 2 | -45 ± 2 | -41 ± 3 | -38 ± 7 | -44 ± 2 | -35 ± 8 | -41 ± 3 | -45 ± 3 | -37 ± 5 | -38 ± 3 |
| **CSA (mm²) †Ω‡** | | | | | | | | | | | |
| Placebo | 16.6 ± 1.7 A | 17.6 ± 1.7 A | 6.3 ± 0.3 CD | 6.1 ± 0.3 CD | 4.5 ± 0.4 CD | 5.0 ± 0.3 CD | 5.5 ± 0.5 CD | 5.8 ± 0.4 CD | 7.8 ± 0.7 C | 4.0 ± 0.2 D | 4.0 ± 0.2 D |
| Indo | 11.8 ± 1.2 B | 12.7 ± 1.0 B | 5.4 ± 0.2 CD | 5.4 ± 0.2 CD | 4.0 ± 0.4 CD | 4.2 ± 0.2 CD | 5.2 ± 0.4 CD | 5.5 ± 0.5 CD | 6.4 ± 0.5 CD | 3.8 ± 0.4 D | 3.4 ± 0.1 D |
| Δ CSA † | -4.7 ± 0.9 B | -4.9 ± 0.8 B | -1 ± 0.2 A | -0.7 ± 0.2 A | -0.6 ± 0.2 A | -0.7 ± 0.2 A | -0.7 ± 0.2 A | -0.3 ± 0.3 A | -1.4 ± 0.4 A | -0.3 ± 0.4 A | -0.5 ± 0.2 A |
| % Δ CSA † | -27.4 ± 4 B | -27 ± 3 B | -14 ± 3 AB | -11 ± 2 AB | -12 ± 4 AB | -14 ± 2.9 AB | -11 ± 4 AB | -6 ± 5 A | -17 ± 3 AB | -5 ± 10 A | -11 ± 6 AB |
| **Mean Velocity (cm/s) †Ω‡** | | | | | | | | | | | |
| Placebo | 26 ± 2 CDE | 26 ± 1 CDE | 40 ± 2 AB | 41 ± 2 A | 26 ± 3 CDE | 32 ± 2.1 BC | 27 ± 2 CDE | 25 ± 1 CDEF | 31 ± 2 CD | 23 ± 1 DEFG | 22 ± 1 EFG |
| Indo | 20 ± 2 EFG | 21 ± 1 EFG | 26 ± 2 CDE | 27 ± 2 CDE | 19 ± 2 EFG | 21 ± 1.3 EFG | 19 ± 1 EFG | 16 ± 1 G | 20 ± 1 EFG | 16 ± 1 G | 16 ± 1 FG |
| Δ Mean velocity † | -6 ± 1 AB | -5 ± 1 A | -14 ± 1 D | -14 ± 1 CD | -7 ± 2 ABC | -11 ± 1 BCD | -8 ± 2 ABC | -9 ± 1 ABCD | -10 ± 1 BCD | -7 ± 1 AB | -7 ± 1 AB |
| % Δ Mean | -24 ± 4 AB | -20 ± 4 A | -35 ± 2 AB | -34 ± 3 AB | -29 ± 7 AB | -35 ± 1.8 AB | -28 ± 5 AB | -38 ± 3 B | -34 ± 3 AB | -32 ± 3 AB | -30 ± 5 AB |

Values are presented as mean ± SE. ICA, internal carotid artery; MCA, middle cerebral artery; ACA, anterior cerebral artery; VA, vertebral artery; BA, basilar artery; PCA, posterior cerebral artery; CVC, cerebral vascular conductance; CSA, cross sectional area; Indo, indomethacin. † Main effect of vessel, means that do not share a letter within a given variable are significantly different; Ω main effect of pill; ‡ vessel x pill interaction, means that do not share a letter within a given variable are significantly different; $p < 0.05$.

**Table 3.6.** Total and regional cerebrovascular responses to hypoxia with and without indomethacin

| | Anterior | Posterior | Total |
|---|---|---|---|
| **Flow (ml/min) †Ω§‡** | | | |
| Placebo | | | |
| *Baseline* | 489 ± 32 | 137 ± 14 | 626 ± 43 |
| *Hypoxia* | 586 ± 47 | 162 ± 20 | 748 ± 65 |
| Indo | | | |
| *Baseline* | 275 ± 19 | 73 ± 6 | 348 ± 22 |
| *Hypoxia* | 352 ± 25 | 94 ± 6 | 447 ± 29 |
| | | | |
| **Δ Flow †** | | | |
| Placebo | 97 ± 20 | 25 ± 8 | 122 ± 27 |
| Indo | 77 ± 11 | 21 ± 4 | 99 ± 14 |
| | | | |
| **% Δ Flow Ω** | | | |
| Placebo | 19 ± 3 | 17 ± 4 | 19 ± 3 |
| Indo | 28 ± 3 | 31 ± 7 | 29 ± 4 |
| | | | |
| **Flow Reactivity (Δflow/ΔS$_P$O$_2$) †** | | | |
| Placebo | 5.3 ± 1.0 | 1.4 ± 0.3 | 6.7 ± 1.2 |
| Indo | 5.6 ± 0.9 | 1.5 ± 0.3 | 7.1 ± 1.2 |
| | | | |
| **% Flow Reactivity (%Δflow/ΔS$_P$O$_2$) Ω** | | | |
| Placebo | 1.1 ± 0.2 | 0.9 ± 0.2 | 1.0 ± 0.2 |
| Indo | 2.0 ± 0.3 | 2.2 ± 0.5 | 2.1 ± 0.3 |
| | | | |
| **CVC (100 ml/min/mmHg) †Ω‡ǂ** | | | |
| Placebo | | | |
| *Baseline* | 518 ± 49 | 145 ± 18 | 663 ± 64 |
| *Hypoxia* | 600 ± 63 | 166 ± 24 | 766 ± 85 |
| Indo | | | |
| *Baseline* | 290 ± 24 | 77 ± 7 | 367 ± 28 |
| *Hypoxia* | 369 ± 30 | 99 ± 8 | 468 ± 36 |
| | | | |
| **Δ CVC †** | | | |
| Placebo | 82 ± 21 | 21 ± 8 | 103 ± 28 |
| Indo | 79 ± 13 | 22 ± 4 | 101 ± 17 |
| | | | |
| **% Δ CVC Ω** | | | |
| Placebo | 15 ± 3 | 13 ± 4 | 15 ± 3 |
| Indo | 28 ± 4 | 30 ± 6 | 28 ± 4 |
| | | | |
| **CVC Reactivity (ΔCVC/ΔS$_P$O$_2$) †** | | | |
| Placebo | 4.4 ± 1.1 | 1.1 ± 0.3 | 5.6 ± 1.4 |
| Indo | 5.7 ± 1.0 | 1.6 ± 0.3 | 7.2 ± 1.3 |
| | | | |
| **% CVC Reactivity (%ΔCVC/ΔS$_P$O$_2$) Ω** | | | |
| Placebo | 0.8 ± 0.2 | 0.7 ± 0.2 | 0.8 ± 0.2 |
| Indo | 2.0 ± 0.3 | 2.2 ± 0.5 | 2.0 ± 0.3 |

Values are presented as mean ± SE. Regional cerebrovascular responses (anterior vs posterior) were analyzed indomethacin. † main effect of region (anterior vs posterior); Ω main effect of pill; § main effect of condition; ‡ region by pill interaction; ǂ region x condition interaction; $p < 0.05$.

**Table 3.7.** Vessel specific cerebrovascular response to hypoxia with and without indomethacin

| | ICA | | MCA | | ACA | | VA | | BA | PCA | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Left | Right | Left | Right | Left | Right | Left | Right | - | Left | Right |
| **Flow (ml/min) †Ω§‡** | | | | | | | | | | | |
|   Placebo | | | | | | | | | | | |
|     *Baseline* | 236 ± 21 | 254 ± 16 | 147 ± 9 | 145 ± 10 | 69 ± 13 | 92 ± 8 | 89 ± 15 | 83 ± 7 | 137 ± 14 | 53 ± 4 | 51 ± 4 |
|     *Hypoxia* | 280 ± 27 | 306 ± 25 | 178 ± 13 | 178 ± 18 | 87 ± 17 | 113 ± 11 | 105 ± 18 | 98 ± 9 | 162 ± 20 | 63 ± 6 | 62 ± 5 |
|   Indo | | | | | | | | | | | |
|     *Baseline* | 129 ± 13 | 146 ± 9 | 81 ± 5 | 84 ± 6 | 46 ± 9 | 51 ± 5 | 58 ± 7 | 48 ± 5 | 73 ± 6 | 34 ± 2 | 32 ± 2 |
|     *Hypoxia* | 166 ± 14 | 186 ± 15 | 108 ± 7 | 106 ± 7 | 51 ± 8 | 69 ± 5 | 63 ± 9 | 57 ± 6 | 94 ± 6 | 35 ± 2 | 37 ± 2 |
| **Δ Flow †Ω** | | | | | | | | | | | |
|   Placebo | 44 ± 9 | 53 ± 11 | 31 ± 7 | 34 ± 9 | 18 ± 5 | 21 ± 5 | 16 ± 4 | 15 ± 4 | 25 ± 8 | 10 ± 3 | 11 ± 2 |
|   Indo | 37 ± 3 | 40 ± 8 | 27 ± 5 | 21 ± 4 | 9 ± 2 | 18 ± 2 | 11 ± 4 | 9 ± 2 | 21 ± 4 | 1 ± 2 | 6 ± 1 |
| **% Δ Flow †Ω** | | | | | | | | | | | |
|   Placebo | 18 ± 3 | 20 ± 3 | 21 ± 4 | 22 ± 4 | 25 ± 3 | 22 ± 4 | 19 ± 2 | 19 ± 5 | 17 ± 4 | 19 ± 5 | 22 ± 4 |
|   Indo | 30 ± 3 | 27 ± 4 | 34 ± 6 | 26 ± 6 | 21 ± 3 | 39 ± 5 | 20 ± 7 | 19 ± 4 | 31 ± 7 | 6 ± 7 | 20 ± 3 |
| **Flow Reactivity (Δflow/ΔS$_P$O$_2$) †** | | | | | | | | | | | |
|   Placebo | 2.4 ± 0.5 | 2.9 ± 0.5 | 1.8 ± 0.4 | 1.9 ± 0.5 | 1.1 ± 0.4 | 1.2 ± 0.3 | 0.9 ± 0.2 | 0.8 ± 0.2 | 1.3 ± 0.3 | 0.6 ± 0.2 | 0.6 ± 0.1 |
|   Indo | 2.7 ± 0.3 | 2.9 ± 0.6 | 1.9 ± 0.4 | 1.5 ± 0.3 | 0.6 ± 0.1 | 1.3 ± 0.1 | 0.8 ± 0.3 | 0.7 ± 0.2 | 1.5 ± 0.3 | 0.1 ± 0.2 | 0.4 ± 0.1 |
| **% Flow Reactivity (%Δflow/ΔS$_P$O$_2$) †Ω‡** | | | | | | | | | | | |
|   Placebo | 1.0 ± 0.1 | 1.1 ± 0.2 | 1.2 ± 0.3 | 1.3 ± 0.2 | 1.4 ± 0.2 | 1.3 ± 0.3 | 1.1 ± 0.2 | 1.0 ± 0.2 | 0.9 ± 0.2 | 1.1 ± 0.3 | 1.3 ± 0.2 |
|   Indo | 2.2 ± 0.3 | 1.9 ± 0.3 | 2.4 ± 0.5 | 1.9 ± 0.5 | 1.4 ± 0.2 | 2.7 ± 0.4 | 1.4 ± 0.5 | 1.4 ± 0.4 | 2.2 ± 0.5 | 0.3 ± 0.5 | 1.4 ± 0.2 |
| **CVC (100 ml/min/mmHg) †Ω§‡** | | | | | | | | | | | |
|   Placebo | | | | | | | | | | | |
|     *Baseline* | 249 ± 26 | 270 ± 27 | 155 ± 13 | 153 ± 14 | 72 ± 14 | 97 ± 11 | 95 ± 18 | 85 ± 7 | 145 ± 18 | 56 ± 5 | 53 ± 5 |
|     *Hypoxia* | 286 ± 33 | 314 ± 35 | 182 ± 18 | 183 ± 22 | 88 ± 18 | 115 ± 14 | 109 ± 20 | 97 ± 9 | 166 ± 24 | 64 ± 7 | 63 ± 6 |
|   Indo | | | | | | | | | | | |
|     *Baseline* | 135 ± 14 | 155 ± 14 | 85 ± 5 | 89 ± 7 | 47 ± 9 | 54 ± 6 | 61 ± 8 | 50 ± 5 | 77 ± 7 | 35 ± 3 | 33 ± 2 |
|     *Hypoxia* | 173 ± 14 | 196 ± 19 | 112 ± 7 | 110 ± 7 | 52 ± 8 | 73 ± 7 | 65 ± 9 | 60 ± 7 | 99 ± 8 | 36 ± 2 | 39 ± 2 |
| **Δ CVC †** | | | | | | | | | | | |
|   Placebo | 38 ± 10 | 44 ± 11 | 27 ± 7 | 30 ± 9 | 16 ± 5 | 18 ± 5 | 14 ± 3 | 12 ± 4 | 21 ± 8 | 9 ± 3 | 10 ± 2 |
|   Indo | 38 ± 4 | 41 ± 9 | 27 ± 5 | 21 ± 4 | 9 ± 2 | 19 ± 2 | 10 ± 4 | 10 ± 2 | 22 ± 4 | 1 ± 3 | 7 ± 1 |

| | ICA | | MCA | | ACA | | VA | | BA | PCA | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Left | Right | Left | Right | Left | Right | Left | Right | - | Left | Right |
| **% Δ CVC Ω** | | | | | | | | | | | |
| Placebo | 15 ± 3 | 16 ± 4 | 17 ± 4 | 18 ± 4 | 21 ± 3 | 18 ± 4 | 16 ± 3 | 14 ± 5 | 13 ± 4 | 15 ± 4 | 18 ± 4 |
| Indo | 30 ± 4 | 27 ± 5 | 33 ± 6 | 26 ± 6 | 22 ± 5 | 39 ± 6 | 18 ± 6 | 19 ± 4 | 30 ± 6 | 7 ± 10 | 22 ± 4 |
| **CVC Reactivity (ΔCVC/ΔS$_P$O$_2$) †** | | | | | | | | | | | |
| Placebo | 2.0 ± 0.5 | 2.4 ± 0.5 | 1.5 ± 0.4 | 1.7 ± 0.5 | 1.0 ± 0.4 | 1.0 ± 0.3 | 0.8 ± 0.2 | 0.6 ± 0.2 | 1.1 ± 0.3 | 0.5 ± 0.2 | 0.5 ± 0.1 |
| Indo | 2.7 ± 0.3 | 2.9 ± 0.7 | 2.0 ± 0.4 | 1.5 ± 0.3 | 0.6 ± 0.1 | 1.3 ± 0.2 | 0.7 ± 0.3 | 0.7 ± 0.2 | 1.6 ± 0.3 | 0.0 ± 0.2 | 0.5 ± 0.1 |
| **% CVC Reactivity (%ΔCVC/ΔS$_P$O$_2$) †Ω‡** | | | | | | | | | | | |
| Placebo | 0.8 ± 0.2 | 0.9 ± 0.2 | 1.0 ± 0.3 | 1.0 ± 0.2 | 1.2 ± 0.2 | 1.1 ± 0.3 | 0.9 ± 0.2 | 0.7 ± 0.3 | 0.7 ± 0.2 | 0.9 ± 0.3 | 1.0 ± 0.2 |
| Indo | 2.1 ± 0.3 | 1.9 ± 0.3 | 2.4 ± 0.4 | 1.8 ± 0.5 | 1.4 ± 0.2 | 2.7 ± 0.4 | 1.3 ± 0.5 | 1.3 ± 0.3 | 2.2 ± 0.5 | 0.3 ± 0.6 | 1.5 ± 0.3 |
| **CSA (mm$^2$) †Ω§‡** | | | | | | | | | | | |
| Placebo | | | | | | | | | | | |
| *Baseline* | 16.6 ± 1.7 | 17.6 ± 1.7 | 6.3 ± 0.3 | 6.1 ± 0.3 | 4.5 ± 0.4 | 5.0 ± 0.3 | 5.5 ± 0.5 | 5.8 ± 0.4 | 7.8 ± 0.7 | 4.0 ± 0.2 | 4.0 ± 0.2 |
| *Hypoxia* | 17.4 ± 1.8 | 18.3 ± 1.9 | 6.9 ± 0.6 | 6.7 ± 0.6 | 5.0 ± 0.8 | 5.6 ± 0.5 | 5.8 ± 0.6 | 6.0 ± 0.4 | 8.3 ± 0.8 | 4.2 ± 0.3 | 4.2 ± 0.3 |
| Indo | | | | | | | | | | | |
| *Baseline* | 11.8 ± 1.2 | 12.7 ± 1.0 | 5.4 ± 0.2 | 5.4 ± 0.2 | 4.0 ± 0.4 | 4.2 ± 0.2 | 5.2 ± 0.4 | 5.5 ± 0.5 | 6.4 ± 0.5 | 3.8 ± 0.4 | 3.4 ± 0.5 |
| *Hypoxia* | 13.2 ± 1.3 | 14.0 ± 1.3 | 6.6 ± 0.8 | 6.1 ± 0.6 | 4.6 ± 0.6 | 5.3 ± 0.3 | 5.7 ± 0.8 | 5.8 ± 0.5 | 6.9 ± 0.7 | 4.0 ± 0.3 | 3.8 ± 0.2 |
| **Δ CSA Ω** | | | | | | | | | | | |
| Placebo | 0.8 ± 0.5 | 0.7 ± 0.4 | 0.6 ± 0.4 | 0.6 ± 0.4 | 0.4 ± 0.4 | 0.6 ± 0.5 | 0.3 ± 0.1 | 0.2 ± 0.2 | 0.5 ± 0.2 | 0.2 ± 0.2 | 0.2 ± 0.2 |
| Indo | 1.3 ± 0.1 | 1.3 ± 0.3 | 1.2 ± 0.7 | 0.8 ± 0.5 | 0.8 ± 0.5 | 1.0 ± 0.4 | 1.0 ± 0.5 | 0.4 ± 0.3 | 0.6 ± 0.3 | 0.0 ± 0.2 | 0.5 ± 0.2 |
| **% Δ CSA Ω** | | | | | | | | | | | |
| Placebo | 5 ± 2 | 4 ± 2 | 9 ± 5 | 10 ± 6 | 6 ± 7 | 13 ± 9 | 5 ± 2 | 4 ± 3 | 6 ± 2 | 5 ± 4 | 4 ± 4 |
| Indo | 11 ± 1 | 10 ± 2 | 21 ± 11 | 13 ± 8 | 19 ± 8 | 27 ± 11 | 18 ± 9 | 8 ± 6 | 8 ± 4 | 2 ± 6 | 14 ± 7 |
| **Diameter (mm$^2$) †Ω§‡** | | | | | | | | | | | |
| Placebo | | | | | | | | | | | |
| *Baseline* | 45.4 ± 2.4 | 47.0 ± 2.1 | 28.3 ± 0.8 | 27.7 ± 0.7 | 23.8 ± 1.1 | 25.1 ± 0.7 | 26.3 ± 1.3 | 27.0 ± 0.9 | 31.3 ± 1.4 | 22.6 ± 0.6 | 22.5 ± 0.6 |
| *Hypoxia* | 46.5 ± 2.4 | 47.8 ± 2.4 | 29.5 ± 1.2 | 29.0 ± 1.2 | 24.6 ± 1.7 | 26.5 ± 1.2 | 26.9 ± 1.4 | 27.5 ± 0.9 | 32.2 ± 1.5 | 23.1 ± 0.9 | 22.9 ± 0.8 |
| Indo | | | | | | | | | | | |
| *Baseline* | 38.4 ± 1.9 | 39.9 ± 1.6 | 26.2 ± 0.5 | 26.1 ± 0.5 | 22.4 ± 1.1 | 23.1 ± 0.6 | 25.4 ± 1.1 | 26.2 ± 1.1 | 28.3 ± 1.1 | 21.9 ± 1.1 | 20.9 ± 0.4 |
| *Hypoxia* | 40.5 ± 2.0 | 41.9 ± 1.9 | 28.6 ± 1.5 | 27.7 ± 1.2 | 23.9 ± 1.4 | 25.8 ± 0.7 | 26.4 ± 2.0 | 27.1 ± 1.1 | 29.5 ± 1.3 | 22.4 ± 0.8 | 22.0 ± 0.6 |
| **Δ Diameter (mm$^2$) Ω** | | | | | | | | | | | |
| Placebo | 1.1 ± 0.6 | 0.9 ± 0.5 | 1.2 ± 0.7 | 1.3 ± 0.8 | 0.8 ± 0.9 | 1.4 ± 1.0 | 0.7 ± 0.3 | 0.5 ± 0.4 | 0.9 ± 0.3 | 0.5 ± 0.5 | 0.4 ± 0.5 |
| Indo | 2.1 ± 0.2 | 1.9 ± 0.4 | 2.4 ± 1.3 | 1.6 ± 0.9 | 2.0 ± 1.0 | 2.6 ± 0.9 | 2.2 ± 1.0 | 0.9 ± 0.7 | 1.1 ± 0.6 | 0.0 ± 0.6 | 1.3 ± 0.7 |

| | ICA | | MCA | | ACA | | VA | | BA | PCA | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Left | Right | Left | Right | Left | Right | Left | Right | - | Left | Right |
| **% Δ Diameter Ω** | | | | | | | | | | | |
| Placebo | 2 ± 1 | 2 ± 1 | 4 ± 2 | 4 ± 3 | 3 ± 3 | 6 ± 4 | 3 ± 1 | 2 ± 1 | 3 ± 1 | 2 ± 2 | 2 ± 2 |
| Indo | 5 ± 0 | 5 ± 1 | 9 ± 5 | 6 ± 3 | 9 ± 4 | 12 ± 4 | 8 ± 4 | 4 ± 3 | 4 ± 2 | 1 ± 3 | 7 ± 3 |
| **Mean velocity (cm/s) †Ω§‡** | | | | | | | | | | | |
| Placebo | | | | | | | | | | | |
| *Baseline* | 26 ± 2 | 26 ± 1 | 40 ± 2 | 41 ± 2 | 26 ± 3 | 32 ± 2 | 27 ± 2 | 25 ± 1 | 31 ± 2 | 23 ± 1 | 22 ± 1 |
| *Hypoxia* | 29 ± 2 | 30 ± 2 | 45 ± 2 | 46 ± 2 | 29 ± 2 | 36 ± 3 | 30 ± 3 | 28 ± 2 | 34 ± 2 | 26 ± 1 | 26 ± 1 |
| Indo | | | | | | | | | | | |
| *Baseline* | 20 ± 2 | 21 ± 1 | 26 ± 2 | 27 ± 2 | 19 ± 2 | 21 ± 1 | 19 ± 1 | 16 ± 1 | 20 ± 1 | 16 ± 1 | 16 ± 1 |
| *Hypoxia* | 23 ± 2 | 24 ± 1 | 30 ± 2 | 31 ± 2 | 19 ± 2 | 23 ± 2 | 19 ± 1 | 17 ± 2 | 25 ± 2 | 16 ± 1 | 17 ± 1 |
| **Δ Mean velocity Ω** | | | | | | | | | | | |
| Placebo | 3 ± 1 | 4 ± 1 | 5 ± 2 | 5 ± 2 | 4 ± 1 | 4 ± 2 | 3 ± 1 | 3 ± 1 | 3 ± 1 | 3 ± 1 | 4 ± 1 |
| Indo | 3 ± 0 | 3 ± 1 | 4 ± 2 | 4 ± 2 | 1 ± 1 | 3 ± 1 | 1 ± 1 | 2 ± 1 | 4 ± 1 | 1 ± 1 | 1 ± 1 |
| **% Δ Mean velocity** | | | | | | | | | | | |
| Placebo | 13 ± 3 | 16 ± 4 | 13 ± 6 | 12 ± 5 | 19 ± 6 | 11 ± 6 | 13 ± 3 | 13 ± 3 | 10 ± 3 | 14 ± 4 | 19 ± 5 |
| Indo | 17 ± 2 | 16 ± 3 | 15 ± 7 | 15 ± 8 | 4 ± 6 | 12 ± 5 | 2 ± 3 | 11 ± 5 | 22 ± 7 | 5 ± 8 | 8 ± 8 |
| **Max velocity (cm/s) †Ω§‡** | | | | | | | | | | | |
| Placebo | | | | | | | | | | | |
| *Baseline* | 58 ± 4 | 62 ± 2 | 89 ± 3 | 88 ± 3 | 70 ± 3 | 79 ± 4 | 65 ± 5 | 61 ± 2 | 71 ± 3 | 63 ± 4 | 61 ± 4 |
| *Hypoxia* | 65 ± 3 | 72 ± 3 | 93 ± 3 | 99 ± 4 | 78 ± 3 | 81 ± 5 | 72 ± 4 | 67 ± 3 | 77 ± 3 | 68 ± 3 | 72 ± 2 |
| Indo | | | | | | | | | | | |
| *Baseline* | 51 ± 3 | 58 ± 2 | 64 ± 3 | 65 ± 2 | 53 ± 3 | 61 ± 4 | 55 ± 3 | 49 ± 4 | 58 ± 3 | 49 ± 3 | 50 ± 4 |
| *Hypoxia* | 55 ± 3 | 65 ± 3 | 76 ± 4 | 78 ± 5 | 63 ± 7 | 68 ± 3 | 60 ± 4 | 50 ± 6 | 65 ± 4 | 55 ± 2 | 58 ± 5 |
| **Δ Max velocity** | | | | | | | | | | | |
| Placebo | 7 ± 3 | 10 ± 3 | 4 ± 3 | 11 ± 3 | 8 ± 4 | 2 ± 2 | 7 ± 2 | 5 ± 1 | 6 ± 2 | 4 ± 3 | 11 ± 4 |
| Indo | 4 ± 2 | 7 ± 4 | 11 ± 3 | 13 ± 4 | 6 ± 5 | 7 ± 4 | 6 ± 3 | 1 ± 3 | 8 ± 5 | 5 ± 4 | 8 ± 6 |
| **% Δ Max velocity** | | | | | | | | | | | |
| Placebo | 14 ± 5 | 17 ± 6 | 6 ± 4 | 12 ± 4 | 14 ± 6 | 2 ± 3 | 12 ± 3 | 9 ± 2 | 9 ± 4 | 9 ± 4 | 22 ± 7 |
| Indo | 10 ± 5 | 13 ± 7 | 18 ± 4 | 19 ± 7 | 9 ± 8 | 15 ± 7 | 11 ± 5 | 2 ± 5 | 15 ± 9 | 13 ± 10 | 18 ± 11 |

Values are presented as mean ± SE. ICA, internal carotid artery; MCA, middle cerebral artery; ACA, anterior cerebral artery; VA, vertebral artery; BA, basilar artery; PCA, posterior cerebral artery; CVC, cerebral vascular conductance; CSA, cross sectional area; Indo, indomethacin. † main effect of vessel; Ω main effect of pill; § main effect of condition; ‡ vessel x pill interaction; $p < 0.05$.

Figures
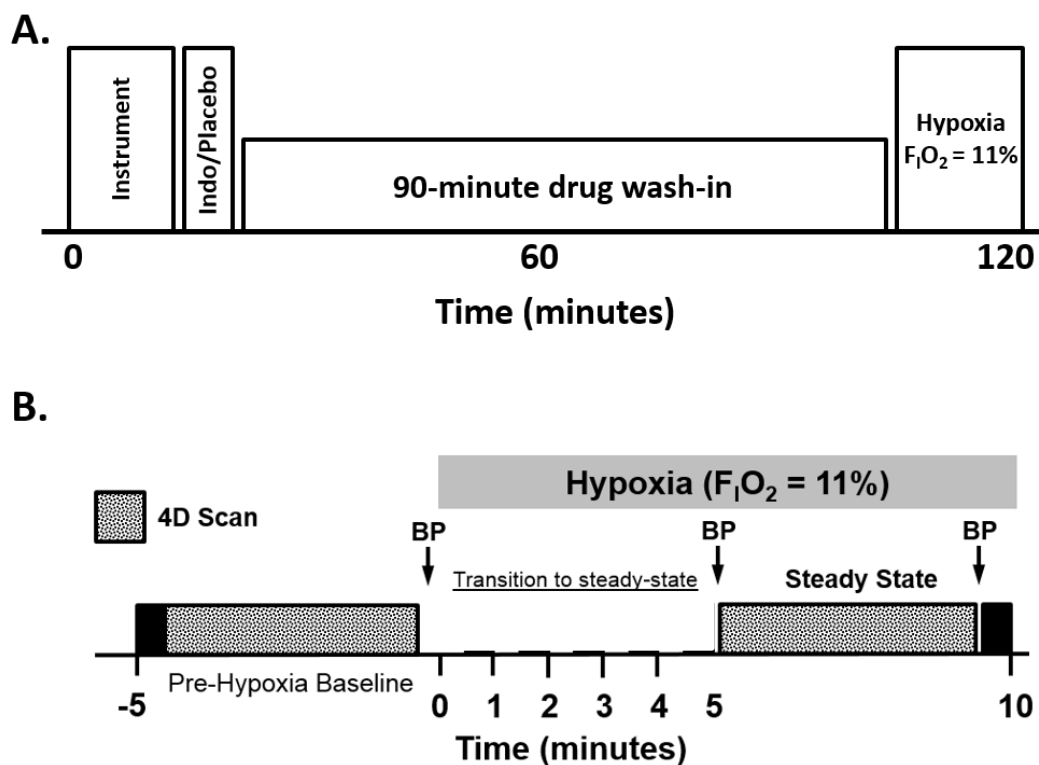
**Figure 3.1**. **Study protocol. A.** Complete study protocol. **B.** Detailed hypoxia protocol. Subjects were studied twice with either placebo or indomethacin in a randomized, double-blinded design. Following baseline scans, subjects received either indomethacin or placebo. Following a 90-minute waiting period the hypoxia scan commenced. Indo, indomethacin, $F_IO_2$, inspired oxygen percentage; BP, blood pressure.
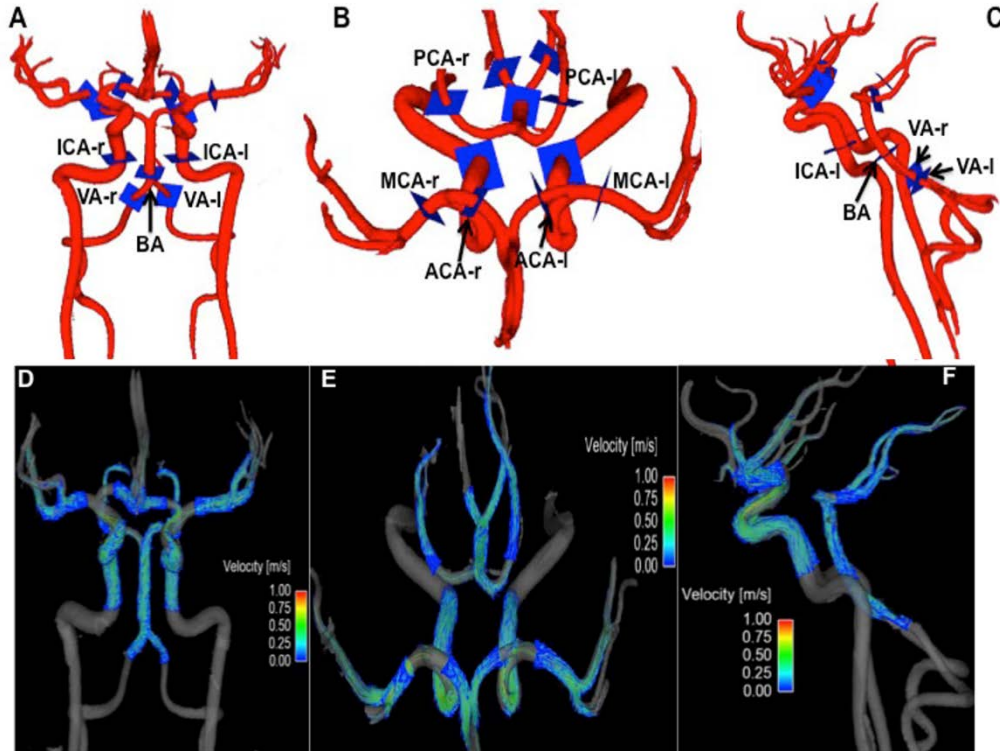
**Figure 3.2. 4-Dimensional flow magnetic resonance imaging (PC-VIPR).** 4a. Coronal PC-VIPR angiogram. 4b. Axial PC-VIPR angiogram. 4c. Sagittal PC-VIPR angiogram. 4d. Coronal PC-VIPR angiogram overlaid with velocity tracings. 4e. Axial PC-VIPR angiogram overlaid with velocity tracings. 4f. Sagittal PC-VIPR angiogram overlaid with velocity tracings. Blue boxes indicate cut-planes for 3.45 mm measurement sections within a given vessel. Arteries included in analysis: VA, vertebral artery (r) right and (l) left; BA, basilar artery; PCA, posterior cerebral artery (r) right and (l) left; internal carotid artery, ICA (r) right and (l) left; MCA, middle cerebral artery (r) right and (l) left; ACA, anterior cerebral artery (r) right and (l) left.

**Figure 3.3. Basal reductions in total and regional cerebrovascular conductance (CVC) with indomethacin.** Regional cerebrovascular responses (anterior vs posterior) were analyzed separately from the total response but are presented together. **A**. Indomethacin decreased absolute CVC and this was greater in the anterior compared to posterior circulation. **B.** Indomethacin decreased relative CVC ~ 45% and this was similar between anterior and posterior circulations. * different from zero, †anterior vs posterior, $p < 0.001$.
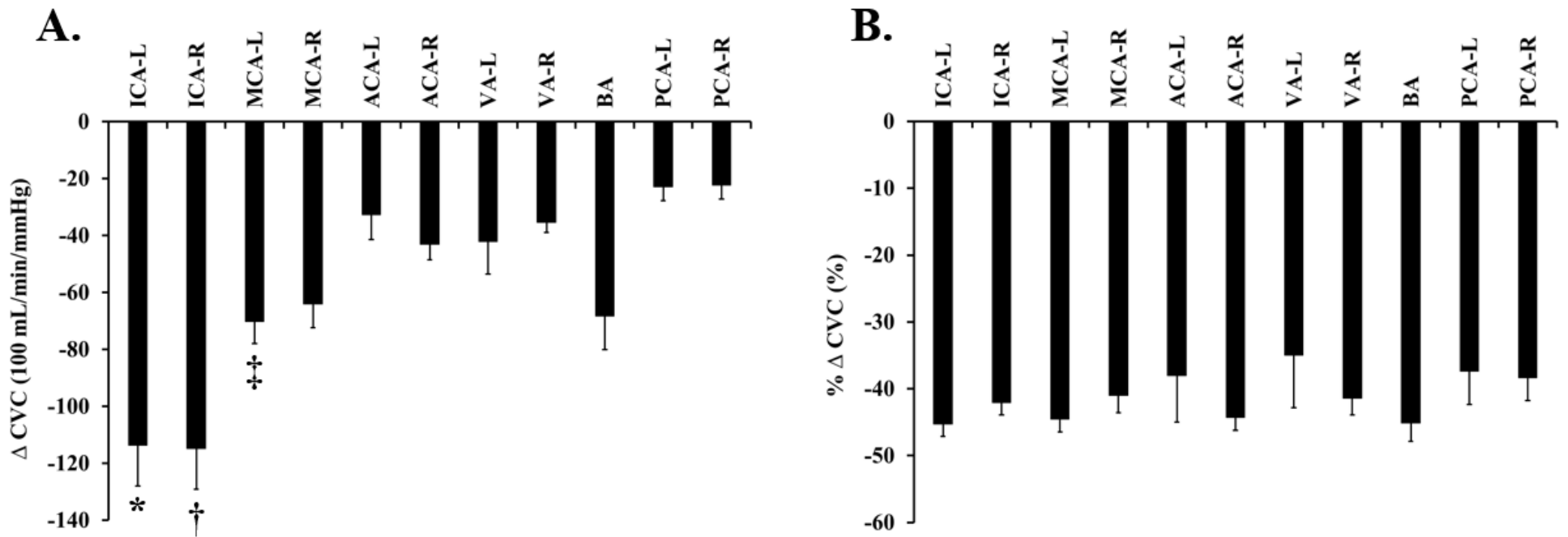
**Figure 3.4. Basal reduction in cerebrovascular conductance (CVC) with indomethacin by vessel. A.** The absolute decrease in CVC with indomethacin was greatest in both internal carotid arteries. **B.** The relative decrease in CVC with indomethacin was similar across all vessels. * vs all other vessels except ICA-R and MCA-L, † vs all other vessels except ICA-L, ‡ vs PCA-L and PCA-R, $p < 0.001$.
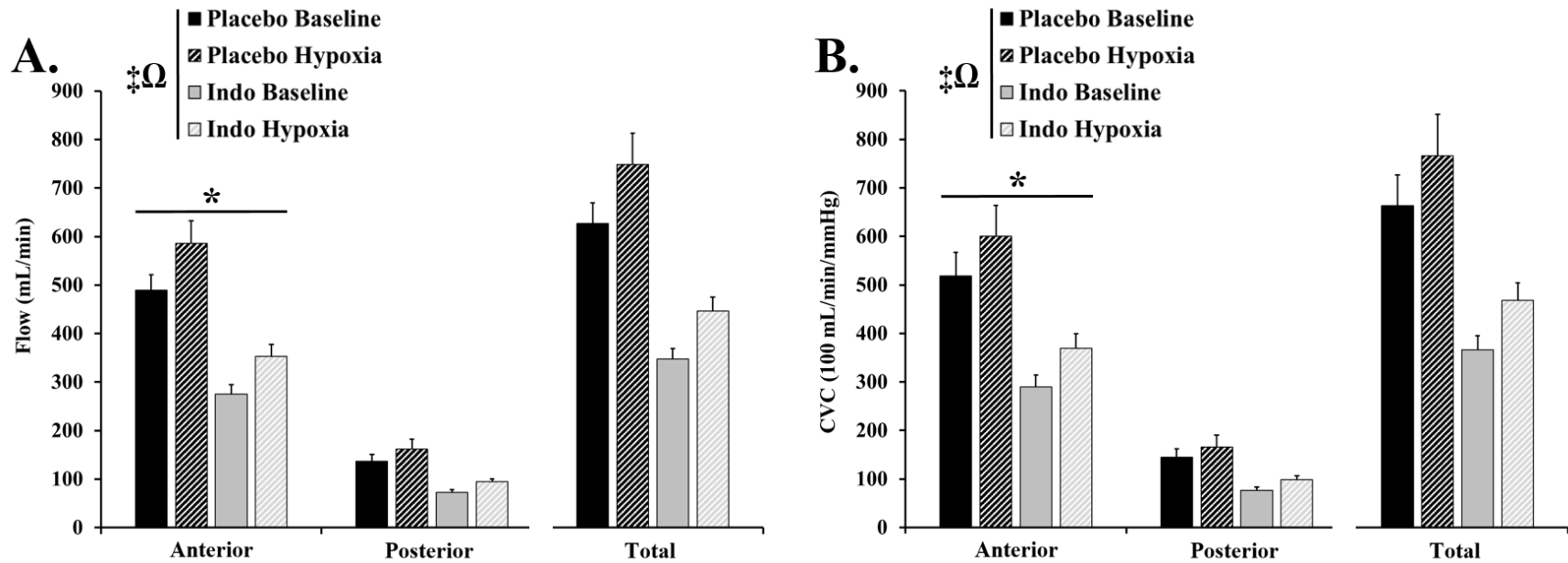
**Figure 3.5. Total and regional cerebral blood flow and cerebrovascular conductance (CVC) at baseline and during hypoxia with placebo and indomethacin (Indo).** Regional cerebrovascular responses (anterior vs posterior) were analyzed separately from the total response but are presented together. **A.** Flow was greater during hypoxia (main effect), was lower with indomethacin (main effect), and was greater in the anterior compared to posterior circulation. **B.** CVC was greater during hypoxia (main effect), was lower with indomethacin (main effect), and was greater in the anterior compared to posterior circulation (main effect). *main effect of region; Ω main effect of pill; ‡ main effect of condition $p < 0.001$.
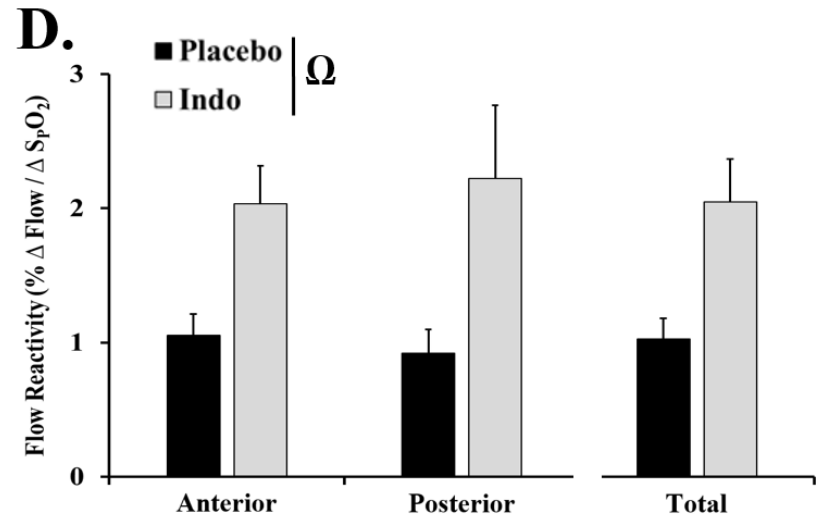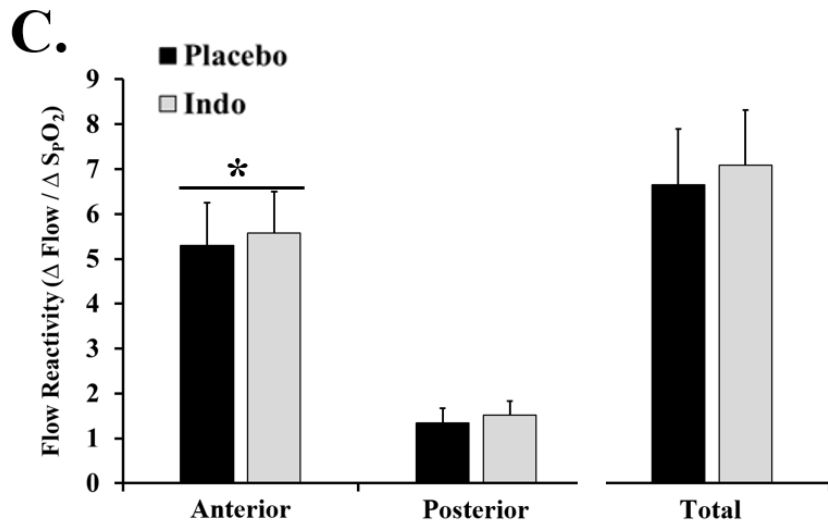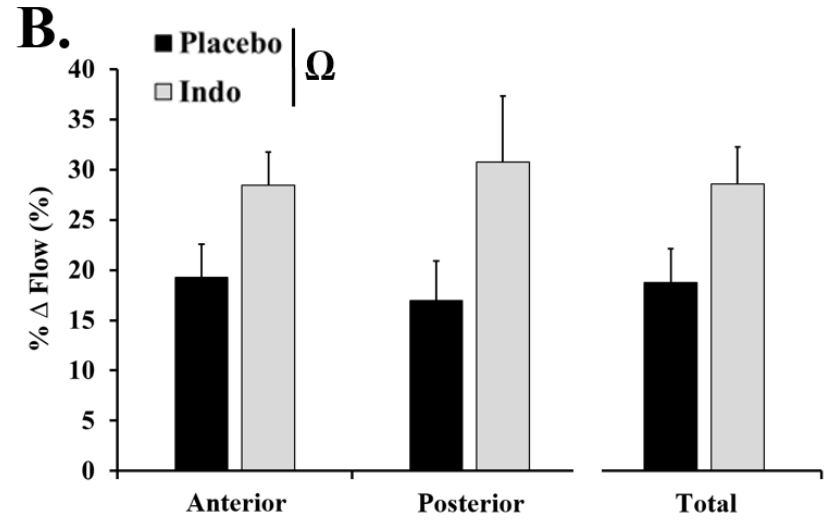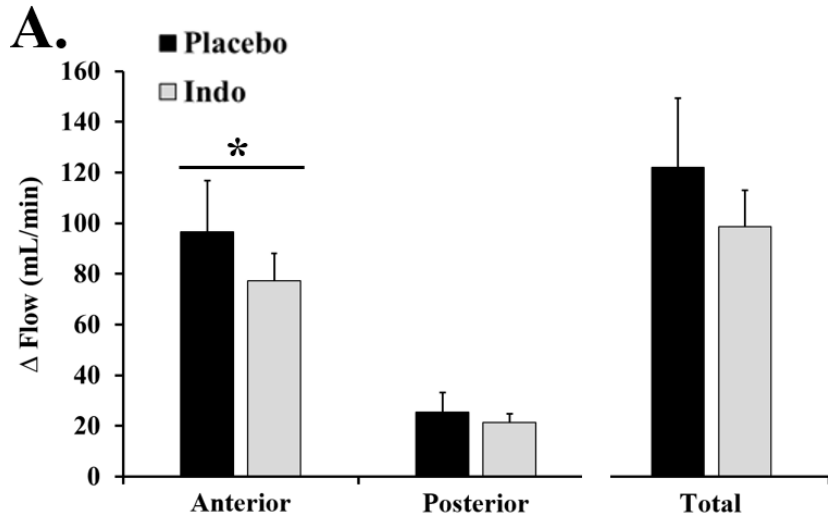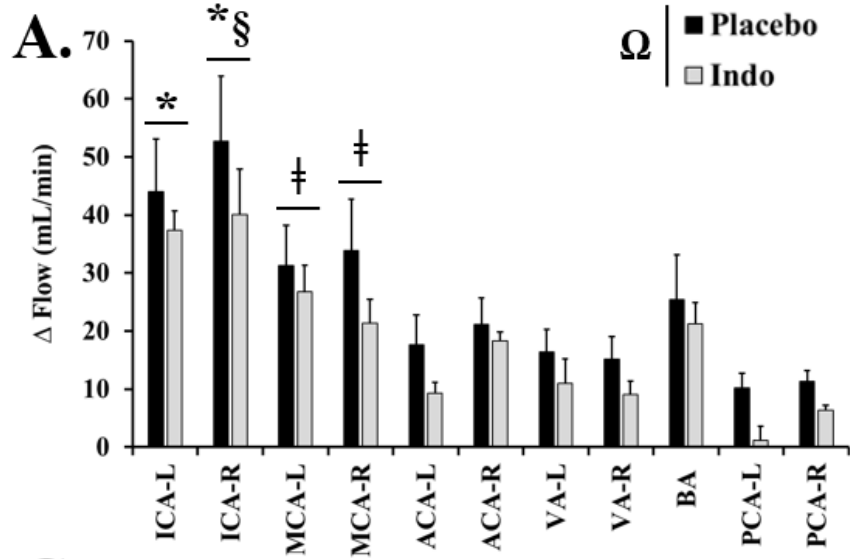
**Figure 3.6.  Changes in hypoxia-mediated total and regional flow and total and regional flow reactivity with and without indomethacin (Indo).**  Regional cerebrovascular responses (anterior vs posterior) were analyzed separately from the total response but are presented together.  **A.**  Hypoxia-mediated increases in absolute flow were similar between indomethacin and placebo and greater in the anterior compared to posterior circulation.  **B.** Hypoxia-mediated increases in relative flow were augmented with indomethacin and similar between anterior posterior circulations.  Note, the greater relative increase in total CBF with Indo trended towards significance ($19 \pm 3$ vs $29 \pm 4$; $p = 0.069$).  **C.** Absolute flow reactivities were similar between indomethacin and placebo and greater in the anterior compared to posterior circulation.  **D.** Relative flow reactivities were augmented with indomethacin and similar between anterior and posterior circulations.  * anterior vs posterior; $\Omega$ main effect of pill; $p < 0.05$.
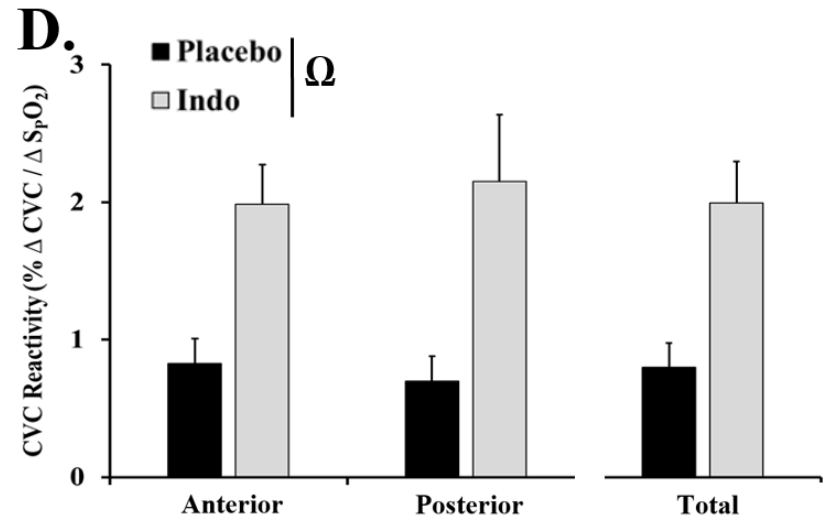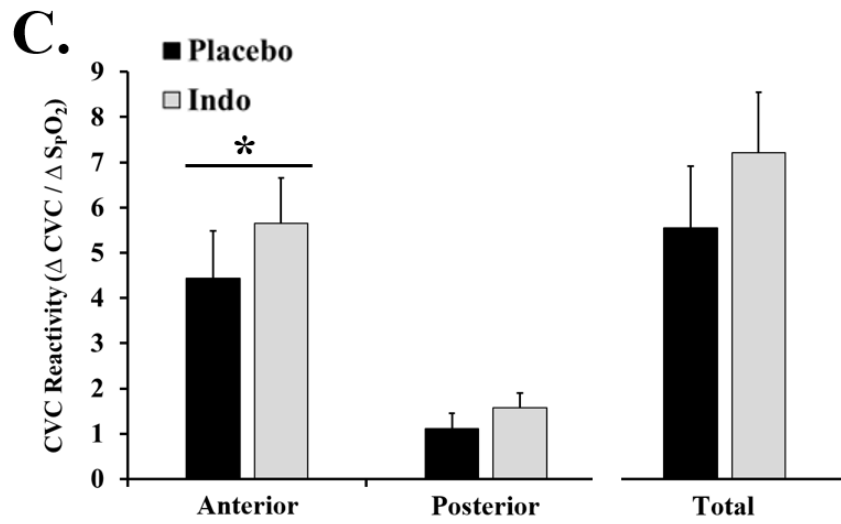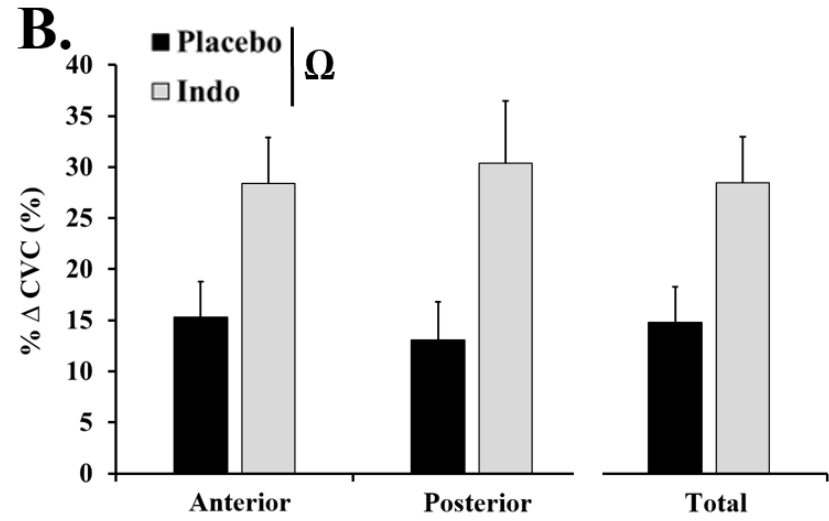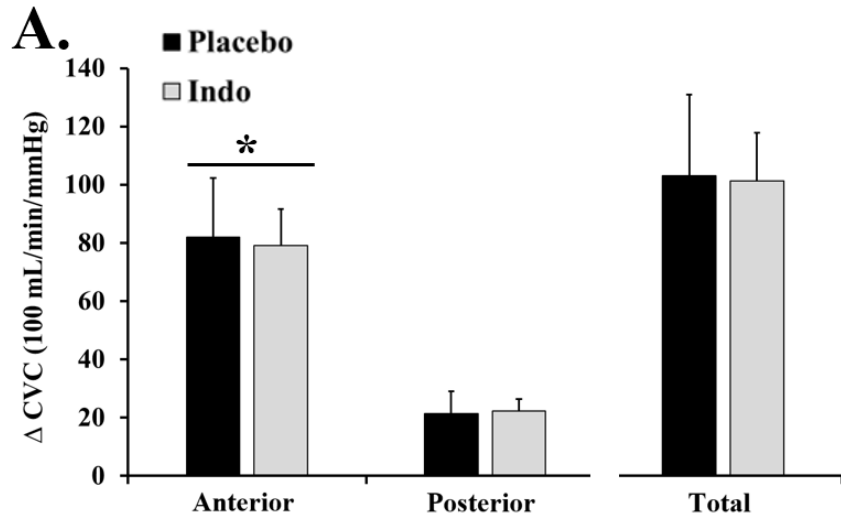
**Figure 3.7. Changes in hypoxia-mediated total and regional cerebrovascular conductance (CVC) and total and regional CVC reactivity with and without indomethacin (Indo).** Regional cerebrovascular responses (anterior vs posterior) were analyzed separately from the total response but are presented together. **A.** Hypoxia-mediated increases in absolute CVC were similar between indomethacin and placebo and greater in the anterior compared to posterior circulation. **B.** Hypoxia-mediated increases in relative CVC were augmented with indomethacin and similar between anterior posterior circulations. **C.** Absolute CVC reactivities were similar between indomethacin and placebo and greater in the anterior compared to posterior circulation. **D.** Relative CVC reactivities were augmented with indomethacin and similar between anterior and posterior circulations. * anterior vs posterior; $\Omega$ main effect of pill; $p < 0.05$.
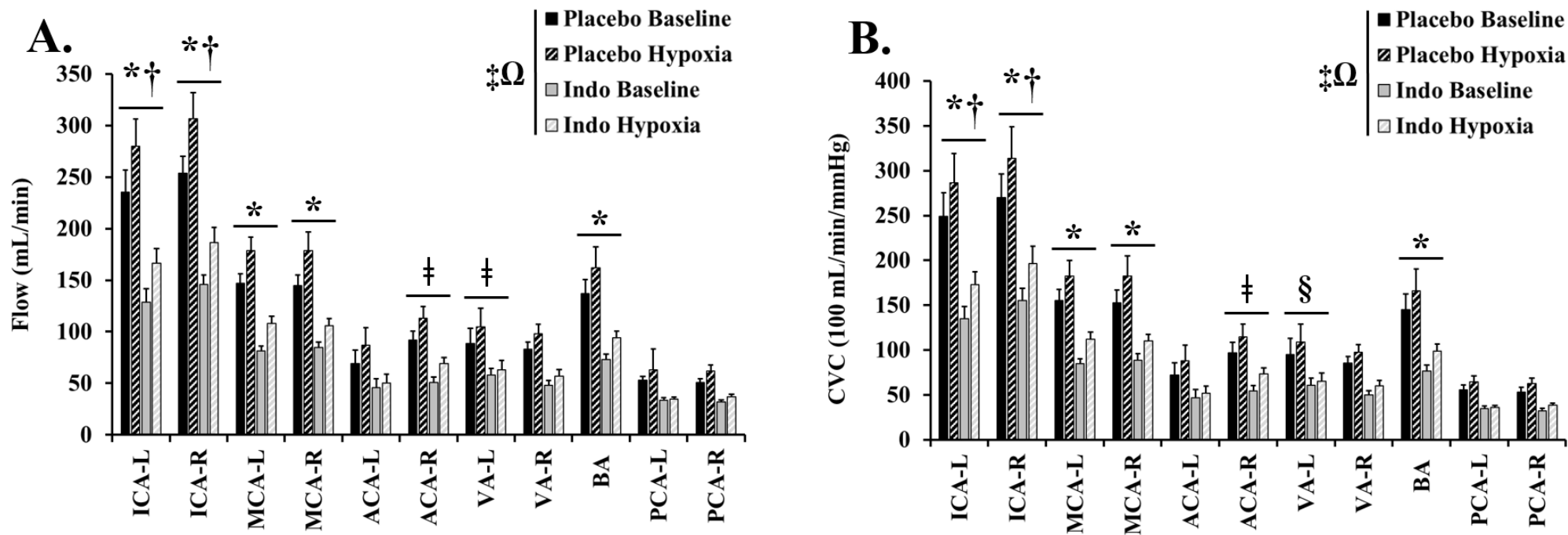
**Figure 3.8. Cerebral blood flow and cerebrovascular conductance (CVC) at baseline and during hypoxia with placebo and indomethacin (Indo). A.** Flow was greater during hypoxia (main effect), was lower with indomethacin (main effect), and was different between vessels (main effect). **B.** CVC was greater during hypoxia (main effect), was lower with indomethacin (main effect), and was different between vessels (main effect). Main effect of vessel: * vs ACAs, VAs, and PCAs, † vs BA and MCAs, ‡ vs PCAs, § vs PCA-R; $\Omega$ main effect of pill; ‡ main effect of condition $p < 0.001$
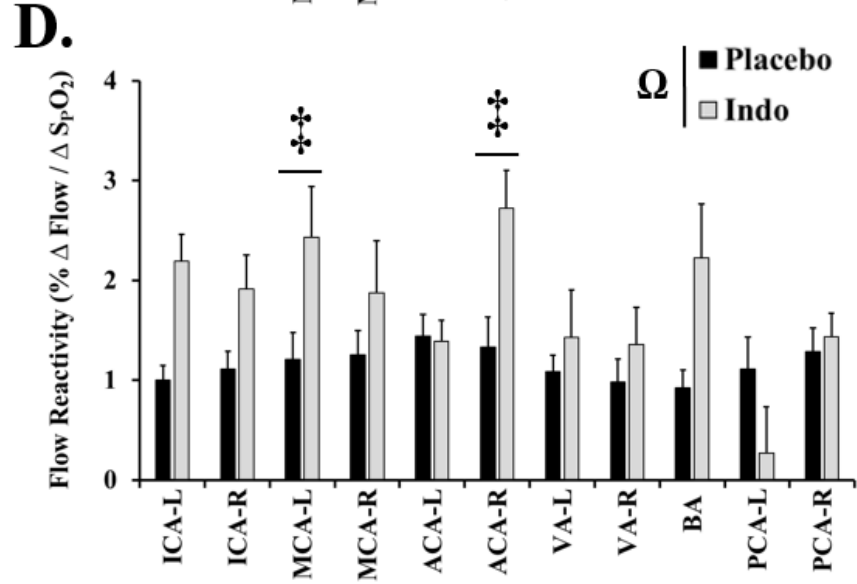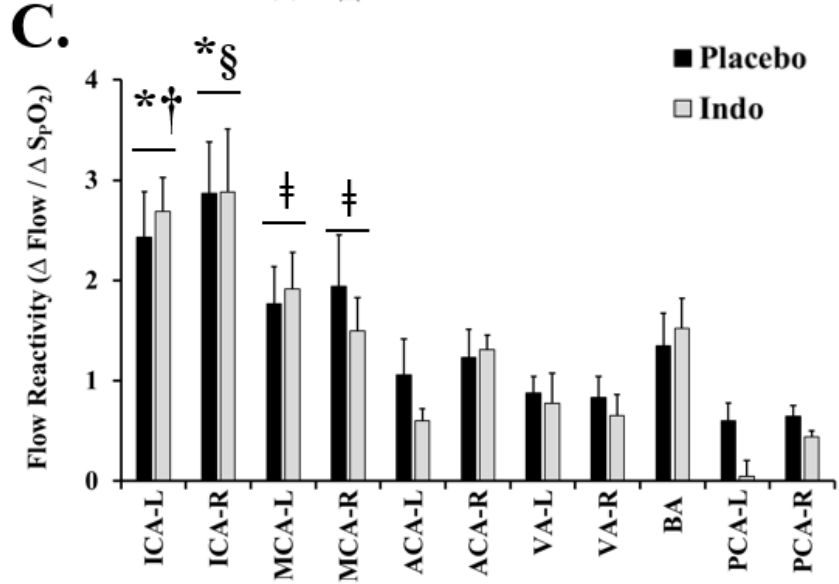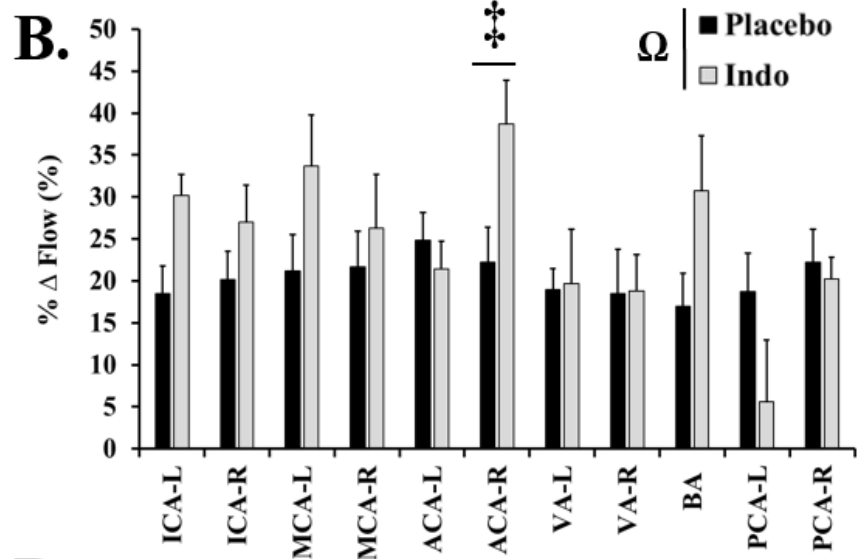
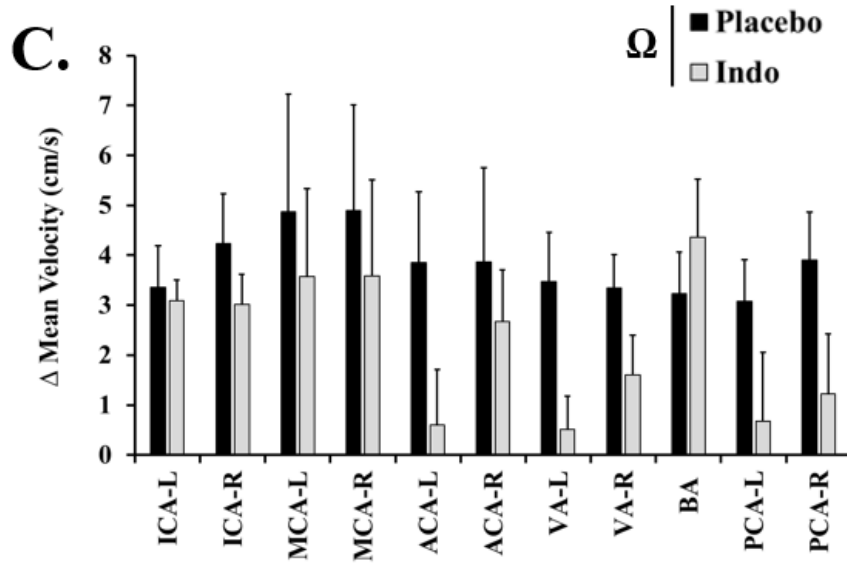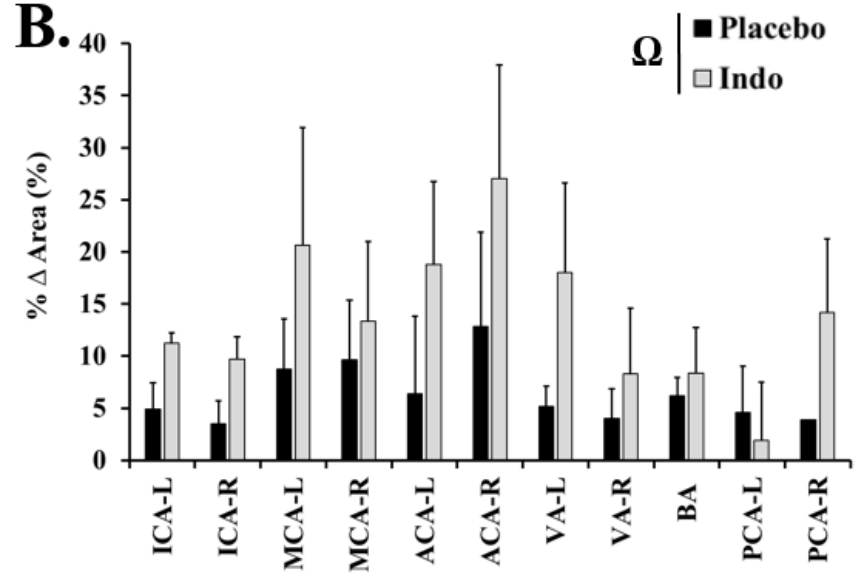**Figure 3.9. Changes in hypoxia-mediated vessel flow and vessel reactivity with and without indomethacin (Indo)**. **A.** Hypoxia-mediated increases in absolute flow were reduced with indomethacin (main effect) and different between vessels (main effect). **B.** Hypoxia-mediated increases in relative flow were augmented with indomethacin (main effect) and different between vessels (main effect). **C.** Absolute flow reactivities were similar between placebo and indomethacin but different between vessels (main effect). **D.** Relative flow reactivities were augmented with indomethacin (main effect) and was different between vessels (main effect). Main effect of vessel: * vs ACAs, VAs, and PCAs, † vs BA, § vs BA and MCA-R, ‡ vs PCAs, ‡ vs PCA-L; Ω main effect of pill; $p < 0.05$.
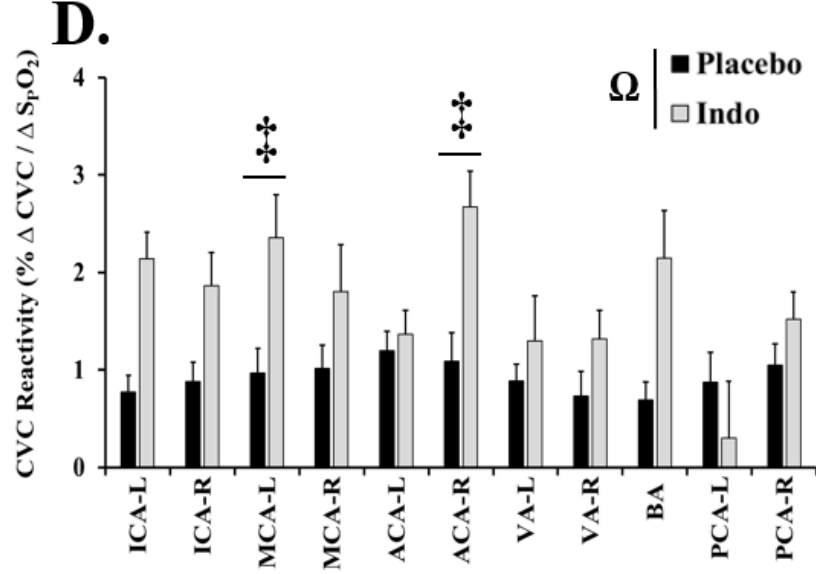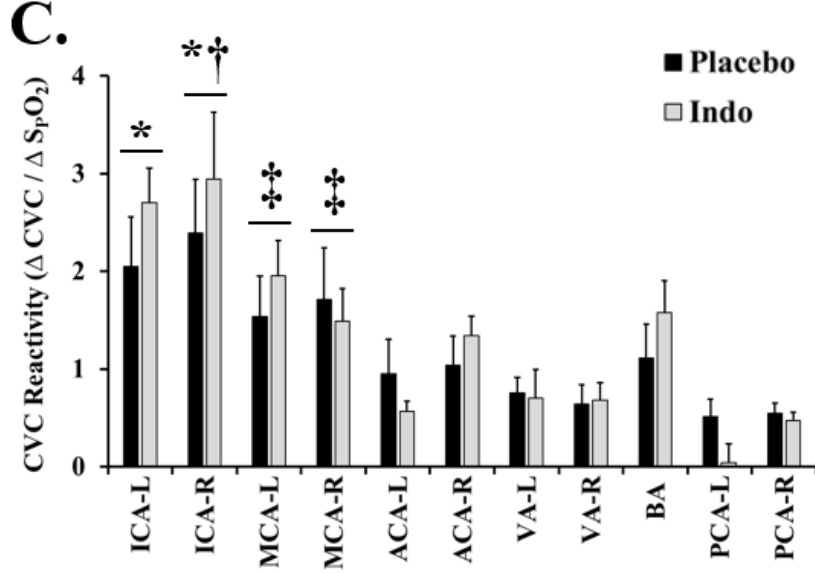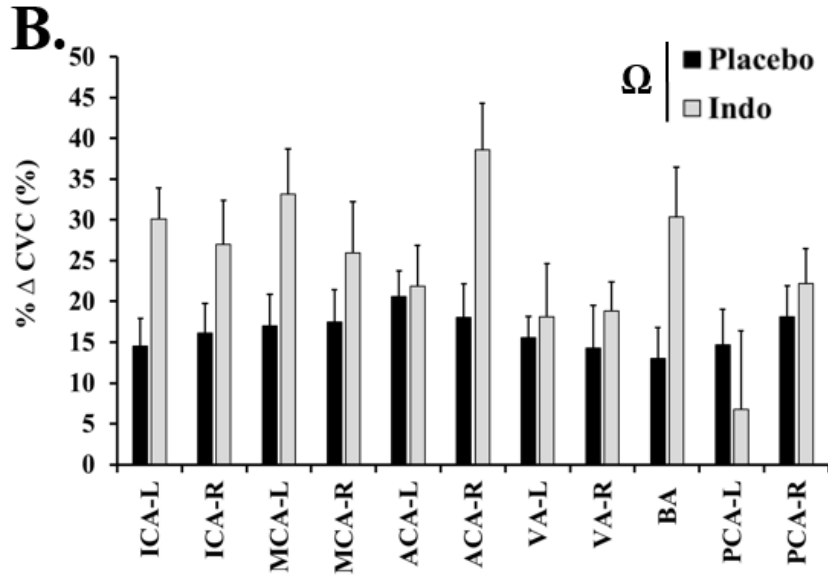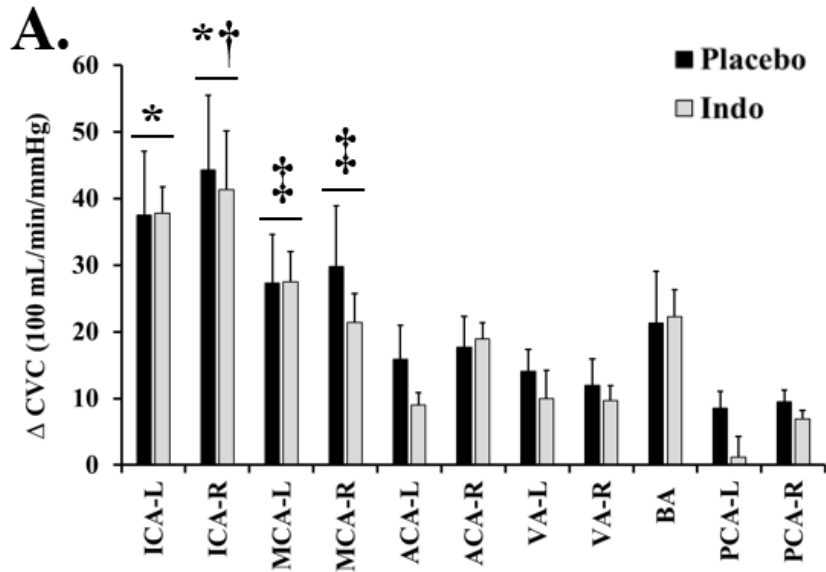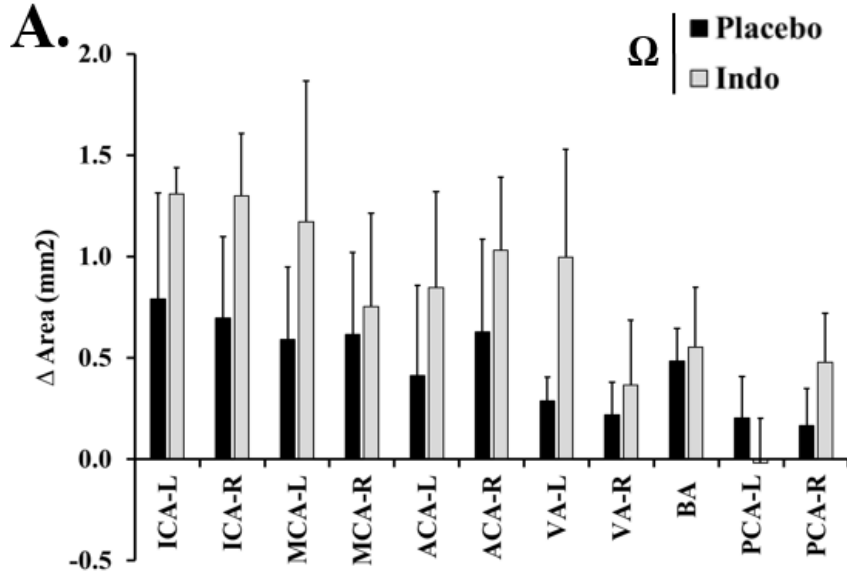
**Figure 3.10. Changes in hypoxia-mediated vessel cerebrovascular conductance (CVC) and vessel reactivity with and without indomethacin (Indo).** **A.** Hypoxia-mediated increases in absolute CVC were similar between placebo and indomethacin but different between vessels (main effect). **B.** Hypoxia-mediated increases in relative CVC were augmented with indomethacin (main effect) and similar between vessels. **C.** Absolute CVC reactivities were similar between placebo and indomethacin but different between vessels (main effect). **D.** Relative CVC reactivities were augmented with indomethacin (main effect) and was different between vessels (main effect). Main effect of vessel: * vs ACAs, VAs, and PCAs, † vs BA, ‡ vs PCA-L; Ω main effect of pill; $p < 0.05$.
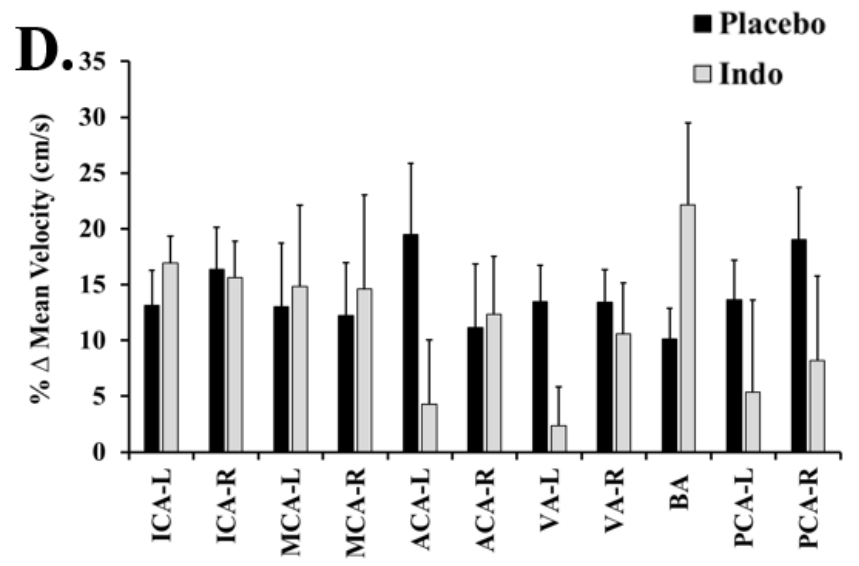
**Figure 3.11. Changes in hypoxia-mediated vessel cross-sectional area and vessel mean velocity with and without indomethacin (Indo). A.** Hypoxia-mediated increases in absolute cross-sectional areas were augmented with indomethacin (main effect) and similar between vessels. **B.** Hypoxia-mediated increases in relative cross-sectional areas were augmented with indomethacin (main effect) and similar between vessels. **C.** Hypoxia-mediated increases in absolute mean velocities were reduced with indomethacin (main effect) and similar between vessels. **D.** Hypoxia-mediated increases in relative mean velocities were similar between placebo and indomethacin as well as between vessels. $\Omega$ main effect of pill; $p < 0.05$

# General Discussion and Conclusion

Identifying mechanisms of cerebrovascular control may aid in uncovering novel therapeutic targets that can be utilized to prevent or slow the progression of cerebrovascular disease and ultimately improve quality of life. With this in mind, this dissertation sought to investigate sex-, menstrual phase-, and region-specific cerebrovascular control in young, healthy adults. We utilized the physiologic stress of hypoxia to elicit robust increases in CBF and tested the mechanistic contribution of COX by oral administration of the non-selective COX inhibitor indomethacin. Specifically, we aimed to:

1.) Determine the sex-specific contribution of COX to hypoxic vasodilation in the MCA.

2.) Determine the menstrual phase-specific contribution of COX to hypoxic vasodilation in the MCA.

3.) Determine the region-specific contribution of COX to hypoxic vasodilation by examining eleven major cerebral arteries.

Based upon these aims we generated the following hypotheses. First, we hypothesized that hypoxic vasodilation in the MCA will be greater in women than men, due in part to a greater contribution of COX. Second, we hypothesized that hypoxic vasodilation in the MCA will be greater during the late follicular versus early follicular phase of the menstrual cycle, due in part to a greater contribution of COX. Lastly, we hypothesized that hypoxic vasodilation will be greater in the posterior circulation (VA, BA, and PCA) compared to the anterior circulation (ICA, MCA, and ACA), due in part to a greater contribution of COX.

Contrary to our hypotheses, our findings demonstrate that hypoxic cerebral vasodilation is not different between men and women, is not different between the early follicular and late follicular phases of the menstrual cycle, and is not region-specific. Additionally, our data suggest that COX does not mediate hypoxic vasodilation, but may restrain hypoxic cerebral vasodilation.

## Contribution of Cyclooxygenase to Basal Cerebral Blood Flow Regulation

The oxygen demand of the brain is about 10x greater than expected given its size, due to its almost complete reliance on oxidative metabolism (42). Thus, the maintenance of CBF is of critical importance. Despite this importance, the mechanism(s) regulating CBF remain poorly understood. COX is one mechanism that has been shown to partially contribute to the maintenance of basal CBF. On average, the non-selective COX inhibitor indomethacin causes an ~30% reduction in MCAv (7, 8, 19, 31, 32, 49-51, 54, 64, 79). This is in agreement with findings from Aim 1 and Aim 2, which demonstrate reductions in MCAv of $31 \pm 2\%$ and $32 \pm 4\%$ with indomethacin. Findings from Aim 3 suggest a substantially greater basal contribution of COX as indomethacin reduced CVC by $44 \pm 2\%$.

As part of this dissertation, we investigated sex-, menstrual phase- and region specific contributions of COX to the maintenance of basal CBF. In Aim 1, we found no sex difference in basal contribution of COX. In Aim 2, we provide evidence that the increase in basal MCAv during the late follicular phase of the menstrual cycle, compared to early follicular phase, is due to a greater basal contribution of COX. Greater basal contributions of COX during the late follicular phase of the menstrual may be explained by the late follicular oscillatory increase in estrogen and subsequent upregulation of vasodilatory $PGI_2$ production. This seems most logical, however it is important to note that this is conjecture as we lack biochemical evidence to support

such conclusions.  Lastly, Aim 3 found no region-specific differences in the basal contribution of COX.  Considered collectively, it appears that the role of COX in the regulation of resting CBF increases during the late follicular compared to early follicular phase of the menstrual cycle, but there are no sex- or region-specific differences.

In Appendix E: Supplementary Data we provide additional, albeit exploratory, analysis on the contribution of COX to basal CBF regulation (Appendix E: Supplementary Data; *Contribution of Cyclooxygenase to Basal Cerebral Blood Flow Regulation*).  This analysis is very exploratory as it applies the vessel cross-sectional areas obtained from Aim 3 to the MCAv values obtained in Aim 1 and Aim 2.  This allows for the calculation of MCA blood flows in both Aim 1 and Aim 2.  This exploratory analysis, despite being extremely limited, does support the lack of sex difference in the contribution of COX to basal CBF regulation.

## Contribution of Cyclooxygenase to Hypoxia Mediated Cerebral Vasodilation

The mechanisms mediating hypoxic cerebral vasodilation remain relatively unknown. One potential mechanism is COX.  In humans, studies have suggested that COX may (51) or may not mediate hypoxic vasodilation (31, 49).  One possible explanation for these equivocal findings is a sex- or region-specific contribution of COX.  With this in mind, Aim 1 investigated the sex-specific contribution of COX to hypoxic cerebral vasodilation in the middle cerebral artery.  Findings from Aim 1 revealed that COX does not contribute to hypoxia-mediated increases in CBF in either sex.

Given that we had a relative even distribution of men and women in Aim 3 (5 men, 4 women) we investigated, in an exploratory fashion, the possibility of sex-differences in hypoxic vasodilation in Aim 3.  Considering all vessels, this analysis suggests that the hypoxia-mediated

increases in relative CVC are greater in women compared to men. Additionally, hypoxia-mediated increases in CVC appear to be unaffected by indomethacin in women, whereas COX restrains hypoxic cerebral vasodilation in men (Appendix F: Supplemental Data Figures; *Relative vessel-specific (Aim III) hypoxia-mediated ($S_PO_2$ = 80%) changes in cerebrovascular conductance (CVC) between sexes with and without indomethacin).* In contrast, looking specifically at the MCA there appears to be no sex-specific hypoxic cerebral vasodilation or sex-specific COX restraint (Appendix F: Supplemental Data Figures; *Re-analysis: Relative hypoxia-mediated ($S_PO_2$ = 80%) changes in middle cerebral artery (MCA) cerebrovascular conductance (CVC) between sexes and menstrual phases with and without indomethacin).* These analyses are limited by the small number of subjects from each sex, and deserve cautious interpretation. However, although exploratory, these findings subtly suggest future studies comparing sex-specific hypoxic cerebral vasodilation with direct quantification of CBF may be warranted.

Given that in Aim 1 we studied women during the early follicular phase of the menstrual cycle, when sex hormones are most similar to men, we rationalized that studying women during the late follicular phase, when estrogen is naturally high may lead to a greater contribution of COX. Data from isolated animal cerebral vessels suggest that estrogen mediates an increase in vasodilatory $PGI_2$ production. Based upon this rationale and expanding upon findings from Aim 1, we investigated the menstrual phase-specific contribution of COX to hypoxic cerebral vasodilation in Aim 2. Despite studying women during the late follicular phase of the menstrual when estrogen is naturally elevated, we found that COX does not contribute to hypoxia-mediated increases in CBF in both early and late follicular phases.

The majority of data to date suggest that COX does not contribute to hypoxia-mediated cerebral vasodilation. However, most studies have utilized TCD to measure blood flow velocity

(an index of CBF) within a given vessel. Utilizing blood flow velocity as an index of CBF is limited in that it assumes a constant vessel diameter. Given that CBF is proportional to vessel radius raised to the 4$^{th}$ power, small changes in diameter can have a profound influence on CBF. Emerging evidence suggests that cerebral vessel diameter increases during hypoxia (66, 144), which would result in velocity measures underestimating hypoxia-mediated increases in CBF. Additionally, little is known about whether indomethacin alters cerebral vessel diameter, which if diameter does change with indomethacin, data interpretation may be additionally limited. TCD is also limited in its ability to quantify flow in multiple vessels, as this methodology only allows for blood flow velocity to be measured concurrently in one or two vessels (often the MCA and PCA/ACA). Therefore, these findings cannot be extrapolated to the entire cerebral circulation. Thus, region-specific differences in hypoxic cerebral vasodilation (140) as well as region-specific contributions of COX (51) may have been overlooked in prior investigations

With this in mind, Aim 3 of this dissertation sought to explore the region-specific contribution of COX to hypoxia-mediated increases in CBF by concurrently examining eleven cerebral arteries. Contrary to our hypothesis, we found no regional differences in hypoxia-mediated increases in CBF. Interestingly, we did find that not only does COX not contribute to hypoxia-mediated cerebral vasodilation, it may be restraining hypoxia-mediated increases in CBF. We show for the first time that COX inhibition resulted in greater relative hypoxia-mediated increases in CBF, primarily mediated through increases in vessel CSA, as mean velocity remained unchanged.

The findings of Aim 3 address the limitations of using MCAv as an index of CBF. Although there was no difference in the relative change in mean velocity between placebo and indomethacin conditions during hypoxia, relative vessel CSA increased more with indomethacin

yielding a greater relative increase in CVC. Thus, depending upon methodology, conclusions may differ. For example, utilizing blood flow velocity as an index of flow, Aim 3 would have led to the conclusion that COX does not contribute to hypoxic cerebral vasodilation, which is consistent with TCD findings in Aim 1 and Aim 2. However, by accounting for changes in vessel CSA by directly quantifying flow, we are able to conclude that not only does COX not mediate hypoxic cerebral vasodilation, it restrains hypoxia-mediated increases in CBF by directly or indirectly increasing vessel tone.

In Appendix E: Supplementary Data we provide additional analysis, albeit exploratory, on the contribution of COX to hypoxia mediated cerebral vasodilation (Appendix E: Supplementary Data; *Contribution of Cyclooxygenase to Hypoxia Mediated Cerebral Vasodilation*). This analysis is very exploratory as it applies the vessel cross-sectional areas obtained from Aim 3 to the MCAv values obtained in Aim 1 and Aim 2. This allows for the calculation of MCA blood flows in both Aim 1 and Aim 2. This exploratory analysis, despite being extremely limited, suggests the potential for sex-differences in hypoxic cerebral vasodilation as well as a sex-specific role of COX. Although highly exploratory, these findings also suggest future studies comparing sex-specific hypoxic cerebral vasodilation with direct quantification of CBF may be warranted.

## Experimental Considerations

This dissertation aimed to answer fundamental questions regarding cerebrovascular control during hypoxia. With each aim, we utilized a unique subject group-specific to the question posed, tightly controlled experimental conditions, and employed a doubled-blind, randomized research design. Despite these strengths, there are limitations that need to be considered.

First, in Aim 1 and Aim 2 we utilized MCAv, as estimation of CBF, with the assumption of constant MCA diameter. Studies suggest that MCA diameter may (144) or may not change (112) change during hypoxia. Although we cannot rule out changes in MCA diameter, with Aim 3 suggesting that both hypoxia and indomethacin change cerebral vessel diameter, there is no current evidence to indicate sex-specific or menstrual phase-specific changes in diameter. If diameter changes were specific to sex or menstrual phase, we may be overlooking potential differences in hypoxic responses and the contribution of COX.

Second, 4D flow MRI methodology in Aim 3 sometimes does not capture data from all arteries, which limits statistical power and thus the confidence in some conclusions. In five subjects at least one or more of the eleven cerebral vessels were not visible. One subject was anatomically missing a VA-R, while a second subject was anatomically missing a VA-L. Additionally, the decrease in resting CVC with indomethacin within some vessels was substantial enough that it fell below the detectable limit of the current methodology. We were still able to collect a complete data set (n = 9) on five cerebral arteries including ICAs, MCAs, and BA. In the remaining vessels, we analyzed a minimum of seven subjects for each vessel.

Third, our study in Aim 3 was powered to detect an expected 50% greater VA relative reactivity compared to the ICA, MCA, and PCA. This power analysis was based upon prior research eliciting and $S_PO_2 = 70\%$ (Willie, Macleod et al. 2012). Given the slightly less severe hypoxia in the current investigation ($S_PO_2 = 81 \pm 1\%$) and inability to quantify CBF in all vessels for all subjects under all conditions, we may have been underpowered to detect regional differences in hypoxic cerebral vasodilation, as well as region-specific contributions of COX. Based upon the current data a sample size of 7 provides 80% power to detect an expected 17% increase in relative CVC during hypoxia. Despite this limitation, we provide novel evidence that

indomethacin restrains hypoxic cerebral vasodilation, which appears to not be different across the cerebral circulation.

Fourth, we utilized an absolute dose of indomethacin and did not measure the concentration of plasma COX metabolites to demonstrate adequate COX inhibition. In Aim 1, this resulted in women receiving a larger relative dose of indomethacin due to a smaller body size. Despite the greater relative dose, there was a similar $32 \pm 3\%$ and $30 \pm 2\%$ decrease in basal MCAv in both men and women, respectively. This similar to that seen with a lower dose of indomethacin administered relative to body weight (50, 51, 79). In a prior study in our lab, the same absolute dose of indomethacin (100 mg) resulted in a large decrease in circulating COX metabolites (49). Combined with our observed large reduction in basal CBF (Aim 1 ($31 \pm 2\%$), Aim 2 ($32 \pm 4\%$) and Aim 3 ($44 \pm 2\%$) and substanial reduction in hypercapnic responses (Aim1 and Aim 2 ~60%) we believe we effectively inhibited COX. From a functional perspective, indomethacin robustly inhibited COX in all three Aims, but we do not have data to support complete inhibition. With this in mind, we may still be underestimating the contribution of COX in CBF regulation.

Fifth, as part of Aim 2, we relied upon the natural sex hormone oscillations associated with the menstrual cycle to attempt to isolate the effects of estrogen in a healthy physiologic context. Given the inherent variability in sex-hormone concentrations, we were unable to completely isolate the effects of estrogen from testosterone. Thus, we cannot conclude that our lack of phase differences is due to a small estrogen effect or testosterone counteracting estrogen.

Lastly, in this dissertation we extensively investigated the role of COX in mediating hypoxic cerebral vasodilation. We providing compelling evidence that COX does not contribute

to hypoxic cerebral vasodilation in a sex-, menstrual phase-, or region-specific manner. Despite this important new knowledge, we were unable to uncover the mechanism(s) contributing to hypoxia-mediated increases in CBF. Future investigations may want to explore additional mechanisms including nitric oxide or endothelial derived hyperpolarizing factor, which have both been suggested to play a role. It may also be worthwhile to explore the idea of compensatory mechanism(s) mediating hypoxic cerebral vasodilation. Given the importance of maintaining $CDO_2$, it seems reasonable that multiple mechanisms would regulate CBF and that potential redundant mechanisms may compensate for the loss of each other. This has been shown to be the case in the peripheral circulation (88) but remains unknown in the cerebral circulation.

## Implications to Human Health

Findings from the current investigation suggest that COX does not contribute to hypoxic cerebral vasodilation and this is not different between sexes, menstrual phases, or cerebrovascular regions. Interestingly, our data indicate that COX restrains hypoxic cerebral vasodilation across the cerebral circulation. Our data also demonstrate that COX substantially (up to $44 \pm 2\%$) contributes to the maintenance of basal CBF, which is not sex- or region-specific, but is menstrual phase-specific.

There are a variety of diseases that are characterized by hypoxia including obstructive sleep apnea, chronic obstructive pulmonary disease (i.e bronchitis and emphysema), and cerebrovascular and cardiovascular ischemic insults (i.e. stroke and heart attack). There are also millions of people who sojourn to high altitudes for their livelihoods or recreational purposes each year. In light of the findings from this dissertation, there are implications to human health.

First, indomethacin should be avoided under conditions of hypoxia. Indomethacin substantially reduces basal CBF (up to $44 \pm 2\%$) and compromises $CDO_2$. Coupled with hypoxia this could be detrimental as the compromised $CDO_2$ during hypoxia results in an OEF that is near the tolerable limit of consciousness in healthy humans, let alone individuals with pre-existing conditions. There are many suitable alternatives to indomethacin that do not affect basal CBF while providing analgesia (54).

Second, indomethacin may serve as a useful headache medicine due to its analgesic effects and ability to regulate basal CBF. Headaches occur for a variety of reasons, but one potential cause is an increase in CBF(121). Given the large reduction in CBF with indomethacin, it may be an effective treatment for headaches. Indomethacin may be especially useful for headaches associated with the increase in CBF associated with the late follicular phase of the menstrual cycle. As shown in Aim 2, the elevated basal CBF in the late follicular phase of the menstrual cycle is mediated by COX.

Third, stroke is the most common cerebrovascular disease in the United States accounting for over $73 billion in annual medical and disability costs. Stroke is known to be both sex-specific and regionally distributed within the brain. Data from Aim 1 suggest that hypoxic cerebral vasodilation is not different between sexes nor is the contribution of COX. Thus, COX likely does not mediate sex-specific stroke risk, and additional cerebrovascular control mechanisms such as nitric oxide synthase or endothelial derived hyperpolarizing factors need to be considered.

## Conclusion

The aim of this dissertation was to investigate the sex-, menstrual phase-, and region-specific contribution of COX in mediating hypoxic increases in CBF. Our findings suggest that

COX does not contribute to hypoxic cerebral vasodilation and this is not different between sexes, menstrual phases, or cerebrovascular regions.  Interestingly, our data suggest that COX restrains hypoxic cerebral vasodilation across the cerebral circulation.  Although not the primary aim of this dissertation, our data also demonstrate that COX substantially contributes (up to $44 \pm 2\%$) to the maintenance of basal CBF, which is not sex- or region-specific, but is menstrual phase-specific.  Additionally, data from Aims 1 and 2 indicate that COX mediates a large portion of hypercapnic vasodilation, which is not sex- or menstrual phase-specific.  Considered collectively, this is the most comprehensive investigation to date exploring the role of COX in the cerebral circulation. These data are the first to suggest COX-mediated restraint of hypoxic cerebral vasodilation and indicate a need for high resolution imaging studies of the human cerebral circulation.  These data will substantially contribute to our knowledge of CBF regulation, specifically the role of COX, with the hope that it will aid in the development of treatments for improvement of the human condition.

# Appendices

Appendix A: Background

*Hagen-Poiseuille Equation*

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

The Hagen-Poiseuille Equation.  A small change in vessel diameter (radius) can have a profound influence of blood flow.  This is indicated by blood flow being proportional to the radius raised to the fourth power.  Q, flow rate; P, perfusion pressure; r, vessel radius; η, blood viscosity; l, vessel length.

*Cyclooxygenase Pathway*
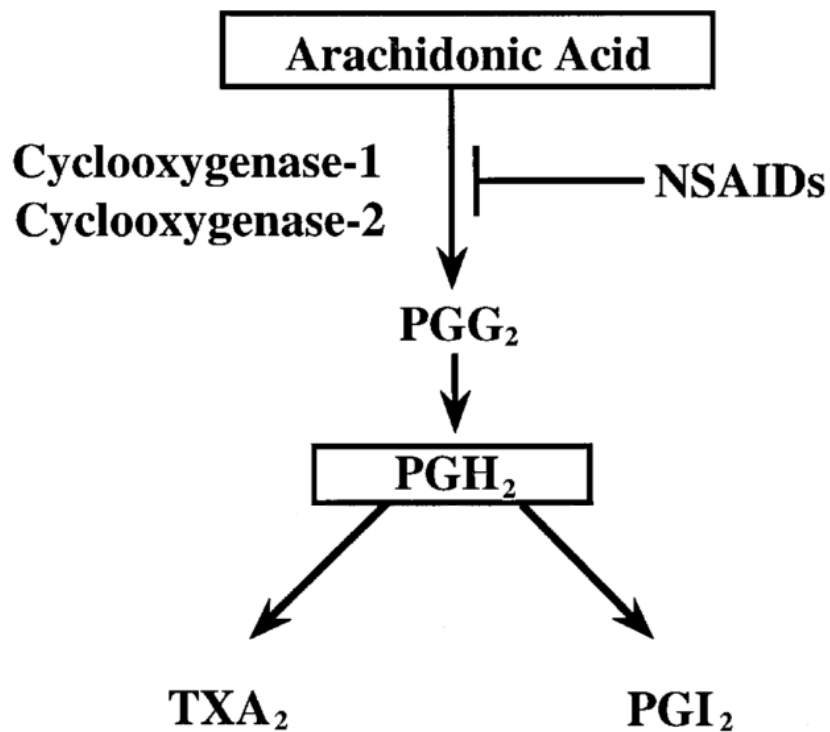


The cyclooxygenase pathway.  Cyclooxygenase is the rate-limiting step in the conversion of arachidonic acid to the vasoactive compounds prostacyclin (PGI2) and thromboxane (TXA2). Figure adapted from Dubois et al. (1998) FASEB J.

*Regional Blood Flow Distribution during Hypoxia*
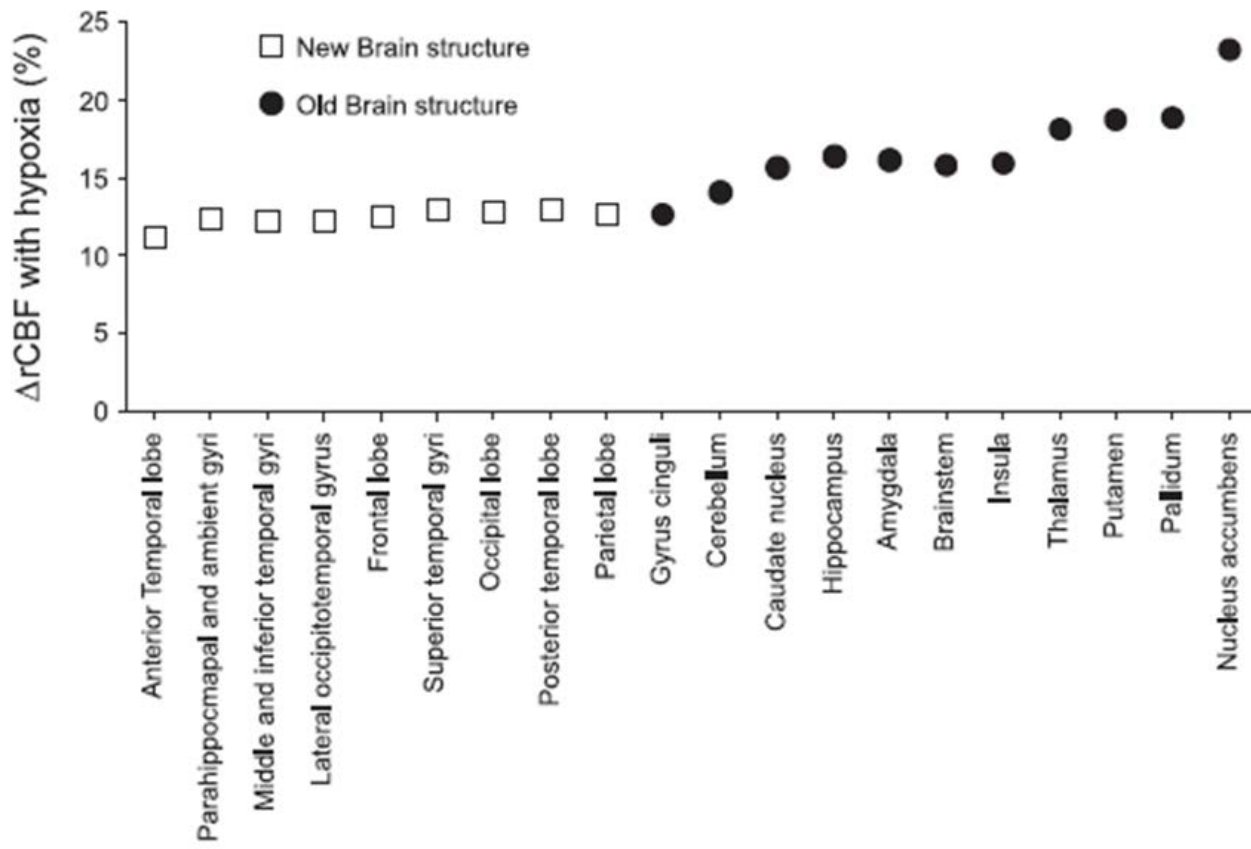


Regional blood flow distribution during hypoxia. "Changes in CBF to different regions of interest during hypoxia. Structures are listed in ascending order of blood flow increase. For clarity, region of interest data shown are the mean of the left and right side of the brain. … Changes in regional CBF($\Delta$rCBF) are expressed as a percent increase above normoxic levels…" (11).

Appendix B: Methods

*4-Dimensional Flow Magnetic Resonance Imaging (PC-VIPR)*



4-Dimensional Flow Magnetic Resonance Imaging (PC-VIPR). 4a. Coronal PC-VIPR angiogram. 4b. Axial PC-VIPR angiogram. 4c. Sagittal PC-VIPR angiogram. 4d. Coronal PC-VIPR angiogram overlaid with velocity tracings. 4e. Axial PC-VIPR angiogram overlaid with velocity tracings. 4f. Sagittal PC-VIPR angiogram overlaid with velocity tracings. Blue boxes indicate cut-planes for 3.45 mm measurement sections within a given vessel. Arteries included in analysis: VA, vertebral artery (r) right and (l) left; BA, basilar artery; PCA, posterior cerebral artery (r) right and (l) left; internal carotid artery, ICA (r) right and (l) left; MCA, middle cerebral artery (r) right and (l) left; ACA, anterior cerebral artery (r) right and (l) left.
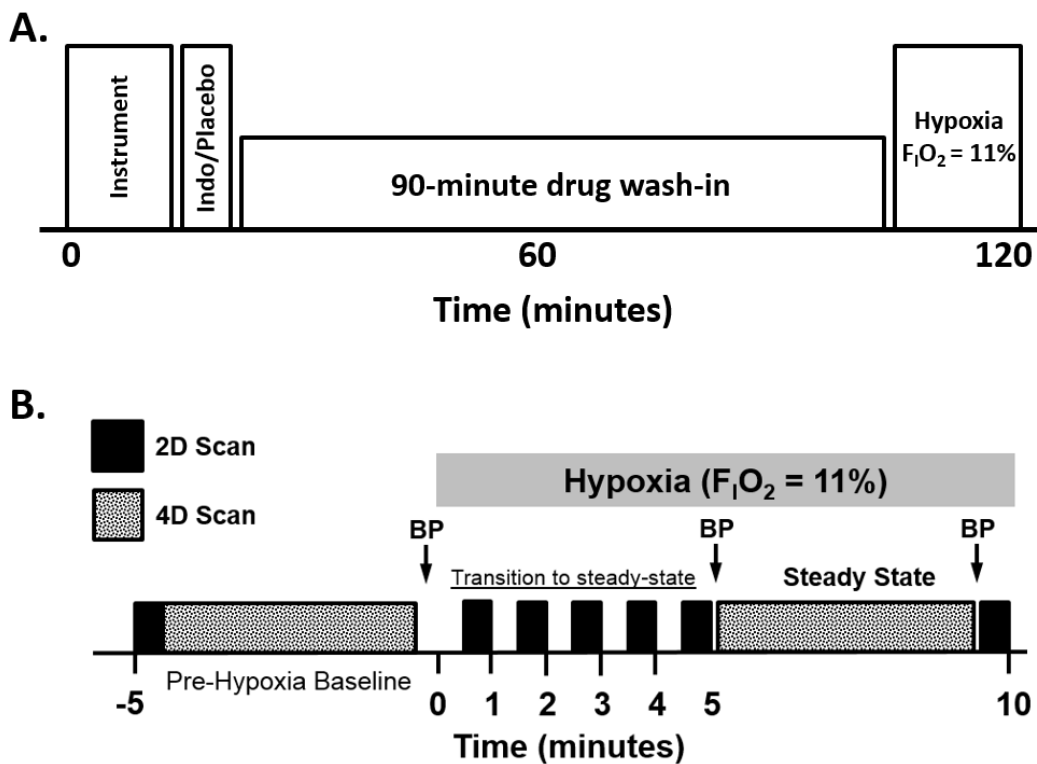
# Appendix C: Experimental Protocol

*Study timeline for addressing Aim 1 and Aim 2*
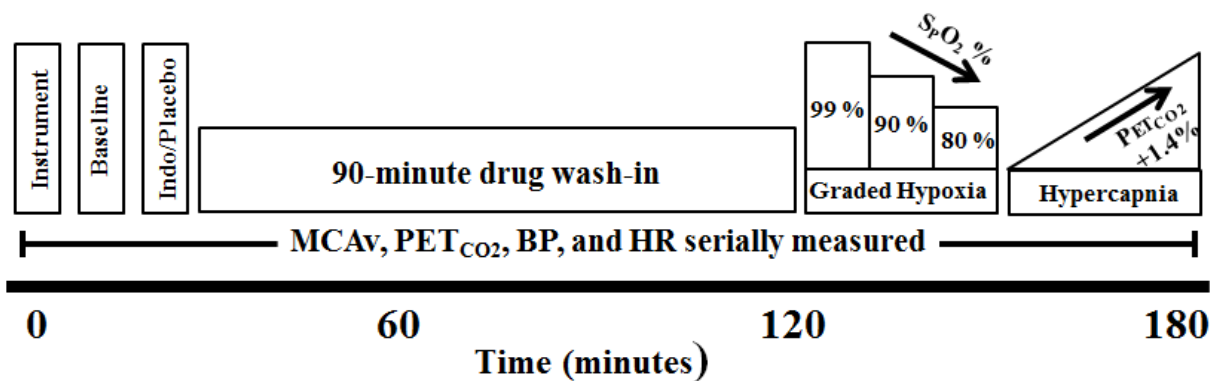


Study timeline for addressing Aim 1 and Aim 2. For Aim 1 subjects were studied twice with either indomethacin or placebo in a randomized, double-blinded design. For Aim 2 subject were studied a total of four times, twice during the early follicular phase and twice during the late follicular phase of the menstrual cycle. Placebo and indomethacin were administered during each of the two phases in a randomized, double-blinded design. Indo, indomethacin, MCAv, middle cerebral artery velocity, $PET_{CO2}$, end-tidal carbon dioxide; BP, blood pressure; HR, heart rate; $S_PO_2$, pulse oximetry oxygen saturation.

*Study timeline for addressing Aim 3*



Study timeline for addressing Aim 3. Subjects were studied twice with either indomethacin or placebo in a randomized, double-blinded design. 4a. Complete study protocol for Aim 3. Following baseline scan, subject received either indomethacin of placebo. Following a 90-minute waiting period the hypoxia scan commenced. 4b. Detailed description of hypoxia portion of protocol. Indo, indomethacin, $F_IO_2$, inspired oxygen percentage; BP, blood pressure.

Appendix D: Data Collection and Analysis

*Aims I and II: Hypoxia Data Collection*
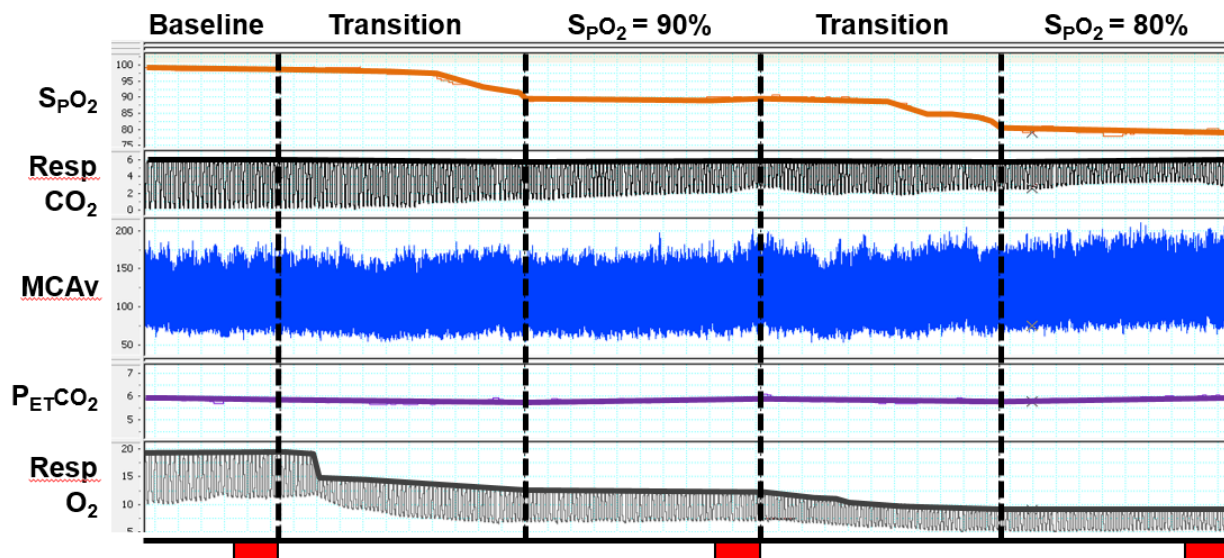


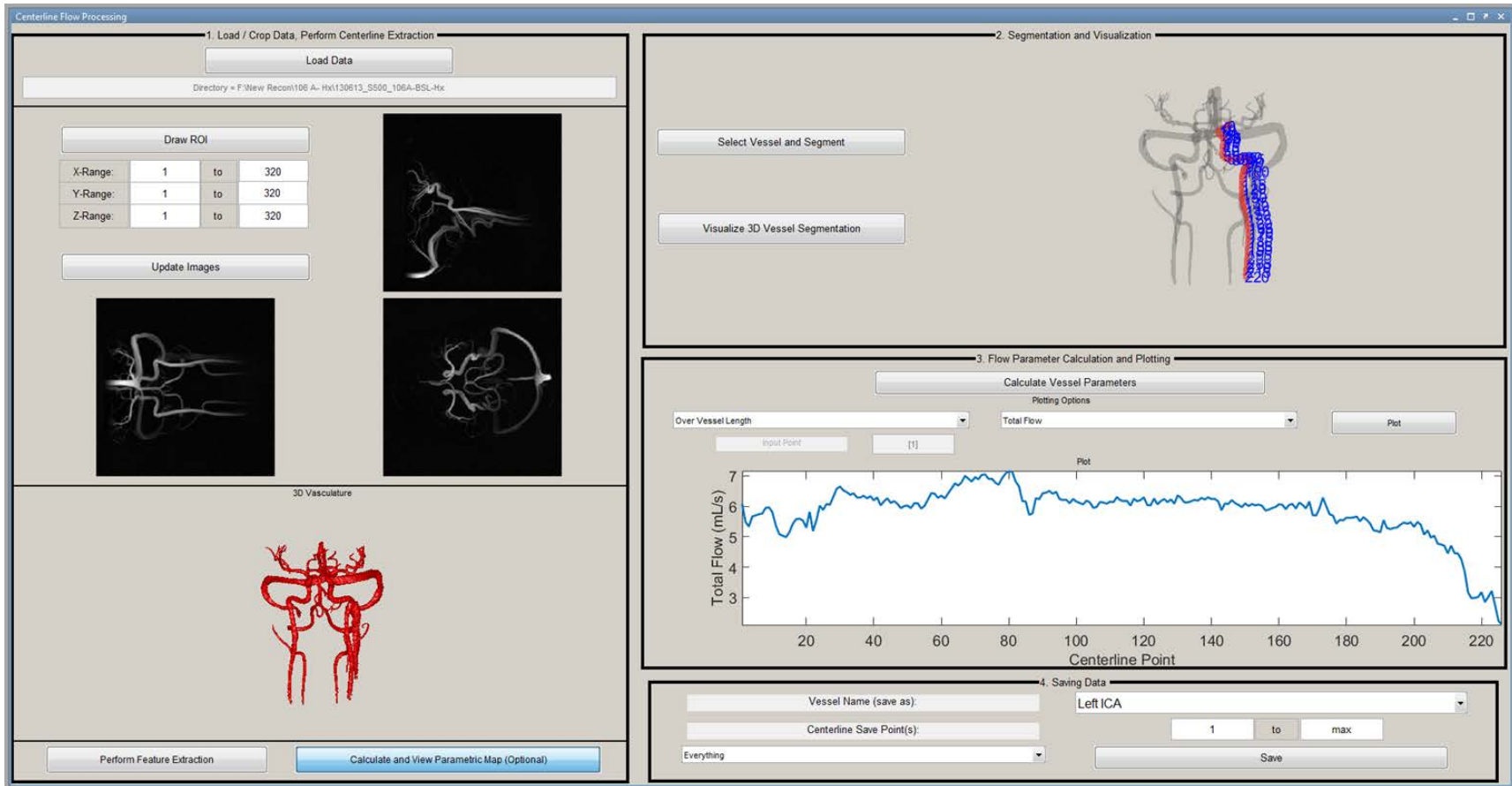30 seconds of averaged data, blood pressure

The above figure is representative of data collected during hypoxia trials for Projects I and II. Each hypoxia trial consisted of 5-minutes of baseline data recording, transition to hypoxia eliciting and $S_PO_2 = 90\%$, 5-minutes of $S_PO_2 = 90\%$, transition to $S_PO_2 = 80\%$, 5-minutes of $S_PO_2 = 80\%$, and removal of hypoxic stimulus. The last 30 seconds of baseline, $S_PO_2 = 90\%$, and, $S_PO_2 = 80\%$ were used for analysis. Variables including pulse oximetry oxygen saturation (SPO2), respiratory $CO_2$ (Resp $CO_2$), respiratory $O_2$ (Resp $O_2$), end-tidal $CO_2$ ($P_{ET}CO_2$), and middle cerebral artery velocity (MCAv) were recorded.

*Aims I and II: Hypercapnia Data Collection*
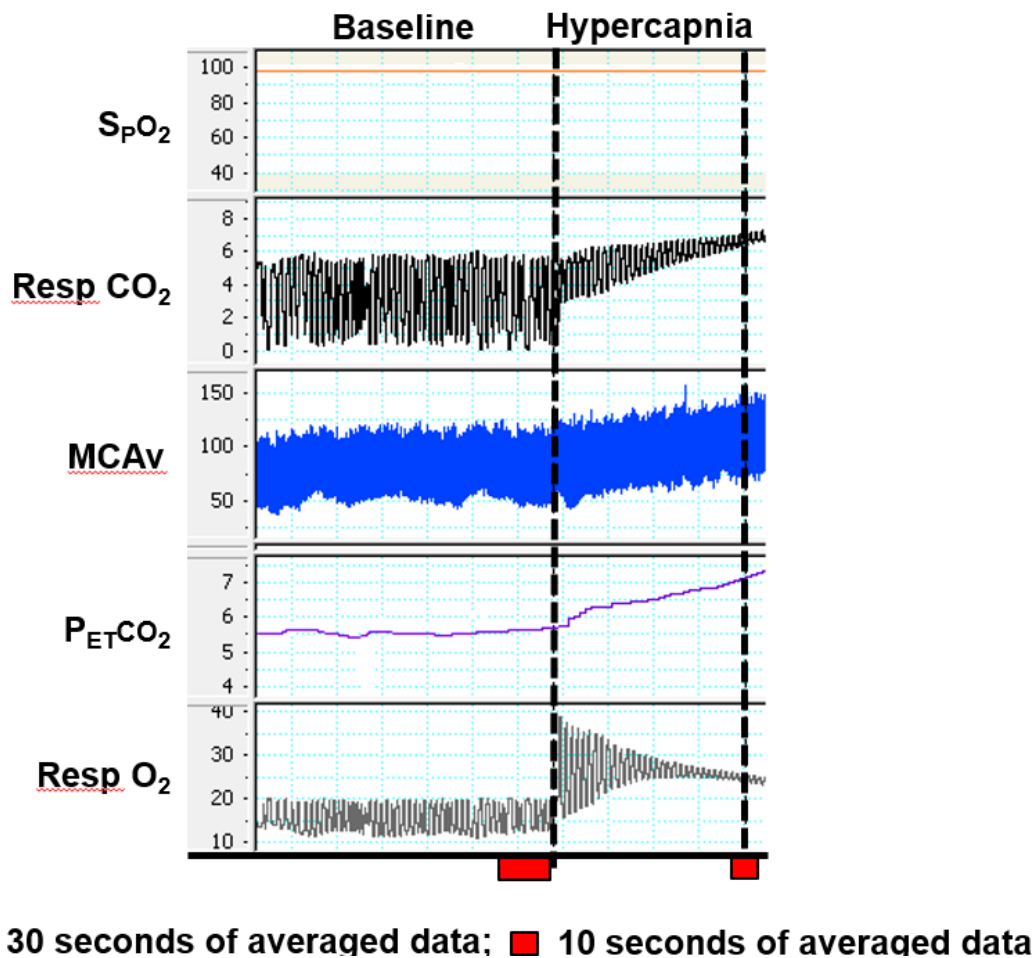


The above figure is representative of data collected during hypercapnic trials for Projects I and II. Each hypercapnic trial consisted of 5-minutes of baseline data recording, hypercapnia eliciting and increase in PETCO2 +10 mmHg, and removal of hypercapnic stimulus. The last 30 seconds of baseline and last 10 seconds of hypercapnia were used for analysis. Variables including pulse oximetry oxygen saturation (SPO2), respiratory $CO_2$ (Resp $CO_2$), respiratory $O_2$ (Resp $O_2$), end-tidal $CO_2$ ($P_{ET}CO_2$), and middle cerebral artery velocity (MCAv) were recorded.

*Aim III: Data Analysis*



The above figure is representative of data analysis for Aim 3. The eleven cerebral vessels of interest were analyzed on an individual

basis, with the left internal carotid artery demonstrated in the above figure. Each hypoxia trial consisted of 5-minutes of baseline data

collection, followed by a transition to hypoxia (<5 minutes), whereupon steady state hypoxia the 4D Flow MRI scan commenced.

Subjects inspired hypoxic gas ($F_IO_2$ = 11%), while ETCO$_2$ was clamped by titrating inspired gas mixture with $CO_2$.

Appendix E: Supplementary Data

*Contribution of Cyclooxygenase to Basal Cerebral Blood Flow Regulation*


*Exploratory Analysis*


The oxygen demand of the brain is about 10x greater than expected given its size, due to its almost complete reliance on oxidative metabolism (42).  Thus, the maintenance of CBF is of critical importance.  Despite this importance, the mechanism(s) regulating CBF remain poorly understood.  COX is one mechanism that has been shown to partially contribute to the maintenance of basal CBF.   On average, the non-selective COX inhibitor indomethacin causes an ~30% reduction in MCAv (7, 8, 19, 31, 32, 49-51, 54, 64, 79).  This is in agreement with findings from Aim 1 and Aim 2, which demonstrate reductions in MCAv of $31 \pm 2\%$ and $32 \pm 4\%$ with indomethacin.  Findings from Aim 3 suggest a substantially greater basal contribution of COX as indomethacin reduced CVC by $44 \pm 2\%$.

Given the greater than expected contribution of COX to the maintenance of basal CBF in Aim 3, we thought it was important to explore whether TCD underestimated the influence of indomethacin in Aim 1 and Aim 2.  We were able to calculate MCA blood flow in Aim 1 and Aim 2 by utilizing the MCAv obtained in these respective Aims, combined with MCA cross-sectional areas obtained in Aim 3 during both placebo and indomethacin conditions.  Aim 3 had a total of nine subjects of which five were men and four were women.  Thus, we had relatively even distribution of sexes. CBF was calculated at the product of mean MCAv (cm/s) and vessel CSA ($cm^2$) and was reported in ml/min. A key assumption in this calculation is the CSA from 9 healthy subjects is reflective of all healthy adults, which limits our interpretation.

$$CBF = mean\ MCA\ velocity * cross\text{-}sectional\ area * 60\ s/min$$

CVC was then calculated as CBF divided by mean arterial blood pressure, multiplied by 100.

$$CVC = CBF / MABP *100$$

Based upon the new calculations for Aim 1 and in agreement with our original findings, we confirm that the contribution of COX to resting basal CBF and CVC is similar between sexes. However, our new calculations also yield a greater contribution of COX to the maintenance of resting CBF. We originally showed a $31 \pm 2\%$ reduction in basal MCA velocity with indomethacin in Aim 1 however, by using cross-sectional areas from Aim 3 and calculating CBF we show an ~43% reduction in CVC in both sexes (Appendix E: Supplementary Data Figures; *Aim I Basal Cerebral Blood Flow Re-Analysis*). This is in agreement with our $44 \pm 2\%$ reduction in basal CVC found in Aim 3.

Similarly, calculating CVC for Aim 2 confirmed our original conclusion that greater CVC in the late follicular phase of the menstrual cycle is abolished with indomethacin. However, in Aim 2 we may have also underestimated the contribution of COX in maintenance of resting CBF. Originally, in Aim 2 we demonstrated a $32 \pm 4\%$ decrease in MCAv with indomethacin, whereas our new analysis with cross-sectional areas obtain from Aim 3 indicates an ~44% reduction in basal CVC with indomethacin (*Appendix E: Supplementary Data Figures; Aim I Basal Cerebral Blood Flow Re-Analysis*). This is again in agreement with our findings from Aim 3. Thus, we conclude that direct quantification of CBF in Aim 3 may provide a more accurate representation of the basal contribution of COX as it accounts for changes in cerebral artery diameter with indomethacin.

As part of this dissertation, we investigated sex-, menstrual phase- and region specific contributions of COX to the maintenance of basal CBF. In Aim 1 we found no sex difference in

basal contribution of COX, which is supported with our re-analysis. In Aim 2, we provide

evidence that the increase in CBF during the late follicular phase of the menstrual cycle,

compared to early follicular phase, is due to a greater basal contribution of COX, which is also

supported by our re-analysis. The re-analysis in Aim 2 is limited in that it is unknown how

menstrual phase influences CSA at rest, with indomethacin, and during hypoxia. For this re-

analysis, we assumed similar CSA changes between the early and late follicular phases of the

menstrual cycle, which could limit our ability to detect menstrual phase differences in the

contribution of COX. Lastly, Aim 3 found no region-specific differences in the basal

contribution of COX. Considered collectively, it appears that the role of COX in the regulation

of resting CBF increases during the late follicular compared to early follicular phase of the

menstrual cycle, but there are no sex or region-specific differences.

It is important to note that our MCA cross-sectional areas obtained from Aim 3 and used

to calculate CBF responses, were obtained from a small number of subjects which included only

5 men and 4 women and therefore this analysis is only exploratory. Additionally, the subjects

from Aim 3 were different from those in Aim 1 or Aim 2 however, the demographics of subjects

were similar between Aims.

*Contribution of Cyclooxygenase to Hypoxia Mediated Cerebral Vasodilation*

*Exploratory Analysis*

Given our lack of sex and menstrual phase differences in Aim 1 and Aim 2, which if there were any, may have been overlooked by our MCAv measures, we went back to our original Aims and calculated the CBF during hypoxia with and without indomethacin. Similar to how we re-analyzed the contribution of COX to resting CBF in Aim 1 and Aim 2 (Appendix E: Supplementary Data; *Contribution of Cyclooxygenase to Basal Cerebral Blood Flow Regulation)*, we re-calculated our sex- and menstrual phase-specific CBF responses in Aim 1 and Aim 2 from the MCA velocities obtained in these respective aims, while using MCA cross-sectional areas obtained from Aim 3 for the respective conditions. CBF was calculated at the product of mean MCAv (cm/s) and vessel CSA (cm$^2$) and was reported in ml/min. CVC was then calculated as CBF divided by mean arterial pressure, multiplied by 100.

In our original Aim 1 we hypothesized that hypoxic cerebral vasodilation would be greater in women than men, due to a greater contribution of COX. Measuring MCAv led us to the conclusion that COX does not mediate hypoxic cerebral vasodilation and this is not different between sexes. However, by calculating CBF and ultimately CVC our conclusions differ. Specifically, this re-analysis shows that hypoxic cerebral vasodilation is greater in women than men, and that COX restrains hypoxic-cerebral vasodilation in men but not in women (Appendix F: Supplemental Data Figures; *Re-analysis: Relative hypoxia-mediated ($S_PO_2 = 80\%$) changes in middle cerebral artery (MCA) cerebrovascular conductance (CVC) between sexes and menstrual phases with and without indomethacin)*. Although this conclusion supports our original

hypothesis, it is important to note that our MCA cross-sectional areas obtained from Aim 3 and used to calculate CBF responses, were obtained from a small number of subjects, which included only 5 men and 4 women. Additionally, the subjects from Aim 3 were different from those in Aim 1 or Aim 2 however, the demographics of subjects were similar between Aims. Given these differing conclusions, one similarity is that COX does not appear to mediate hypoxic cerebral vasodilation. These data estimates are limited to the MCA, indicating a need for a well-powered study to directly test for sex differences in hypoxic cerebral vasodilation (and mechanisms)

In Aim 2 we hypothesized that hypoxic cerebral vasodilation would be greater in the late follicular compared to the early follicular phase of the menstrual cycle, due to a greater contribution of COX. Using MCAv as an index of CBF, we concluded that hypoxic cerebral vasodilation is similar between menstrual phases and unaffected by indomethacin. Calculating CBF from MCA cross-sectional areas obtained in Aim 3, identical conclusions were reached that the late follicular phase does not increase hypoxic vasodilation compared to early follicular phase of the menstrual cycle (Appendix F: Supplemental Data Figures; *Re-analysis: Relative hypoxia-mediated ($S_PO_2$ = 80%) changes in middle cerebral artery (MCA) cerebrovascular conductance (CVC) between sexes and menstrual phases with and without indomethacin)*. It is worth noting that we did not have MCA cross-sectional areas from the late follicular phase of the menstrual cycle, as the women in Aim 3 were all studied in the early follicular phase of the menstrual cycle. Therefore, we used the same MCA cross-sectional areas for the early and late follicular phases. There is no available evidence to suggest that indomethacin- or hypoxia-mediated changes in MCA cross-sectional are menstrual phase-specific, but this remains a possibility as late phase increases in CSA would lead us to underestimate phase differences.

Considering that our re-analysis in Aim1 suggests that hypoxic cerebral vasodilation is sex-specific as is the COX-mediated restraint of hypoxic cerebral vasodilation, we investigated the possibility of sex differences further in Aim 3. Considering all vessels, the hypoxia-mediated increase in relative CVC is greater in women compared to men. Additionally, hypoxia-mediated increases in CVC are unaffected by indomethacin in women, whereas COX restrains hypoxic cerebral vasodilation in men (Appendix F: Supplemental Data Figures; *Relative vessel-specific (Aim III) hypoxia-mediated ($S_PO_2 = 80\%$) changes in cerebrovascular conductance (CVC) between sexes with and without indomethacin.).* In contrast, looking specifically at the MCA there appears to be no sex-specific hypoxic cerebral vasodilation or sex-specific COX restraint (Appendix F: Supplemental Data Figures; *Re-analysis: Relative hypoxia-mediated ($S_PO_2 = 80\%$) changes in middle cerebral artery (MCA) cerebrovascular conductance (CVC) between sexes and menstrual phases with and without indomethacin).* However, these findings are most likely limited by the small number of subjects in from each sex (5 men and 4 women).

In light of this sex-specific exploration and re-analyses, data suggest the possibility of sex-specific hypoxic cerebral vasodilation and sex-specific COX restraint of hypoxic cerebral vasodilation. Re-analyzing data from Aim 1 and combined with data from Aim 3 support the notion that hypoxic cerebral vasodilation in greater in women than men. Additionally, re-analyzing data from both Aim 1 and Aim 2 and combined with data from Aim 3 suggests hypoxic cerebral vasodilation is greater in women than men, and that COX restrains hypoxic-cerebral vasodilation in men but not in women (Appendix F: Supplemental Data Figures; *Combined re-analysis: Relative hypoxia-mediated ($S_PO_2 = 80\%$) changes in middle cerebral artery (MCA) cerebrovascular conductance (CVC) between sexes with and without indomethacin).* Although these data are exploratory at best, future studies utilizing advanced

imaging technology are warranted to explore sex-specific hypoxic cerebral vasodilation and sex-specific COX restraint of hypoxic cerebral vasodilation.

Appendix F: Supplementary Data Figures

*Aim I Basal Cerebral Blood Flow Re-Analysis*
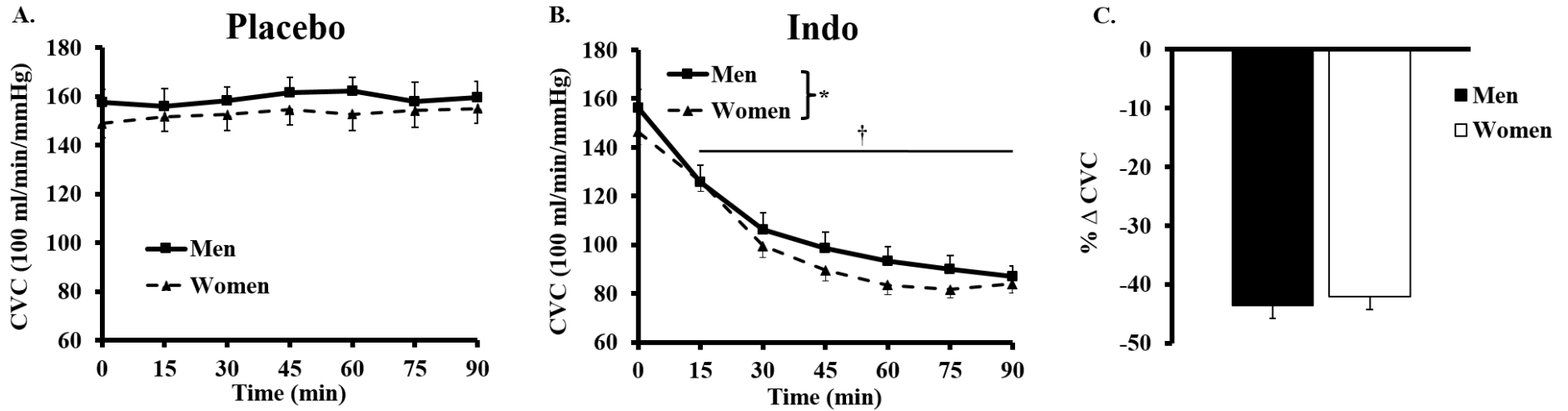


Basal cerebrovascular conductance (CVC) following placebo or indomethacin (Indo) administration. **A.** CVC was not different between sexes over the 90-minutes of placebo wash-in. **B.** MCAv decreased by 15-minutes of Indo wash-in, and was less in women. **C.** The relative change in CVC 90-minutes following Indo was similar between sexes and resulted in an ~43% decrease in CVC. * Main effect of sex; † main effect of Indo; $p < 0.05$.

*Aim II Basal Cerebral Blood Flow Re-Analysis*
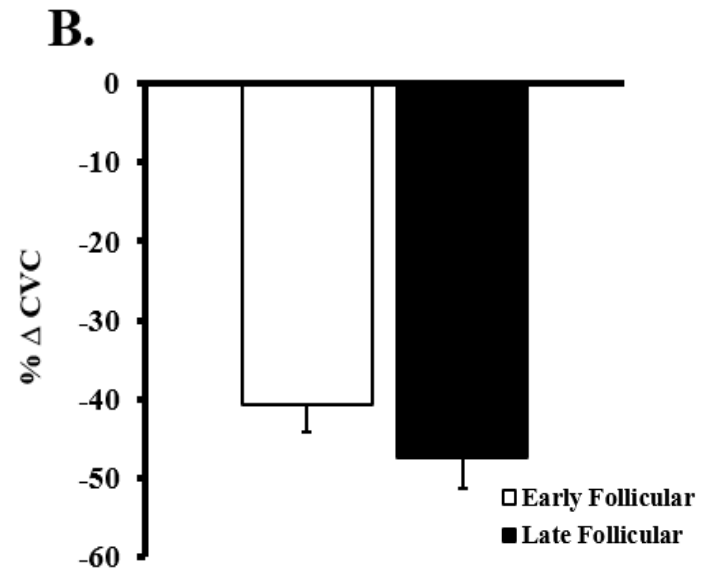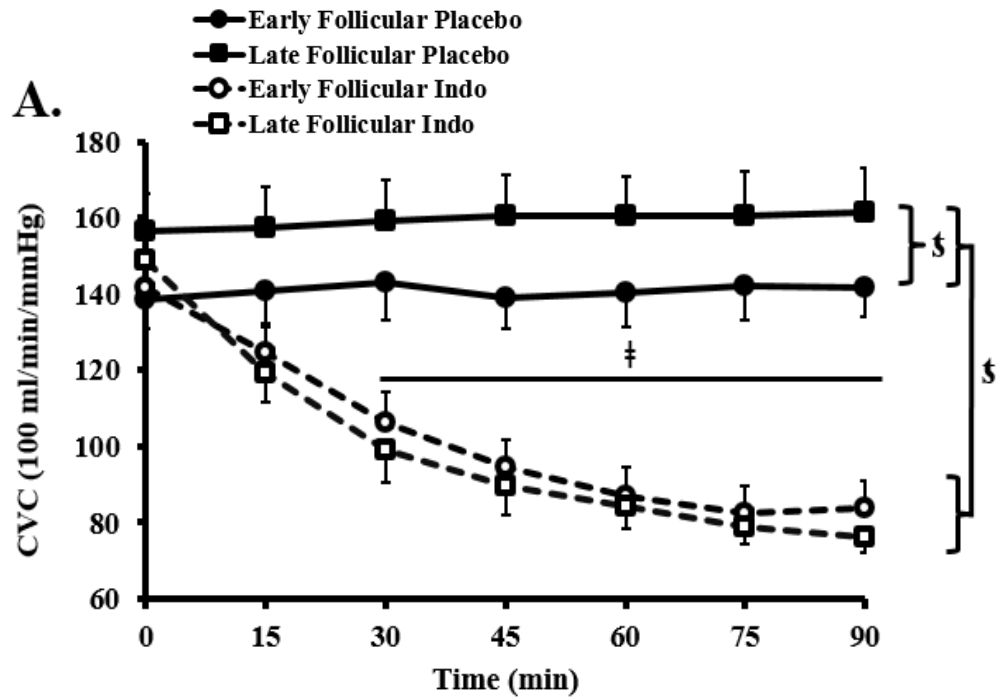


Basal cerebrovascular conductance (CVC) during early and late follicular phases of the menstrual cycle with placebo or indomethacin (Indo). **A.** During placebo CVC was greater in the late follicular phase compared to the early follicular phase of the menstrual cycle (phase x drug, P=0.001). Administration of indomethacin decreased CVCi by the 30-minute time point (drug x time, P<0.001) and

abolished the phase difference in basal CVCi (phase x drug, P=0.001).  **B**. The relative decrease in CVC was similar between the early

and late follicular phase of the menstrual cycle with indomethacin decreasing CVC by ~ 44%.  ꙅ phase x drug interaction; ‡ drug x

time interaction, vs time-point 0; $p < 0.05$.

*Re-analysis: Relative hypoxia-mediated ($S_PO_2$ = 80%) changes in middle cerebral artery (MCA) cerebrovascular conductance (CVC) between sexes and menstrual phases with and without indomethacin.*
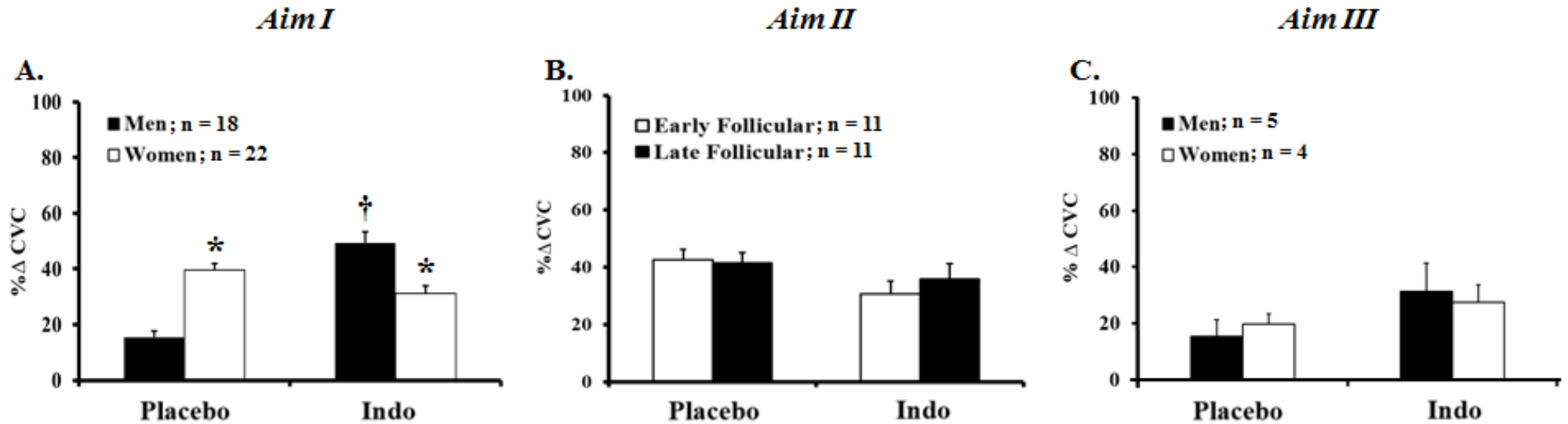


Relative hypoxia-mediated ($S_PO_2$ = 80%) changes in middle cerebral artery (MCA) cerebrovascular conductance (CVC) betw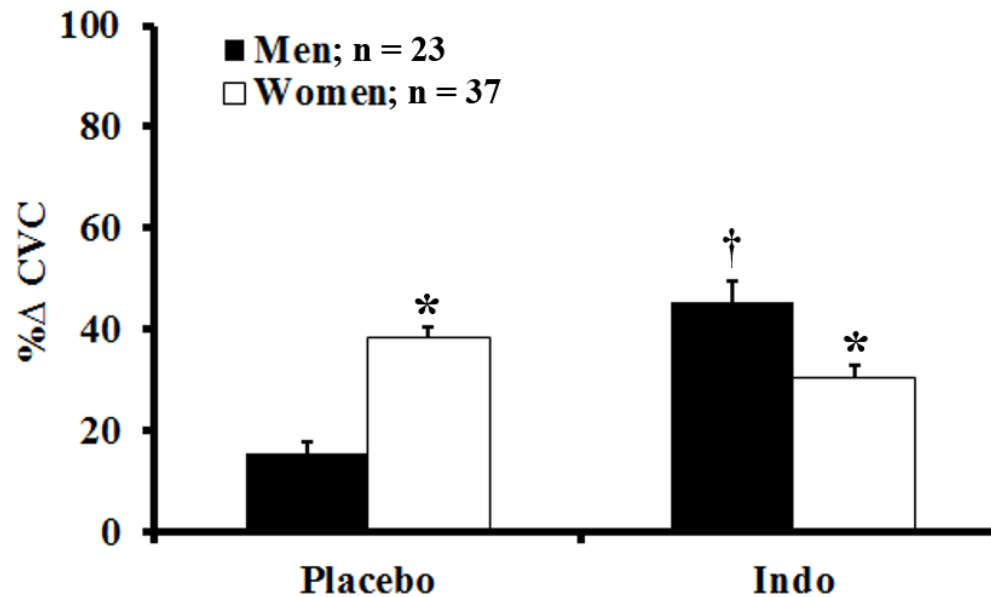een sexes and menstrual phases with and without indomethacin (Indo). CVC was calculated from MCA velocities obtained in Aims I and II via MCA cross-sectional areas obtained in Aim 3. CVC was calculated as CVC = [(mean MCA velocity * MCA cross-sectional area * 60) / (mean arterial blood pressure) * 100]. **A.** The relative hypoxia-mediated increase in CVC was greater in women than men with placebo and greater in men than women with indomethacin. Indomethacin augmented the relative hypoxia-mediated increase in

CVC in men but had no effect in women. **B.** The relative hypoxia-mediated increase in CVC was similar between the early and late follicular phases of the menstrual cycle and was unaffected by indomethacin. **C.** The relative hypoxia-mediated increase in CVC was similar between sexes and there was a trend for the relative hypoxia-mediated increase in CVC being augmented with indomethacin.

* vs men within condition, † vs placebo within sex; group x pill interaction, $p < 0.001$

*Combined re-analysis: Relative hypoxia-mediated (S$_P$O$_2$ = 80%) changes in middle cerebral artery (MCA) cerebrovascular conductance (CVC) between sexes with and without indomethacin.*



Combined relative hypoxia-mediated (S$_P$O$_2$ = 80%) changes in middle cerebral artery (MCA) cerebrovascular conductance (CVC) between sexes with and without indomethacin from Aims 1, 2, and 3. CVC was calculated from MCA velocities obtained in Aims 1 and 2 via MCA cross-sectional areas obtained in Aim 3. CVC was calculated as CVC = [(mean MCA velocity * MCA cross-sectional area * 60) / (mean arterial blood pressure) * 100]. The relative hypoxia-mediated increase in CVC was greater in women than men

with placebo and greater in men than women with indomethacin. Indomethacin augmented the relative hypoxia-mediated increase in

CVC in men but had no effect in women. * vs men within condition, † vs placebo within sex; group x pill interaction, p < 0.001.

*Relative vessel-specific (Aim III) hypoxia-mediated (S$_P$O$_2$ = 80%) changes in cerebrovascular conductance (CVC) between sexes with and without indomethacin.*



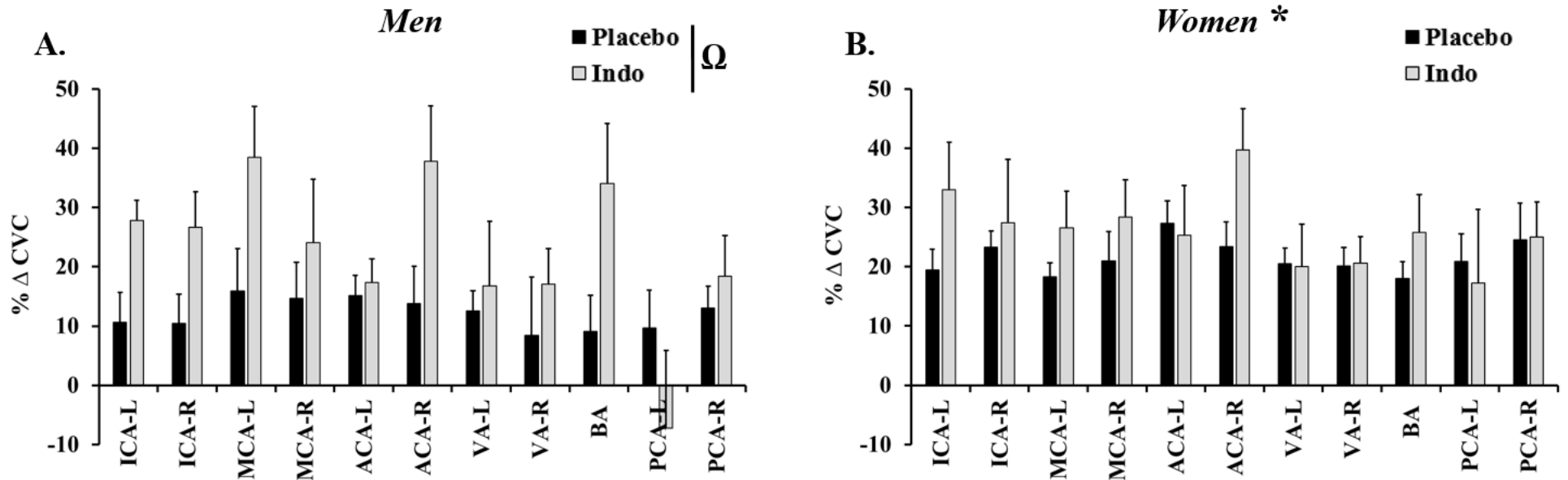Relative vessel-specific (Aim III) hypoxia-mediated (S$_P$O$_2$ = 80%) changes in cerebrovascular conductance (CVC) between sexes with and without indomethacin (Indo). **A.** The relative hypoxia-mediated increases in CVC were augmented with indomethacin in men and not different between vessels. **B.** The relative hypoxia-mediated increases in CVC were greater in women compared to men and the relative hypoxia-mediated increases in CVC in women were unaffected by indomethacin and similar between vessels. Ω main effect of pill; * main effect of sex; $p < 0.01$.

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