CARBON DIOXIDE MEDIATED VASOMOTION OF EXTRA-CRANIAL CEREBRAL ARTERIES: A ROLE FOR PROSTAGLANDINS?

by

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Abstract

Cerebrovascular regulation during perturbations in arterial CO₂ is thought to occur solely at the level of the pial vessels. However, recent evidence implicates large extra-cranial cerebral blood vessels in this regulatory process. Although the mechanisms governing CO₂ mediated vasomotion remain unclear, animal and human studies support a large role of prostaglandins. Thus, we examined two hypotheses: 1) vasomotion of the internal carotid artery (ICA) would occur in response to both hyper and hypocapnia; and 2) pharmacological inhibition of prostaglandin synthesis with Indomethacin (INDO; a non-selective cyclooxygenase inhibitor) would reduce the vasomotor response of the ICA to changes in end-tidal PCO₂ (P<sub>ET</sub>CO₂).

Using a randomized single-blind placebo controlled study, subjects (n=10) were tested on two occasions. Before and 90-minutes following either oral INDO (1.2mg/kg) or placebo capsule, concurrent measures of beat-by-beat blood flow, velocity and diameter of the ICA were made at rest and during steady state stages (4 min) of iso-oxic hypercapnia (+3, +6, +9mmHg above baseline) and hypocapnia (-3, -6, -9mmHg below baseline). End-tidal forcing was employed for the control of blood gases. To examine if INDO affected ICA vasomotion in a cyclooxygenase inhibition independent manner, a subset of subjects (n=5) were tested before and 45-minutes following oral Ketorolac (0.25mg/kg). During pre-drug testing in the INDO trial, the ICA dilated during hypercapnia at +6mmHg (4.72±0.45 vs. 4.95±0.51mm; P<0.001) and +9mmHg (4.72±0.45 vs. 5.12±0.47mm; P<0.001), and constricted during hypocapnia at -6mmHg (4.95±0.33 vs. 4.88±0.27mm; P<0.05) and -9mmHg (4.95±0.33 vs. 4.82±0.27mm; P<0.001). Following INDO, dilation of the ICA was still observed at +6mmHg (4.50±0.54 vs. 4.57±0.52mm; P<0.05) and +9mmHg (4.50±0.54 vs. 4.61±0.50mm; P<0.01); however, INDO reduced the vasomotor responsiveness by 67±28% (0.045±0.015 vs. 0.015±0.012mm ∙ mmHgP<sub>ET</sub>CO₂⁻¹). In the Ketorolac condition, there was no effect of the drug on the vasomotor response to hyper or hypocapnia. We conclude that: 1) changes in P<sub>ET</sub>CO₂ mediate vasomotion of the ICA, 2) inhibition of non-selective prostaglandin synthesis via INDO markedly reduces the vasomotor response to changes in P<sub>ET</sub>CO₂; and 3) INDO may be acting via a mechanism(s) independent of cyclooxygenase inhibition to reduce CO₂ mediated vasomotion.
Preface

Chapter 1. Aspects from Chapter 1 (see “1.4 Measurement of cerebral blood flow”) have been published elsewhere: Ainslie PN., Hoiland RL. (2014) Transcranial Doppler Ultrasound: Valid, Invalid, or Both? Journal of Applied Physiology. 117(10), 1081-1083. Both the editorial first draft and figure 1 were created in cooperation with Prof. Ainslie. I completed the revisions following the editor’s comments in cooperation with Prof. Ainslie. ©Permission was not required for reproduction of figures and text upon request. I wrote Chapter 1, and received extensive feedback from Prof. Ainslie through several editing processes.

Chapter 2. Chapter 2 will be submitted to J Physiol for publication. Prof. Ainslie and myself planned the experiment. Data collection was completed with the assistance of Mike Tymko, Anthony Bain, and Kevin Wildfong. Dr. Brad Monteleone provided useful discussions for the selection of our drug interventions. This study was completed at The University of British Columbia – Okanagan Campus. I completed all data analysis and wrote the manuscript. Prof. Ainslie provided extensive feedback and critically reviewed the manuscript for appropriate data interpretation and content. All co-authors edited the manuscript. This study received ethical approval from The University of British Columbia Clinical Research Ethics Board (ID: H11-02657).

Chapter 3. I wrote chapter 3 and received extensive feedback from Prof. Ainslie prior to finalization.
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<th>Definition</th>
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<tbody>
<tr>
<td>20-HETE</td>
<td>20-hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ACA</td>
<td>Anterior cerebral artery</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BA</td>
<td>Basilar artery</td>
</tr>
<tr>
<td>BOLD</td>
<td>Blood oxygen level dependent</td>
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<tr>
<td>$\text{Ca}^{2+}$</td>
<td>Calcium</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CoV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CSA</td>
<td>Cross-sectional area</td>
</tr>
<tr>
<td>EET</td>
<td>Epoxyeicosatrienoic acid</td>
</tr>
<tr>
<td>FiCO$_2$</td>
<td>Fractional inspired carbon dioxide</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>ICA</td>
<td>Internal carotid artery</td>
</tr>
<tr>
<td>ICAv</td>
<td>Internal carotid artery blood velocity</td>
</tr>
<tr>
<td>INDO</td>
<td>Indomethacin</td>
</tr>
<tr>
<td>$K^+$</td>
<td>Potassium</td>
</tr>
<tr>
<td>$K_{\text{ATP}}$ channels</td>
<td>Adenosine triphosphate sensitive potassium channels</td>
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<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
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<tr>
<td>MCA</td>
<td>Middle cerebral artery</td>
</tr>
<tr>
<td>MCAv</td>
<td>Middle cerebral artery blood velocity</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NVC</td>
<td>Neurovascular coupling</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>P₆CO₂</td>
<td>Partial pressure of arterial carbon dioxide</td>
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<tr>
<td>P₆O₂</td>
<td>Partial pressure of arterial oxygen</td>
</tr>
<tr>
<td>PCA</td>
<td>Posterior cerebral artery</td>
</tr>
<tr>
<td>PCAV</td>
<td>Posterior cerebral artery blood velocity</td>
</tr>
<tr>
<td>PₑTₐCO₂</td>
<td>Partial pressure of end-tidal carbon dioxide</td>
</tr>
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<td>PₑTₐO₂</td>
<td>Partial pressure of end-tidal oxygen</td>
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<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGD₂</td>
<td>Prostaglandin D₂</td>
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<td>PGE₂</td>
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<tr>
<td>PGF₂α</td>
<td>Prostaglandin F₂α</td>
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<td>PGH₂</td>
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<td>PGI₂</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PGG₂</td>
<td>Prostaglandin G₂</td>
</tr>
<tr>
<td>PKA</td>
<td>Cyclic adenosine monophosphate dependent protein kinase</td>
</tr>
<tr>
<td>PKG</td>
<td>Cyclic guanosine monophosphate dependent protein kinase</td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>QICA</td>
<td>Internal carotid artery blood flow</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>TCD</td>
<td>Transcranial Doppler ultrasound</td>
</tr>
<tr>
<td>VA</td>
<td>Vertebral artery</td>
</tr>
<tr>
<td>Vₘₙₙₓ</td>
<td>Maximum blood velocity</td>
</tr>
</tbody>
</table>
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For Ivar and Marcelle

Here lies the product of 23 years hard work
Chapter 1: Introduction

1.1. An overview of cerebrovascular regulation.

The human brain is distinctly different from other organs in that it possesses a very high metabolic demand with limited intra-cellular energy stores, thus necessitating precisely controlled blood flow to match metabolic demand. A disproportionately high metabolic demand relative to its size (~2% of total body weight) necessitates the direction of approximately 15% of resting cardiac output to the brain. Ultimately, the brain is responsible for ~20% of resting metabolism (Kety & Schmidt, 1948a). As a result, maintenance of optimal oxygen (O$_2$) and nutrient supply is paramount in maintaining neuronal function and consciousness (Lennox et al., 1935; Van Lieshout et al., 2003). Complete cessation of cerebral blood flow (CBF) results in syncope in as little as four seconds, which can quickly progresses to more serious outcomes such as seizure, permanent neurological damage, and eventually death if cerebral perfusion is not restored within a few minutes (Smith et al., 2011). Precise control of CBF involves the interplay of key and often overlapping or redundant regulatory mechanisms. The four primary regulators of CBF, which encompass their own respectively complex regulation, are arterial blood gases [i.e., O$_2$ and carbon dioxide (CO$_2$); Kety & Schmidt, 1948b; Willie et al., 2012; Wolff & Lennox, 1930], cerebral metabolism [i.e., neurovascular coupling (NVC); Roy & Sherrington, 1890; Attwell et al., 2010], cerebral perfusion pressure (i.e., cerebral autoregulation; Lucas et al., 2010; Tzeng & Ainslie, 2014; Numan et al., 2014), and to a lesser extent regulation by the autonomic nervous system (e.g., Umeyama et al., 1995).

The brain’s high blood supply is delivered through two bilateral pairs of large arteries: two internal carotid arteries (ICA), and two vertebral arteries (VA). The VA’s run cephalad off of the subclavian artery, through processes in the vertebral column starting at the 6$^{th}$ cervical vertebrae, and ramify to form the basilar artery (BA) at the base of the brain. The ICA’s run cephalad from the common carotid bifurcation to the base of the brain, where they along with communicating arteries, the BA, and the posterior cerebral arteries (PCA) ramify into an anastomotic ring, called the Circle of Willis. This unique anastomotic ring was first reported
to exist in 1664 in the doctoral works of Sir Thomas Willis titled: *Cerebri Anatome* (Willis, 1664).

The circle of Willis gives rise to three pairs of large cerebral arteries: The anterior cerebral arteries (ACA), middle cerebral arteries (MCA), and the aforementioned PCA’s. These large cerebral arteries run outward towards the surface of the cerebral cortex, exiting the brain parenchyma where they then enter into the pia mater. At this point, they are referred to as pial vessels, which were previously thought to be the exclusive regulators of cerebrovascular resistance (Wolff & Lennox, 1930). However, it is now known that while pial vessels may be the primary regulator of cerebrovascular tone, vasomotion of large cerebral arteries also plays a role in mediating cerebrovascular resistance in both animals (Heistad *et al.*, 1978; Faraci & Heistad, 1990) and humans (Willie *et al.*, 2014; Lewis *et al.*, 2015). These pial vessels, which transition into penetrating arterioles, dive down into the brain parynchema through what is known as the ‘Virchow Robin’ space. Penetrating arterioles become encapsulated in astrocytic end-feet and pericytes, and transition into parynchemal arterioles. The regulation of CBF by arterial blood gases, metabolism, cerebral perfusion pressure, and the autonomic nervous system will be discussed next.

1.1.1. CBF regulation by arterial blood gases.

The cerebral vasculature is highly sensitive to changes in the partial pressure of arterial carbon dioxide, and to a lesser extent, oxygen (P$_{a}$CO$_2$ & P$_{a}$O$_2$, respectively). Changes in P$_{a}$CO$_2$ bidirectionally influence CBF as elevations in P$_{a}$CO$_2$ (hypercapnia) cause an increase in CBF and reductions in P$_{a}$CO$_2$ (hypocapnia) cause a decrease in CBF (Ainslie & Duffin, 2009; Willie *et al.*, 2012). While P$_{a}$O$_2$ is not as potent a regulator of CBF as P$_{a}$CO$_2$, a marked reduction in P$_{a}$O$_2$ (i.e., to below 55mmHg) results in a compensatory increase in CBF to maintain cerebral O$_2$ delivery during both normobaric (see: Figure 1 in: Ainslie *et al.*, 2014) and hypobaric (see: Figure 2 in: Ainslie & Subudhi, 2014) hypoxia. Hyperoxia (elevated P$_{a}$O$_2$) can have a small constrictive influence on CBF, that is largely mediated via hyperventilation (Willie *et al.*, 2012; Smith *et al.*, 2012). With respect to the influence of blood gases on blood flow regulation, the cerebral vasculature is distinctly different from that
of the peripheral circulation (Lennox & Gibbs, 1932; Ainslie et al., 2005) where there is a limited influence of arterial blood gases. This impact of changes in arterial blood gases on cerebrovascular regulation was observed nearly a century ago in cats (Wolff & Lennox, 1930), soon thereafter investigated in humans (Lennox & Gibbs, 1932), and then comprehensively characterized several decades later (Shapiro et al., 1966). Cerebrovascular reactivity to changes in $P_aCO_2$ is important in maintaining constancy of central pH and hence breathing stability (Ainslie & Duffin, 2009; Xie et al., 2009; Fan et al., 2010). For example, impaired (reduced) cerebrovascular reactivity to $P_aCO_2$ is implicated in central sleep apnea, in both patients with congestive heart failure (Xie et al., 2005; Javaheri & Dempsey, 2013) and in otherwise healthy volunteers at high altitude (Burgess et al., 2014). Moreover, impaired cerebrovascular reactivity to $P_aCO_2$ is associated with an increased risk for stroke (Markus & Cullinane, 2001) and all cause mortality (Portegies et al., 2014). Therefore, in addition to maintaining $O_2$ delivery during hypoxia, it is obvious that cerebrovascular reactivity to blood gases is essential in maintaining homeostatic function in several capacities.

1.1.2. CBF regulation by cerebral metabolic processes.

Increases in neural activity induce metabolic demand for $O_2$ and glucose, and are coupled to increases in CBF (Roy & Sherrington, 1890; Attwell et al., 2010; Willie et al., 2011b). Much of the data informing scientific knowledge of the coupling between cerebral metabolism and CBF have been derived from various gas perturbations and pharmacological blockades in vitro using brain tissue slices, while a relatively smaller number of studies have sought to investigate the regulation of metabolic and CBF coupling on a global scale (i.e., total brain). The phenomena of metabolism and flow coupling, both in tissue bath preparations and in vivo is termed - and will henceforth be referred to as - neurovascular coupling (NVC). This energy consumption of the brain, which drives NVC, stems primarily from maintenance of ionic gradients (related to action potentials, resting potentials, etc.), with metabolic demand increasing in concert with increased neuronal firing (Attwell & Laughlin, 2001).
Neural activity causes an immediate increase in dendritic glycolysis with the metabolic response in astrocytes delayed slightly (Kasischke et al., 2004). The immediate increase in dendritic activity is driven primarily by oxidative metabolism, and seemingly depletes O$_2$ stores, which results in the astrocytic response being primarily dependent on anaerobic glycolysis (Kasischke et al., 2004). The NVC occurs in mere seconds, and acts to mitigate and effectively eliminate the immediate drop in tissue O$_2$ associated with the onset of neuronal activity (Vanzetta & Grinvald, 1999; Offenhauser et al., 2005). Larger increases in neural activity elicit larger reductions in tissue O$_2$ and concomitant compensatory increases in CBF, while impairments in NVC potentiate the drop in tissue O$_2$ (Offenhauser et al., 2005). Astrocytes seem to be the primary regulator of the NVC response, with the ability to modulate pre-synaptic, post-synaptic, and direct vascular signaling due to their involvement in what is known as the ‘tripartite synapse’ and their direct apposition to microvasculature (Perea et al., 2009; Kowiański et al., 2013).

The current leading theory is that NVC acts as a feed forward mechanism, as synaptic release of glutamate and post-synaptic metabotropic glutamate receptor binding (i.e., simulated neural activity) results in an increase in intracellular astrocytic Ca$^{2+}$ leading to adjacent microvascular dilation (Attwell et al., 2010). At a fundamental level, the signal mediating both neural activity and NVC is one in the same (glutamate receptor binding), highlighting the efficacy of the system mediating NVC. Several primary factors have been proffered to regulate NVC including: 1) prostaglandins (PG), 2) adenosine, 3) nitric oxide (NO), and 4) the prevailing level of O$_2$ tension [reviewed in: (Attwell et al., 2010)]. Briefly, increased intracellular Ca$^{2+}$, from metabatropic glutamate receptor binding or photolysis of caged Ca$^{2+}$, signals the conversion of membrane phospholipids into arachidonic acid and subsequently prostaglandin E$_2$ (PGE$_2$). This PGE$_2$ moves out of the astrocyte and into the perivascular space where it binds to prostaglandin EP receptors on adjacent vascular smooth muscle, resulting in smooth muscle relaxation (Gordon et al., 2008). In instances where astrocytic O$_2$ levels are reduced (i.e., increased glycolysis), adenosine triphosphate (ATP) production decreases increasing the intracellular astrocytic adenosine concentration. Consequently, adenosine is released to the perivascular space where it binds to adenosine A$_{2A}$ receptors on adjacent smooth muscle cells, with the consequent signal transduction inhibiting Ca$^{2+}$
channel mediated increases in intracellular vascular smooth muscle Ca\textsuperscript{2+}, thus inhibiting constriction (Gordon et al., 2008). Lastly, the prevailing level of O\textsubscript{2} has been demonstrated to dictate the directionality that these mechanisms conform to in response to neuronal activation. In the brain-slice preparation used by Gordon et al., 2008, hyperoxia resulted in neuronal activation mediated microvascular vasoconstrictions whereas physiological levels of O\textsubscript{2} (i.e., 20% O\textsubscript{2} in tissue bath) ‘switched’ the NVC response to mediate microvascular vasodilation (Gordon et al., 2008)

While the mechanistic underpinnings of NVC are primarily determined in vitro, NVC has been quantified in vivo in humans using several measurement techniques including blood oxygen level dependent (BOLD) magnetic resonance imaging (MRI; Bruhn et al., 2001), arterial spin tagging MRI (St Lawrence et al., 2003), and transcranial Doppler ultrasound (TCD; Willie et al., 2011). Little work has been done to determine the specific cellular pathways contributing to NVC in living humans. Therefore, the in vivo origins and regulation of NVC remains poorly understood.

1.1.3. CBF regulation by cerebral perfusion pressure.

Early study of the cerebrovascular response to changes in blood pressure dates back nearly one hundred years. In the late 1930’s Mogens Fog published two papers that reported decreases in blood pressure lead to increases in pial arteriolar diameter (Fog, 1937), while increases in blood pressure resulted in decreases in pial arteriolar diameter (Fog, 1939). These changes occur due to active vasomotion (smooth muscle cell contraction / relaxation), with each response taking ~30-60 seconds to initiate. This counteractive effect of pial arterial vasomotion in response to changes in blood pressure was later misconstrued to maintain constancy of CBF during changes in cerebral perfusion pressure and coined “cerebral autoregulation” by Niels Lassen in 1959 (Lassen, 1959). This theory is still to this day misconstrued and published in high impact journals (i.e., Meng & Gelb, 2015) despite our much improved understanding of cerebral pressure flow relationships (Tzeng & Ainslie, 2014). In actuality, CBF is dependent on cerebral perfusion pressure in a primarily passive manner (Figure 1.0). While changes in pial arteriolar caliber may act to protect the cerebral
microvasculature from deleterious changes in intravascular pressure, CBF changes in concert with cerebral perfusion pressure in both steady state (Lucas et al., 2010; Numan et al., 2014) and dynamic (Tzeng et al., 2010; Tan, 2012) instances. The presence of segmentally specific regulation of vasomotor tone by blood pressure, at least in animals, provides evidence for this teleological perspective of protecting the microvasculature. For example, during increases in blood pressure, the larger pial vessels constrict progressively in a concomitant fashion, while the smallest pial vessels dilate (Kontos et al., 1978). Conversely, larger pial vessels dilate initially upon a reduction in blood pressure, while the smaller arterioles begin to dilate at a lower pressure threshold (Kontos et al., 1978). This highlights that there is not one site of cerebrovascular resistance governing the relationship between pressure and flow, and that cerebrovascular regulation during blood pressure perturbations is a highly complex and integrative process. Overall, the cerebrovasculature seems to be more sensitive to decreases than increases in CBF (Tzeng et al., 2010; Numan et al., 2014; Figure 1.0), which may involve elevations in cerebral specific sympathetic nervous activity (SNA) during increases in blood pressure (Cassaglia et al., 2008).

Figure 1.0. The steady state cerebral pressure-flow relationship in humans. Changes in mean arterial pressure (MAP) lead to pressure passive changes in cerebral blood flow (CBF) in humans. The above figure was synthesized from 40 peer-reviewed articles with a total of 49 separate experiments. Thin lines represent the mean cerebral pressure-flow relationship of individual studies while the weighted mean of all studies is depicted by the thick black lines. These data collectively depict that CBF changes in concert with MAP, while the cerebrovasculature is less sensitive to increases in MAP (Numan et al., 2014). From: Numan et al., Med Eng & Phys, 2014 with permission.
1.1.4. CBF regulation by the autonomic nervous system.

The human cerebral circulation is highly innervated with perivascular nerves, with the highest density of nerves located in the posterior arteries (Bleys et al., 1996). Previous study has shown that ganglionic blockade at rest in healthy individuals has no effect on MCA blood velocity (MCAv; Ide et al., 2000; Zhang & Levine, 2007) or extra-cranial vessel (ICA & VA) diameter as assessed with MRI (Kang et al., 2010). However, these findings are not universal as CBF has been reported to increase with ganglionic blockade when measured with single photon emission computed tomography (Umeyama et al., 1995). Thus, while the role of the autonomic nervous system in regulating CBF remains controversial, it is likely quite a negligible factor at rest. To the contrary, there is some evidence that the autonomic nervous system, specifically, the sympathetic nervous system is integral in regulating CBF during surges in blood pressure. As mentioned previously (see section “1.1.3 CBF regulation by cerebral perfusion pressure”), increases in blood pressure result in less of a CBF response than do decreases in blood pressure. It is thought that while increases in systemic blood pressure activate the baroreflex and actively reduce sympathetic output to the periphery, that there is a cerebral specific increase in SNA (Cassaglia et al., 2008), although this has only been demonstrated in animals.

1.2. Cerebrovascular regulation by arterial carbon dioxide.

Of the aforementioned regulators of CBF (metabolism, perfusion pressure, arterial blood gases and autonomic nervous activity), $P_aCO_2$ is seemingly the most potent regulator of cerebrovascular tone. The cerebrovascular response to CO$_2$ is characterized as the unit (i.e., mL/min), or percent, change in CBF per unit change in either $P_{ET}CO_2$ or $P_aCO_2$ – termed cerebrovascular CO$_2$ reactivity. Changes in $P_aCO_2$ mediate alterations in CBF locally via changes in extravascular pH (Kontos et al., 1977a), but not intraluminal CO$_2$ (Kontos et al., 1977b) or pH (Lambertsen et al., 1961; Harper & Bell, 1963). For example, in cats, pretreatment with intravenous bicarbonate to maintain normal pH nearly abolishes pial vessel dilation in response to hypercapnia (Kontos et al., 1977b) highlighting the dependency of this response on pH. Therefore, movement of CO$_2$ through the vessel wall and consequent
alteration of extravascular pH is seemingly necessary to alter vasomotor tone. While previously a contentious matter (Serrador et al., 2000; Giller, 2003), evidence continues to emerge supporting the theory that CO₂ is vasoactive throughout the entire cerebral vascular tree (see Figure 1.1) implicating both large cerebral arteries through to cerebral arterioles in the regulation of cerebrovascular resistance during PₐCO₂ perturbations (Wolff & Lennox, 1930; Willie et al., 2012; Verbree et al., 2014; Coverdale et al., 2014, 2015). Previous animal data corroborates this recent paradigm shift in human cerebrovascular regulation (Heistad et al., 1978; Faraci & Heistad, 1990).

![Figure 1.1](image-url)

**Figure 1.1. Carbon dioxide mediated cerebral vasomotion occurs throughout the entire cerebral vascular tree.** A. Classic data from Wolff & Lennox (Wolff & Lennox, 1930) depicting hypercapnic vasodilation of pial arterioles in anesthetized cats. Two vessels can be seen; the one on the left is an image during craniotomy in the resting state, while the one on the right is during hypercapnia. The difference in diameter can be visualized by the addition of the dotted line to the diameter of the vessel in the right image. These data, along with extrapolation from others (Serrador et al., 2000), led to the belief that CBF regulation during changes in PₐCO₂ was mediated solely by pial arteries / arterioles. Adapted from Wolff & Lennox, 1930 - ©permission not required upon request. B. Vasomotion of the MCA as assessed by high resolution (3T) MRI in humans during changes in end-tidal carbon dioxide (PₑT CO₂). Reproduced from Coverdale et al., 2014 - ©permission not required upon request. C. Data depicting vasomotion of the internal carotid artery (ICA) in humans throughout a wide range of arterial PCO₂ (PₐCO₂). It is now, therefore, established that vasomotion of large intra- and extra- cranial cerebral arteries occurs in response to changes in PₐCO₂ along with changes at the pial artery and arteriolar level. Reproduced from Willie et al., 2012 - ©permission received.

1.2.1. Response characteristics.

Cerebrovascular CO₂ reactivity is a relatively linear response during both hypo and hypercapnia (Skow et al., 2013), with the magnitude of reactivity during hypercapnia near double that during hypocapnia (i.e., hypercapnia ~ Δ4% CBF/mmHgPₐCO₂ vs. hypocapnia ~ Δ2%CBF/mmHgPₐCO₂; Ainslie & Duffin, 2009; Willie et al., 2012; Figure 1.2-B). There are
regional differences in the magnitude of reactivity between the anterior and posterior circulation, and between grey and white matter. Specifically, grey matter possesses an approximate 3-fold greater CO₂ reactivity compared to white matter (Ramsay et al., 1993), likely due to lower vascularization.

When assessed using duplex ultrasound, absolute CBF reactivity of the anterior circulation through the ICA is approximately double that of posterior reactivity through the VA during hypocapnia (Willie et al., 2012) with the difference being even greater during hypercapnia (Willie et al., 2012; Hoiland et al., 2015). Differences in absolute MCAv and posterior cerebral artery blood velocity (PCAv) reactivity are not apparent during end-tidal forcing through an extreme range of changes in P₅CO₂ (i.e., 15-65mmHg; Willie et al., 2012) or during hyperventilation and changes in fractional inspired CO₂ (Ogawa et al., 1988). However, apparent differences in hypercapnic CBF reactivity between anterior and posterior circulation (ICA vs. VA) are corroborated by MCAv and PCAv reactivity measures during hyperoxic rebreathing (Skow et al., 2013). Differences between Skow et al., 2013 and Willie et al., 2012 may be due to differences in statistical power (i.e., sample size), while Ogawa et al., 1988 utilized non-linear analysis parameters as opposed to the more commonly used linear analysis approach. Thus it is fair to conclude the anterior circulation has a higher absolute reactivity than that of the posterior circulation, at least as observed using duplex ultrasound.
In relative terms (i.e., $\%\Delta CBF \cdot \text{mmHg}P_a\text{CO}_2^{-1}$), the majority of data indicate that both ICA and VA flow/velocity reactivity and MCAv and PCAv reactivity do not differ during hypercapnia (Hauge *et al.*, 1980; Willie *et al.*, 2012; Skow *et al.*, 2013; Hoiland *et al.*, 2015), with very minimal evidence to the contrary (Sato *et al.*, 2012). Similarly, both MCAv and PCAv and ICA flow reactivity do not differ during hypocapnia; however, VA flow reactivity has been reported as both greater than (Hauge *et al.*, 1980; Willie *et al.*, 2012) and similar (Sato *et al.*, 2012) to that of the ICA during hypocapnia. Between-study differences likely relate to differing experimental paradigms, manipulations of blood gases, and measurement technique standardization and analysis. The specifics of measuring CBF with ultrasound and different methods for blood gas manipulation are discussed in Section 1.4. *Measurement of cerebral blood flow*; and Section 1.5. *Experimental methods to assess cerebrovascular CO_2 reactivity.*
1.2.2. Factors influencing cerebrovascular CO₂ reactivity.

Cerebrovascular CO₂ reactivity is subject to between test and between subject variability and can be altered by a myriad of potential concurrent physiological effectors. Consideration of potential interactions with other primary regulators of CBF (hypoxia, neuronal activity, blood pressure, and autonomic inputs) is pivotal in fully understanding CO₂ induced vasomotion. It has been previously demonstrated that alterations in PₐO₂ will interact with PₐCO₂ perturbations to collectively dictate cerebrovascular reactivity. Specifically, hypoxia will elevate CBF across a wide range of PₐCO₂ in both the hypo and hypercapnic CO₂ range (Ainslie & Poulin, 2004; Mardimae et al., 2012). In instances of severe hypoxia (i.e., PₐO₂≈40mmHg), CBF remains above baseline despite pronounced hypocapnia (Mardimae et al., 2012).

Elevations in PₐCO₂ result in concomitant increases in blood pressure due to increased SNA (Ainslie et al., 2005). When the elevations in blood pressure are sustained, they are generally related to the magnitude of the flow response to hypercapnia (Willie et al., 2012; Regan et al., 2014). Thus, it is important to consider the influence of systemic blood pressure in the cerebrovascular response to CO₂. A seminal study conducted in 1965 by Harper & Glass, showed that progressive decreases in mean arterial pressure (MAP) up to a reduction of 66% below baseline, causes marked reductions in cerebrovascular CO₂ reactivity to hypo and hypercapnia (Harper & Glass, 1965). It is unclear if progressive elevations in blood pressure also affect the cerebrovascular response to changes in PₐCO₂.

Lastly, chemoreflex activation via acidosis increases SNA (Steinback et al., 2009, 2010b, 2010a) and may, therefore, be implicated in modulating cerebrovascular CO₂ reactivity. Increasing SNA through various techniques does not alter reactivity in either the hyper and hypocapnic range (LeMarbre et al., 2003; Ainslie et al., 2005); however, pharmacological blockade of SNA has been reported to reduce both hypocapnic (Peebles et al., 2012) and hypercapnic (Przybyłowski et al., 2003) reactivity. A reduction in hypercapnic reactivity via pharmacological SNA blockade is likely mediated by an abolished pressor response (Przybyłowski et al., 2003) which, as mentioned, normally contributes to the flow response
(Willie et al., 2012; Regan et al., 2014). There is, however, data indicating that the pressor response is augmented by ganglionic blockade (Jordan et al., 2000). This relationship between SNA and CO₂, and cerebrovascular CO₂ reactivity has yet to be investigated using volumetric flow measurement techniques. Collectively, it is apparent that while cerebrovascular CO₂ reactivity is often viewed as an independent effector of CBF, it is in fact an integrative response that interacts with many other physiological variables.

1.2.3. The potential cellular mechanisms mediating cerebrovascular CO₂ reactivity.

Regulation of CBF at the cellular level in response to varying stimuli is a multifaceted and complex process. See Figure 1.3 for a generalized overview of cerebrovascular smooth muscle cell regulation by CO₂. Overall, potassium channels and vasoactive factors such as adenosine, NO, epoxyeicosatrienoic acids (EET’s), and PG’s all potentially mediate cerebrovascular vasomotion in response to alterations in $P_aCO_2$. Each of these factors will be briefly overviewed below, while the role of PG’s will be reviewed in detail.
Figure 1.3. The Putative mechanisms governing cerebrovascular smooth muscle cell relaxation during CO$_2$ perturbations. Changes in potassium channel conductance and alterations in vascular smooth muscle cell calcium sensitivity mediated via cyclic nucleotides govern vascular tone. Increased potassium channel conductance results in potassium efflux from, and a hyperpolarization of, smooth muscle cells leading to relaxation, while reductions in conductance lead to contraction (Faraci & Sobey, 1998). Calcium sensitivity is regulated primarily by cyclic nucleotide activity, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). For example, increases in cAMP activate cAMP dependent protein kinase (PKA), which inhibits smooth muscle cell contraction (Kerrick & Hoar, 1981) through phosphorylation (and deactivation) of myosin light chain kinase (MLCK; Adelstein & Conti, 1978). Moreover, cyclic nucleotides increase conductance through potassium channels (Song & Simard, 1995). Prostaglandins (PGE$_2$ & PGI$_2$), epoxyeicosatrienoic acids (EET’s), nitric oxide (NO), and adenosine are all capable of modulating cyclic nucleotide activity and / or potassium channel conductance. In the above figure a (+) symbol represent activation of a downstream signal, whereas (-) represents deactivation. AA, Arachidonic Acid; PKG, cGMP dependent protein kinase; PL$_{A2}$, Phospholipase A$_2$.

1.2.3.1. Potassium channels.

Potassium (K$^+$) channels are present in cerebrovascular smooth muscle cells and their impact on membrane potential is an important regulator of vascular tone (Faraci & Sobey, 1998). Activation of K$^+$ channels increases K$^+$ conductance resulting in efflux of K$^+$ from vascular smooth muscle cells and consequent hyperpolarization and relaxation. Inhibition of K$^+$
channels contrastingly reduces $K^+$ conductance resulting in depolarization and constriction. Although not collected in cerebrovascular smooth muscle cells, data in mesenteric smooth muscle cells of rats in vitro indicate that a reduction in pH activates ATP sensitive potassium channels ($K_{ATP}$ channels; Wang et al., 2003). During hypercapnia in humans, inhibition of $K_{ATP}$ channels with glibenclamide has no effect on the blood velocity response through the MCA (Bayerle-Eder et al., 2000); however, as $K_{ATP}$ channels directly effect vascular tone it is difficult to extrapolate velocity measures to represent volumetric CBF in this instance. Overall potassium channels regulate cerebrovascular smooth muscle tone (at least in animals) downstream of several vasoactive factors which are implicated in $CO_2$ mediated cerebrovascular vasomotion: NO [via cyclic guanosine monophosphate (cGMP)], Adenosine, EETs, and PGs [via cyclic adenosine monophosphate (cAMP)]. Signal transduction and the role of these aforementioned vasoactive factors in $CO_2$ mediated vascular regulation are discussed below.

1.2.3.2. Adenosine.

A potential role for adenosine in the regulation of CBF during hypercapnia has been previously demonstrated in rats as adenosine receptor antagonism via caffeine reduces the CBF response to hypercapnia (Phillis & DeLong, 1987). Moreover, the response to hypercapnia is potentiated by both the administration of dipyridamole and adenosine deaminase inhibitors, which both increase adenosine concentrations (Phillis & DeLong, 1987). In humans, treatment with caffeine (i.e., adenosine receptor antagonism) has been shown to reduce baseline CBF, but possess no effect on cerebrovascular $CO_2$ reactivity (Blaha et al., 2007). However, as the half life of caffeine is approximately 5.5 hours (Statland & Demas, 1980), and subjects were only asked to refrain from caffeine containing beverages for 6 hours, pre-existing caffeine may have contaminated baseline recordings making it difficult to draw strong conclusions from the study by Blaha et al., 2007. Therefore, the potential role of adenosine in modulating $P_aCO_2$ mediated cerebrovascular vasomotion in humans remains unclear.
1.2.3.3. Nitric oxide.

Hypercapnia induces increased release of cGMP (Parfenova et al., 1994) a second messenger downstream of NO activity. Moreover, inhibiting nitric oxide synthase (NOS) non-selectively or by selectively inhibiting neuronal NOS (nNOS) significantly blunts the CBF response to hypercapnia in rats (Wang et al., 1994a; Smith et al., 1997), while addition of NO donors restores hypercapnia mediated increases in CBF (Iadecola & Zhang, 1996). Contrary to animal studies, some (White et al., 1998; Ide et al., 2007), but not all (Schmetterer et al., 1997) evidence in humans suggests NO may not appreciably impact cerebrovascular reactivity to hypercapnia; however, these data are based upon velocity and not flow indices of CBF. Increases in CBF in response to hypercapnia have been reported to take ~30seconds to complete and reach steady state (Shapiro et al., 1966), which may be a result of the time needed for larger cerebral vessel dilation to occur and, therefore, indicate a role for endothelial released NO. Such is the case in the periphery, where shear stress mediated dilation takes ~30seconds to occur (Black et al., 2008) - a response driven primarily by NO (Green et al., 2014). However, despite similar time dependent response characteristics between peripheral and cerebral responses it is unknown if NO is a key regulator of CBF during alterations in PₐCO₂.

1.2.3.4. Epoxyeicosatrienoic acids.

Metabolic byproducts of the arachidonic acid pathway called EET’s are formed via epoxygenase activity (Figure 1.4). There are four different EETs (5,6-EET; 8,9-EET; 11,12-EET; and 14,15-EET), which all vasodilate the cerebrovasculature in animals (Leffler & Fedinec, 1997), although a dilatory effect of all EETs has not been consistently demonstrated, with evidence that only 5,6-EET may be vasoactive (Ellis et al., 1990). Interestingly, the presence of prostacyclin (PGI₂) seems to facilitate EET mediated dilation of pial vessels (Leffler & Fedinec, 1997). However, the direct impact of EETs on cerebral vasomotion in response to PₐCO₂ alterations has not been investigated in humans.
1.3. The prostaglandin pathway

In the 1930’s PGs were discovered by Kurzok and Lieb (Kurzrok & Lieb, 1930). However, PGs were not isolated in their pure form until the mid 1960’s (Samuelsson, 2012). The presence of PGI$_2$ was later determined in 1976 (Moncada et al., 1976). Since both peripheral (Busse et al., 1984; Nicholson et al., 2009), and cerebral (Davis et al., 2004) vascular smooth muscle relax in response to PG’s, they have been implicated as an important factor in vascular function and health. These PGs are produced as an end product of the arachidonic acid pathway (Weksler et al., 1977), which is mediated in part by cyclooxygenase (COX) activity (Smith, 1992). This makes COX an ideal target to inhibit for the investigation of PG mediated functions, including cerebrovascular regulation.

A downstream product of the arachidonic acid pathway (Weksler et al., 1977), PG production results from COX conversion of arachidonic acid through to prostaglandin H$_2$ (PGH$_2$; Smith et al., 1991; Smith, 1992). Specifically, this pathway begins with the conversion of arachidonic acid to prostaglandin-G$_2$ (PGG$_2$) by COX. This PG$_2$ is then reduced to prostaglandin endoperoxide-H$_2$ (PGH$_2$; also commonly referred to as PG synthase) by peroxidase. Finally, specific isomerization or reduction of PGH$_2$ produces one of either prostaglandin-D$_2$, E$_2$, F$_{2\alpha}$ (PGD$_2$, PGE$_2$, & PGF$_{2\alpha}$, respectively), PGI$_2$, or thromboxane-A$_2$ (Smith, 1992). Figure 1.4 depicts a simplified outline of PG production as well as the co-existing lipoxygenase and epoxygenase pathways which are likely also implicated in the control of CBF via flux through the arachidonic acid pathway (Attwell et al., 2010). The production of PG’s is seemingly endothelial dependent in both peripheral (Weksler et al., 1977; Messina et al., 1992) and cerebrovascular (Hsu et al., 1993) blood vessels. Post mortem studies have demonstrated the ability of cerebral vascular tissue to produce PG’s, primarily PGI$_2$ and also PGE$_2$ to a lower extent (Abdel-halim et al., 1980), highlighting that they are present in - and likely vasoactive regulators of - the cerebrovascular circulation. As PGI$_2$ has a relatively short half life (~30seconds) and the more stable PG’s are quickly metabolized, it is likely that PG’s primarily mediate effects localized to their production site, thus necessitating this production via cerebral vessels (Narumiya et al., 1999). On a whole body scale, the production of PG’s is ubiquitous. The potential role for PG’s as regulators of cerebrovascular CO$_2$ reactivity will now be
extensively reviewed. First, animal data In Vitro and In Vivo will be summarized followed by an overview of In Vitro and In Vivo work in humans.

**Figure 1.4. The arachidonic acid pathway.** Arachidonic acid is produced from membrane phospholipids through the activity of phospholipase A. It is then converted into one of three compounds: PGG₂, epoxyeicosatrienoic acid (EET's), and 20- hydroxyeicosatetraenoic acid (20-HETE). Inhibition of cyclooxygenase is commonly used to inhibit the production of PG’s from arachidonic acid. PG, Prostaglandin; PGG₂, Prostaglandin G₂; PGH₂, Prostaglandin H₂; PGE₂, Prostaglandin E₂; PGD₂, Prostaglandin D₂; PGI₂, Prostacyclin; PGG₂, Prostaglandin G₂; PGH₂, Prostaglandin H₂; PGF₂α, Prostaglandin F₂α; TXA₂, Thromboxane A₂

1.3.1. Prostaglandins and animal cerebral vasomotor tone: In Vitro

The putative role of PG’s in the cerebrovascular regulation of animals is based on the production of PG’s In Vitro in rats (Gecse et al., 1982), guinea pigs (Gecse et al., 1982), and pigs (Parfenova et al., 1995b). Supportive evidence is also based on the presence of PG synthases (Boullin et al., 1979). Synthesis of PG’s likely occurs in both smooth muscle (Parfenova et al., 1995a) and endothelial cells (Hsu et al., 1993). Addition of exogenous PG’s to In Vitro tissue baths, such as PGE₂, PGI₂ and iloprost (a synthetic PGI₂ analogue) dilate the MCA and BA of cats (Whalley et al., 1989; Parsons & Whalley, 1989) – these responses are unaffected by the application of Indomethacin (INDO; Whalley et al., 1989).
This finding is indicative of the action of these agents downstream of COX (i.e., smooth muscle receptor binding). The VA and BA of baboons have also been demonstrated to relax upon induction of PGI₂ into a tissue bath (Boullin et al., 1979). While PGE₂ is largely considered to be a vasodilator, there is data to the contrary in dogs, indicating PGE₂ induced contraction of MCA and BA (Toda & Miyazaki, 1978) highlighting the likelihood of between species response variation. Consistent with the majority of reports exemplifying PG mediated dilation of large cerebral arteries, it has been repeatedly observed that the smaller pial vessels of the brain dilate significantly in response to numerous PG’s (Welch et al., 1974; Ellis et al., 1979). Collectively, In Vitro animal data indicate that PG’s are likely an important factor in regulating CBF, at least in these aforementioned animals studies and related preparations.

1.3.2. Prostaglandins, animal CBF and CO₂ reactivity: In Vivo

Topical application of arachidonic acid (e.g., 200µg/mL in artificial cerebrospinal fluid) stimulates increased cerebral PG production (Kontos et al., 1985; Ellis et al., 1990; Leffler et al., 1993) and subsequent pial vessel dilation in cats (Wei et al., 1980; Kontos et al., 1984), rabbits (Ellis et al., 1990), and newborn pigs (Leffler et al., 1990, 1993). Direct application of specific prostanoids, such as PGE₂ and PGI₂, causes dose dependent dilation of pial vessels in newborn pigs (Leffler & Busija, 1985, 1987) and cats (Wahl et al., 1973, 1989). Iloprost has also been demonstrated to induce pial arteriolar dilation in newborn pigs, with this dilation blunted by the administration of INDO (Parfenova et al., 1995b). This marked blunting of dilation in response to PGI₂ receptor agonism (via iloprost) is indicative that, in addition to COX inhibition, INDO also inhibits PGI₂ receptor agonism mediated signal transduction and consequent vasodilation (Parfenova et al., 1995b). Moreover, the selectivity of INDO’s effect on PGI₂ receptors was confirmed when INDO was shown to have no effect on β-adrenoreceptor mediated dilation of pial vessels (Parfenova et al., 1995b). This finding is in contrast to the In Vitro studies where INDO does not affect the vasomotor response to topically applied PG’s (Whalley et al., 1989). These discrepancies highlight the potential limitations of comparing In Vitro and In Vivo data regarding PG mediated cerebral vasomotion.
Much like topical application of arachidonic acid, hypercapnia has been reported to increase cerebral PG synthesis in newborn pigs (Leffler et al., 1993; Parfenova et al., 1994) and cultured endothelial cells (Parfenova & Leffler, 1996). Inhibition of PG synthesis with the administration of INDO results in a marked reduction of cerebrovascular CO$_2$ reactivity in baboons (Pickard & MacKenzie, 1973) and newborn pigs (Leffler et al., 1993, 1994). However, this response seems to be selective to INDO in some species as aspirin does not inhibit hypercapnic pial vessel dilation in newborn pigs (Parfenova et al., 1995b), but not in others as cyclooxygenase-1 (COX$_1$) inhibition also blunts the hypercapnic CBF response in rats (Niwa et al., 2001). Drug dependent differences are further apparent in that INDO selectively reduces CBF in newborn pigs, whereas Aspirin, Ibuprofen, and Naproxen do not (Chemtob et al., 1991).

1.3.3. Prostaglandins and human cerebral vasomotor tone: In Vitro

Evidence for a role of PG’s, at least when using *In Vitro* tissue baths, in mediating cerebral vasomotion in humans was reported in 1979 due to both its endogenous production in the cerebrovasculature and vasomotor influence (Boullin et al., 1979; Hagen et al., 1979). The two most common vasodilator PG’s - PGE$_2$ and PGI$_2$ - are both capable of causing vasodilation of human cerebral arteries. Specifically, PGE$_2$ and iloprost both dilate human MCA segments pre-contracted with phenylephrine (Davis et al., 2004). In addition to the PGI$_2$ mediated dilation of the MCA, *In Vitro* application of PGI$_2$ also dilates the human BA (Boullin et al., 1979; Paul et al., 1982; Parsons & Whalley, 1989), indicating that PGI$_2$ acts as a vasodilatory agent in both the anterior and posterior cerebral circulation. However, the vasodilatory effect of PGE$_2$ appears exclusive to the anterior circulation as it dilates MCA segments (Davis et al., 2004), but constricts BA segments (Toda & Miyazaki, 1978; Parsons & Whalley, 1989). These regional differences are likely due to differences in dish preparation, pre-contractile agents, and concentrations of PGI$_2$ (or related analogues) used (Uski et al., 1983); such differences could also be indicative of regionally specific cerebrovascular regulation by PG’s.
Binding of PG receptors (i.e., IP and EP<sub>4</sub> receptors; Figure 1.5) occurs on the vascular smooth muscle cell membrane with the resultant signal transduction independent of the endothelium (Davis et al., 2004). While exogenous PG’s clearly possess vasomotor effects on the cerebral circulation as outlined above, their relevance to endogenous vasomotor regulation is limited. However, PGI<sub>2</sub> synthase in human BA and VA’s has been demonstrated (Boullin et al., 1979), providing evidence that endogenously produced PG’s play a role in cerebrovascular regulation.

**Figure 1.5. Putative pathways for prostanoid mediated signal transduction.** Binding of prostacyclin and PGE<sub>2</sub> to IP and EP<sub>4</sub>/EP<sub>2</sub> receptors, respectively, on the vascular smooth muscle cell wall leads to increases in intracellular cAMP via stimulation of adenylate cyclase (Narumiya et al., 1999). Increases in cAMP and subsequent increases in cAMP dependent protein kinase lead to smooth muscle relaxation (Narumiya et al., 1999; Kerrick & Hoar, 1981). This is achieved via cAMP dependent protein kinase mediated phosphorylation of myosin light chain kinase, which reduces its activity (Adelstein et al., 1978), thus reducing phosphorylation of myosin and it’s binding with actin. Indomethacin (INDO) inhibits cAMP dependent protein kinase activity (Kantor & Hampton, 1978), which would lead to increased phosphorylation of myosin light chain kinase (or reduced inhibition via cAMP dependent protein kinase); therefore, increasing phosphorylation of myosin and binding with actin. Moreover, *In Vivo* it has been shown that INDO also reduces prostacyclin receptor (IP) mediated increases in cAMP (Parfenova et al., 1995).
While PGI₂ and PGE₂ primarily possess vasodilatory roles in the cerebral circulation, their upstream substrate, PG endoperoxides (PGG₂ & PGH₂) cause constriction of human BA’s (Boullin et al., 1979). It is therefore plausible that cerebrovascular constriction and reductions in CBF due to PG inhibition (via INDO) are resultant from an increased PG endoperoxide concentration (from reduced flux through arachidonic acid pathway). These latter changes occur in addition to the reduced production of vasodilatory PG’s.

1.3.4. Prostaglandins and human CBF: In Vivo

Irrespective of the measurement technique used, non-selective COX inhibition with INDO causes an approximate 20-30% reduction in resting CBF. This has been exemplified in studies utilizing TCD (Markus et al., 1994; Xie et al., 2006; Fan et al., 2010), the Kety & Schmidt technique (Eriksson et al., 1983; Wennmalm et al., 1984), and varying MRI techniques (Bruhn et al., 2001; St Lawrence et al., 2002). However, this effect is generally exclusive to INDO as resting CBF is largely unaffected by a wide array of other COX inhibitors such as acetylsalicate (Eriksson et al., 1983; Wennmalm et al., 1984; Markus et al., 1994; Bruhn et al., 2001; Table 1.0), naproxen (Eriksson et al., 1983; Wennmalm et al., 1984), and sulindac (Markus et al., 1994). A recent contrasting study reported a reduction in baseline CBF in response to Naproxen; however, this study used peak systolic MCAv as its index of flow, which is a poor indication of mean velocity over an entire cardiac cycle (Szabo et al., 2014). Infusion of epoprostenol (PGI₂) counter-intuitively causes a slight reduction in CBF (~8%), however, it is unclear if this effect was due to the concurrent reductions in MAP (Brown & Pickles, 1982; Pickles et al., 1984) versus that of a direct PGI₂ mediated vasoconstriction.
Cyclooxygenase, an important enzyme responsible for prostanoid synthesis has two specific isoforms, COX₁ and COX₂. Drugs differ as to whether they inhibit only one isoform, or both, or in their relative affinity for each COX isoform (Brune & Patrignani, 2015). This table summarizes in a simplistic nature which isoform previously used drugs inhibit.

<table>
<thead>
<tr>
<th>Drug</th>
<th>COX-1</th>
<th>COX-2</th>
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<tr>
<td>Indomethacin</td>
<td>✔</td>
<td>✔</td>
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<tr>
<td>Naproxen</td>
<td>✔</td>
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<td>Ketorolac</td>
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<td>Ibuprofen</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Acetylsalicylic Acid</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Sulindac</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Celebrex</td>
<td>✔</td>
<td>✔</td>
</tr>
</tbody>
</table>

1.3.5. Prostaglandins and human cerebrovascular CO₂ reactivity: In Vivo

Cerebrovascular CO₂ reactivity is reduced by approximately 40-60% following INDO (Wennmalm et al., 1981; Eriksson et al., 1983; Xie et al., 2006; Hoiland et al., 2014; Figure 1.6), with impairments likely greater in grey matter as opposed to white matter (St Lawrence et al., 2002). Similar to the effect reported on resting CBF, reductions in cerebrovascular CO₂ reactivity following COX inhibition are exclusive to the use of INDO, despite other drugs (i.e., aspirin & naproxen) efficaciously inhibiting PG synthesis (Eriksson et al., 1983). Upon chronic use of INDO (e.g., three days) CO₂ reactivity is restored to normal (Pickles et al., 1984). However, chronic treatment with INDO for one week has been contrastingly shown to reduce CO₂ reactivity (Eriksson et al., 1983). Whether this difference over time (i.e., three days versus one week) is a result of methodological differences between studies or a time dependent effect of INDO is unclear. Due to the unique ability of INDO to reduce CO₂ reactivity it has been proposed - although not clearly established - that INDO induced impairments in resting CBF and CO₂ reactivity are due to a mechanism independent of PG synthesis inhibition (Eriksson et al., 1983; Markus et al., 1994). Despite a differential effect on CBF when compared with other COX inhibitors (i.e., Aspirin, Ibuprofen, Naproxen, etc.; Table 1.1), INDO completely inhibits platelet aggregation in response to arachidonate...
application similar to both aspirin and naproxen indicating that its level of PG synthesis inhibition does not differ from other COX inhibitors (Eriksson et al., 1983). Whilst an alternate mechanism, if indeed present, remains to be elucidated, there is evidence that INDO effects cerebrovascular CO₂ reactivity by reducing brain tissue extracellular pH mediated signal transduction in rats (Wang et al., 1993) or by inhibiting smooth muscle cyclic adenosine monophosphate (cAMP) and cAMP dependent protein kinase activity (Kantor & Hampton, 1978; Parfenova et al., 1995b), or both.

![Image](91x91.png)

**Figure 1.6. Absolute cerebrovascular CO₂ reactivity during hyperoxic hypercapnia pre- and post 1.45mg/kg Indomethacin in humans.** Individual (○) and mean (■) values. Mean cerebrovascular data plotted against steady-state iso-oxic hypercapnia steps (0, +3, +6 and +9 PₑTCO₂). A. Absolute MCAv responses pre- and post-INDO (cm/s · mmHg PₑTCO₂⁻¹; n=13); B. Absolute PCAv responses pre- and post-INDO (cm/s · mmHgPₑTCO₂⁻¹); C. Absolute ICA responses pre- and post-INDO (ml/min · mmHgPₑTCO₂⁻¹); D. Absolute VA responses pre- and post-INDO (ml/min · mmHg PₑTCO₂⁻¹). *denotes a significant change from baseline post INDO administration (P<0.05). Reproduced from Hoiland et al., 2015 with ©permission.

Assessing the potential role of PG’s in mediating cerebral vasodilation during alterations in PₑCO₂ is a complex task for two main reasons. First, PG production results from flux through
the arachidonic acid pathway, a pathway that produces three different end substrates; PG’s (via COX); 20-hydroxyeicosatetraenoic acid (20-HETE; via lipoxygenases); and EET’s (via epoxygenases). The potential for changes in exoxygenase and lipoxygenase activity during inhibition of COX, due to increased substrate availability, poses as a difficulty (especially in humans) when attempting to specifically partition the role of PG’s from other vasoactive factors. Both 20-HETE and EET’s are vasoactive (Attwell et al., 2010), possessing constrictive (Wagerle & Mishra, 1988) and dilatory (Leffler & Fedinec, 1997) effects, respectively. Second, the inhibition of COX has a tendency to be ineffective in reducing cerebrovascular CO$_2$ reactivity unless inhibited by INDO as previously stated. Other non-selective COX inhibitors (i.e., ibuprofen, naproxen) have no effect on CBF reactivity to changes in P$_3$CO$_2$ (see Table 1.1). Thus, it is imperative to consider both the complexity of the signaling pathway and specific pharmacology when assessing the role of PG’s in mediating cerebrovascular CO$_2$ reactivity and vasomotion. Additional consideration of both the arachidonic acid pathway and several COX inhibitors and their effect on cerebral vasodilation are now provided. This includes discussion of vasoactive factors such as reactive oxygen species (ROS) and NO, as well as pharmacological effects on cell signaling and Ca$^{2+}$ sensitivity.
Table 1.1 The effect of prostaglandin synthesis inhibitors on resting cerebral blood flow and cerebrovascular CO₂ reactivity in humans.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Study</th>
<th>Population</th>
<th>Measurement Technique</th>
<th>Δ CBF - Rest</th>
<th>Δ Reactivity - Hypercapnia</th>
<th>Δ Reactivity - Hypocapnia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin (COX 1 &amp; 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100mg (oral)</td>
<td>Markus et al., Stroke, 1994</td>
<td>Both Sexes (33±6years)</td>
<td>TCD</td>
<td>-30%*</td>
<td>-34% (NS)</td>
<td>-45%*</td>
</tr>
<tr>
<td>100mg (oral)</td>
<td>Kastrup et al., J Neurol Sci, 1998</td>
<td>Females (28±2years)</td>
<td>TCD</td>
<td>-17%*</td>
<td>-66%*</td>
<td></td>
</tr>
<tr>
<td>100mg (oral)</td>
<td>Kastrup et al., J Neurol Sci, 1998</td>
<td>Males (31±4years)</td>
<td>TCD</td>
<td>-19%</td>
<td>-56*</td>
<td></td>
</tr>
<tr>
<td>0.2mg/kg (intravenous)</td>
<td>St. Lawrence et al., J Magn Reson Im, 2002</td>
<td>Both Sexes (35±8years)</td>
<td>MRI (Arterial Spin Labeling)</td>
<td>-36%*</td>
<td>-63%*</td>
<td></td>
</tr>
<tr>
<td>0.2mg/kg (intravenous)</td>
<td>Bruhn et al., J Magn Reson Im, 2001</td>
<td>Both Sexes (range: 22-31, mean: 26 years)</td>
<td>MRI (Blood Oxygen Level Dependent)</td>
<td>-40%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5mg/kg (oral)</td>
<td>Eriksson et al., Acta Physiol Scand, 1983</td>
<td>Males (range: 21-32years)</td>
<td>Kety-Schmidt Technique</td>
<td>-20%*</td>
<td>-54%*</td>
<td></td>
</tr>
<tr>
<td>0.8mg/kg (oral)</td>
<td>Wennmalm et al., Arch Toxicol Suppl, 1984</td>
<td>Male (range: 20-32years)</td>
<td>Kety-Schmidt Technique</td>
<td>-35%</td>
<td>-50%*</td>
<td></td>
</tr>
<tr>
<td>100mg (rectal)</td>
<td>Jensen et al., J Neurosurg Anesth, 1996</td>
<td>Both Sexes (range: 18-48years)</td>
<td>133Xe scintillation detectors</td>
<td>-35%*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100mg (rectal)</td>
<td>Wennmalm et al., Clin Physiology, 1981</td>
<td>Male (range: 20-40years)</td>
<td>Kety-Schmidt Technique</td>
<td>-35%</td>
<td>-72%*</td>
<td></td>
</tr>
<tr>
<td>25-50mg (oral)</td>
<td>Okabe et al., J Cereb Blood Flow &amp; Metab, 1983</td>
<td>Both sexes (44±15years)</td>
<td>133Xe scintillation detectors</td>
<td>-21%*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin (COX 1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1200mg (oral)</td>
<td>Markus et al., Stroke, 1994</td>
<td>Both Sexes (35±8years)</td>
<td>TCD</td>
<td>-7% (NS)</td>
<td>5% (NS)*</td>
<td>5% (NS)*</td>
</tr>
<tr>
<td>0.5g over 2 minutes (intravenous)</td>
<td>Bruhn et al., J Magn Reson Im, 2001</td>
<td>Both Sexes (range: 22-31, mean: 26 years)</td>
<td>MRI (Blood Oxygen Level Dependent)</td>
<td>No change</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45mg/kg (oral)</td>
<td>Eriksson et al., Acta Physiol Scand, 1983</td>
<td>Both Sexes (range: 21-31years)</td>
<td>Kety-Schmidt Technique</td>
<td>-7% (NS)</td>
<td>-1% (NS)</td>
<td></td>
</tr>
<tr>
<td>45mg/kg (oral)</td>
<td>Wennmalm et al., Arch Toxicol Suppl, 1984</td>
<td>Male (range: 20-32years)</td>
<td>Kety-Schmidt Technique</td>
<td>No change</td>
<td>9% (NS)</td>
<td></td>
</tr>
<tr>
<td>Naproxen (COX 1 &amp; 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4mg/kg (oral)</td>
<td>Eriksson et al., Acta Physiol Scand, 1983</td>
<td>Males (range: 24-48years)</td>
<td>Kety-Schmidt Technique</td>
<td>-1% (NS)</td>
<td>-8% (NS)*</td>
<td></td>
</tr>
<tr>
<td>4mg/kg (oral)</td>
<td>Wennmalm et al., Arch Toxicol Suppl, 1984</td>
<td>Male (range: 20-32years)</td>
<td>Kety-Schmidt Technique</td>
<td>-8% (NS)</td>
<td>-14% (NS)</td>
<td></td>
</tr>
<tr>
<td>Sulindac (COX 1 &amp; 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>300mg (oral)</td>
<td>Markus et al., Stroke, 1994</td>
<td>Both Sexes (34±8years)</td>
<td>TCD</td>
<td>1% (NS)</td>
<td>&lt;1% (NS)</td>
<td>-10% (NS)</td>
</tr>
</tbody>
</table>

Data are arranged to show the effect of multiple prostaglandin synthesis inhibitors across studies using various measurement techniques. Collectively, the presented data highlight that only indomethacin is capable of significantly reducing CBF and cerebrovascular CO₂ reactivity. Due to several studies failing to report specific values for drug effects they have been estimated from presented graphs and figures. These studies have been noted with an * symbol.
1.3.6. Prostaglandin pathway: cyclic adenosine monophosphate production

*In vitro*, both cerebral microvascular smooth muscle and endothelial cells produce low levels of cAMP (Parfenova & Leffler, 1996) with PGI₂ and PGE₂ application markedly increasing cAMP production/concentrations above basal levels (Parfenova *et al.*, 1995a). Topically applied cAMP results in pial arteriolar dilation in newborn pigs, with pial arterial dilation strongly related to the increases in cAMP concentration (*r*>0.80; Parfenova *et al.*, 1993). Moreover, hypercapnia (Parfenova *et al.*, 1993, 1994), and both PGE₂ (Parfenova *et al.*, 1995a) and PGI₂ receptor agonism (Parfenova *et al.*, 1994, 1995b) increase cerebrovascular cAMP production. These findings provide compelling evidence that hypercapnia induced increases in cAMP are in part PGE₂/PGI₂ receptor agonism dependent (see Figure 1.5). This likely dependence of cAMP production during hypercapnia on PG activity (i.e. IP or EP receptor binding), is apparent in that INDO concurrently reduces cAMP production and vasomotor responses subsequent to PGI₂ receptor agonism (via iloprost) by 50-75% (Parfenova *et al.*, 1995b). Parfenova *et al.*, 1995 were able to more specifically highlight that the reduced cAMP production is a direct consequence of PGI₂ receptor agonism and not COX inhibition, as aspirin (also a COX inhibitor) does not inhibit PGI₂ receptor mediated vasomotor responses and had no effect on iloprost induced cAMP production (Parfenova *et al.*, 1995b). Thus, where other COX inhibitors have no effect, it seems INDO has a specific effect (that is not COX related) relative to inhibition of PGI₂ receptor mediated responses, potentially explaining its potent ability to inhibit cerebrovascular responses to CO₂. However, the exact location of this effect in the post-receptor signaling cascade is not clear. Thus, there is likely some effect of INDO on post receptor activation of adenylate cyclase or adenylate cyclase mediated production of cAMP, or both. These effects are independent to the downstream INDO induced inhibition of cAMP dependent protein kinase (Kantor & Hampton, 1978) which acts to increase smooth muscle cell Ca²⁺ sensitivity and blunt vessel dilation (Figure 1.5).

While increased production of cAMP is a consequent effect of PGI₂ receptor agonism via iloprost, hypercapnia also induces increases in cerebral cAMP (Parfenova *et al.*, 1993, 1995b). This PGI₂ receptor mediated production of cAMP during hypercapnia is an attractive
hypothesis to explain hypercapnic vasodilation as reductions in pH may increase PGI₂ receptor affinity (Hashimoto et al., 1990), making PGI₂ a potent vasoactive agent in an acidic milieu. It is plausible that only an extremely low amount of PGI₂ is necessary to trigger this hypercapnic PGI₂ receptor mediated production of cAMP. For example, near complete (i.e. >70-80%) inhibition of PGI₂ production via Aspirin has no effect on cAMP production subsequent to iloprost infusion or hypercapnia in newborn pigs (Parfenova et al., 1995b) and PGI₂ mediated facilitation of hypercapnic dilation is not dose dependent (Leffler et al., 1999). Therefore, PG’s may still be mediating dilation despite apparent abolished synthesis with other COX inhibitors. However, whether only a low level of PGI₂ is necessary to elicit hypercapnic dilation, or there are PG independent factors increasing cAMP (Parfenova & Leffler, 1996) in vivo remains unknown. Taken together, the production of cAMP during hypercapnia, and inhibition of PGI₂ receptor mediated cAMP production via INDO, likely explains how INDO reduces cerebrovascular CO₂ reactivity in animals (Pickard & MacKenzie, 1973; Parfenova et al., 1995b) and humans (Eriksson et al., 1983). Therefore, despite the apparent inability to detect hypercapnia induced PG synthesis in humans at baseline (Eriksson et al., 1983) or in animals treated to block PG synthesis (Leffler et al., 1993), PG’s may still be mediating responses at an extremely low plasma concentration.

In summary, in addition to its efficacy in inhibiting PG synthesis, INDO is likely inhibiting a post-receptor signal cascade that is reliant on only minimal PGI₂ activity (Parfenova et al., 1995b). Moreover, it is important to consider that downstream of changes in cAMP production, INDO also inhibits cAMP dependent protein kinase activity (Kantor & Hampton, 1978). These finding suggest that INDO may be acting in two additional manners compared to other COX inhibitors: 1) a prostaglandin dependent mechanism whereby IP receptor stimulation by PGI₂ increases smooth muscle cell cAMP levels, independent of COX inhibition, and 2) a prostaglandin independent mechanism whereby the inhibition of cAMP-dependent protein kinase results in increased smooth muscle cell calcium sensitivity.
1.3.7. Prostaglandin pathway: reactive oxygen species production

Increased PG production, induced via topical application of arachidonic acid within a cranial window, is associated with ROS production in cats (Kontos et al., 1985). The production of ROS resulting from the application of arachidonic acid is inhibited to the same extent by superoxide dismutase and INDO, suggesting that the increase in ROS is mediated via COX activity (Kontos et al., 1985). In COX1 null mice, superoxide dismutase has no effect on bradykinin induced dilation, indicating COX1 is responsible for ROS release during flux through the arachidonic acid pathway in this species (Niwa et al., 2001). In addition to their similar effects on ROS production, superoxide dismutase and INDO equally block vasodilation to both 5,6-EET and arachidonic acid (Ellis et al., 1990). This 5,6-EET is a COX substrate providing further evidence that COX produced ROS results in cerebral vasodilation in both cats and rabbits (Ellis et al., 1990). While the implications of concurrent ROS production during PG synthesis in regulating cerebrovascular tone is unknown in humans, it has been shown that ROS inhibition with superoxide dismutase and catalase nearly abolishes the vasodilatory response to topical arachidonic acid application in cats (Kontos et al., 1984) and rabbits (Ellis et al., 1990). Further, pial arterioles of cats (Wei et al., 1985), mice (Rosenblum, 1983) and newborn pigs (Leffler et al., 1990) dilate in response to increased ROS production. Taken together, at least at rest in animals, these studies indicate that pial vessel dilation in response to flux through the arachidonic acid pathway is largely due to ROS production secondary to COX activity.

Contrary to topical administration of arachidonic acid, combined superoxide dismutase and catalase do not attenuate the vasodilatory response to increased \( P_{a}CO_2 \) in cats (Kontos et al., 1984), newborn pigs (Wagerle & Mishra, 1988; Leffler et al., 1991) and mice (Rosenblum, 1983). However, supporting data in cats and rabbits specifically may not be relevant for hypothetical translation to humans as INDO administration in these animals also does not attenuate the CBF response to hypercapnia (Wei et al., 1980; Busija, 1983; Busija & Heistad, 1983) signifying hypercapnic vasodilation is not a PG dependent response in these species. Similar to topical application of arachidonic acid, hypercapnia also increases flux through the arachidonic acid pathway and consequent PG synthesis (Leffler et al., 1991); however, this
finding is not universal (Ellis et al., 1980). Thus reductions in hypercapnia mediated dilation in response to treatment with COX inhibitors are likely attributable to a reduction in PG’s but not the concurrent reduction in COX mediated ROS production (Leffler et al., 1991). For example, superoxide dismutase has no effect on the CBF response to hypercapnia in both normal and COX1 null mice (Niwa et al., 2001). Interestingly, superoxide dismutase greatly reduced (~50%) the cerebral dilation response to topical application of bradykinin (a peptide that causes downstream PG production and COX activity, in normal mice, but not COX1 null mice). These findings, in combination with the lack of effect during hypercapnia in both groups of mice, highlights that whilst ROS are an important mediator of dilation in conjunction with COX activity, this mechanism likely has no appreciable effect during hypercapnic challenges (Niwa et al., 2001). Further, it is unlikely that ROS effect hypercapnia induced PG production (Leffler et al., 1991). These studies highlight that the effects of ROS on vascular tone during increased PG synthesis at rest cannot be extrapolated to hypercapnic challenges, as COX mediated hypercapnic dilation is seemingly independent of the mediation of vasomotor tone by ROS in a multitude of species. This phenomenon remains to be studied in humans.

1.3.8. Prostaglandin pathway: interaction with nitric oxide

As both PG and NO formation are predominantly endothelium dependent they are commonly thought to interact. However, little evidence exists as to the potential synergistic or facilitative effects of NO and PG’s in the cerebrovascular response to changes in $P_{aCO_2}$. As hypercapnia leads to increases in cGMP a second messenger in the NO signal transduction pathway (Parfenova et al., 1993, 1994) concurrent to the aforementioned increases in cAMP this highlights the potential for these signaling pathways to modulate the other. Moreover, while iloprost and PGE$_2$ lead to increases in cerebral endothelial production of cGMP (Parfenova et al., 1995b), INDO markedly attenuates the cGMP response to hypercapnia (Parfenova et al., 1994). This potential interaction is also apparent in that INDO inhibits the hyperemic effect of applied L-arginine in rats during hypercapnia (Wang et al., 1994b). While the lack of data in animals precludes any conclusion as to potential NO and PG interactions during hypercapnia, the extremely limited study of NO in the regulation of
human cerebrovascular CO₂ reactivity (White et al., 1998; Ide et al., 2007) highlights how little is known as to the interaction of these systems in healthy humans.

1.4. Measurement of cerebral blood flow

Although considerable anatomical knowledge was acquired by anatomists three centuries ago, little was known about the brain’s circulatory control until the latter half of the nineteenth century. At this time, the first measurements of CBF were made by the Italian physiologist, Angelo Mosso. Mosso, in a select population of patients with skull defects, was able to measure cerebral volume changes, or brain pulsations, in response to emotional and cognitive stimuli, subsequent to his development of a specialized plethysmograph (Mosso, 1880). This provided the first insight into cerebrovascular regulation in humans, and signifies the starting point in the development of a wide array of CBF measurement techniques occurring over the last ~150 years. Some 50 years after Mosso’s original work, Kety and Schmidt developed an invasive method incorporating nitrous oxide as a tracer to measure CBF (Kety & Schmidt, 1948a). This technique was predominant in the cerebrovascular research world until Aaslid and colleagues, in 1982, demonstrated the utility of the TCD for the non-invasive assessment of CBF (Aaslid et al., 1982). This technique, which has been used across multiple fields for the assessment of numerous pathologies (i.e., stenosis, anemia, brain death, cerebral vasospasm, etc.), has largely shaped contemporary knowledge of cerebrovascular physiology.

Within the past decade, more advanced ultrasound techniques have been introduced into the field of cerebrovascular physiology. These techniques include both duplex ultrasound, and transcranial colour coded Doppler. Detailed discussions on other advanced imaging techniques such as MRI and PET are beyond the scope of this thesis and are reviewed elsewhere (Rota Kops et al., 1990; Zwanenburg et al., 2011). Since this thesis is based upon the use of TCD and peripheral duplex ultrasound, the primary principles of these techniques will now be discussed in detail.
1.4.1. Transcranial Doppler ultrasound.

The utility of Doppler ultrasound for the measurement of blood velocity in peripheral blood vessels was reported as early as 1965 (Miyazaki & Kato, 1965), while its applicability within the cerebral circulation was first described in 1982 (Aaslid et al., 1982). Aaslid and colleagues (1982) demonstrated that thin areas of the skull (or “acoustic windows”) allow for transmission of low frequency (2MHz) ultrasound for the insonation of various intra-cranial vessels. Thus, measurement of cerebral blood velocity in the large intra-cranial cerebral arteries of the brain (ACA, MCA, PCA & BA) was made possible in real time and on a beat-to-beat basis and, therefore, at a much greater temporal resolution than previous steady state techniques allowed for (Kety & Schmidt, 1948a). The primary limitation of TCD is that it does not provide an absolute measurement of volumetric blood flow, but rather measures the speed of red blood cells travelling through the insonated vessel. Therefore, the potential for changes in arterial diameter during alterations in P\textsubscript{a}O\textsubscript{2} (Wilson et al., 2011), P\textsubscript{a}CO\textsubscript{2} (Giller et al., 1993; Ainslie & Hoiland, 2014; Verbree et al., 2014; Coverdale et al., 2014, 2015; Figure 1.7), and blood pressure (Lewis et al., 2015) must be accounted for when interpreting TCD data. For example, traditionally it had been assumed that the MCA does not change in diameter during alterations in P\textsubscript{a}CO\textsubscript{2}, with this assumption largely based upon a low resolution (1.5T) magnetic resonance imaging (MRI) study performed in the year 2000 by Serrador and colleagues. However, recent high resolution (3 & 7T) MRI and ultrasound data have contested these previous assumptions, making it apparent that MCA diameter does change during P\textsubscript{a}CO\textsubscript{2} perturbations greater than approximately 7.5mmHg above or below baseline values (Willie et al., 2012; Verbree et al., 2014; Coverdale et al., 2014, 2015). Thus, the use of TCD will lead to underestimation of flow during hypercapnia, and overestimations of flow during hypocapnia. Such limitations have encouraged the measurement of CBF via Duplex ultrasound through the extra-cranial blood vessels (ICA & VA). This approach permits the quantification of both velocity and diameter of the vessel of interest.
Figure 1.7. The impact of MCA vasomotion on the discrepancy between volumetric and velocity indices of CBF. Previously reported changes in middle cerebral artery (MCA) diameter (left y-axis) and their calculated impact on the discrepancy between flow and velocity measures (right y-axis) during changes in end-tidal PCO2 (P\textsubscript{ET}CO\textsubscript{2}). To highlight the effects of MCA vasomotion we estimated the potential difference between CBF and velocity changes using the following. For example, cross-sectional area (CSA; cm\textsuperscript{2}) * Velocity (cm/s) * 60s = Flow (mL/min). Assuming a baseline MCA velocity of 60cm/s (which was done for all studies to facilitate diameter effect comparisons) coupled with the observed alterations in CSA with hyper- or hypocapnia (Coverdale \textit{et al.}, 2014), we calculated a representative baseline MCA flow value: 5.6mm\textsuperscript{2} * 60cm/s *60 s = Flow; therefore 0.056cm\textsuperscript{2} *60cm/s * 60s = 201.6 mL/min. Assuming previously reported values of cerebrovascular reactivity (Willie \textit{et al.}, 2012), MCA velocity increases ~4%/mmHg increase in P\textsubscript{ET}CO\textsubscript{2}. Assuming this as vessel reactivity (for all studies), we can estimate the volumetric MCA flow during hypercapnia using the reported CSA: 6.5mm\textsuperscript{2} * 84cm/s * 60s = Flow; therefore, 0.065cm\textsuperscript{2} * 84 cm/s * 60s = 327.6 mL/min. As such, the percent difference for flow between baseline and hypercapnia is [(327.6-201.6)/201.6]*100 = 62.5%. The percent difference in velocity between baseline and hypercapnia is [(84-60)/60]*100 = 40%, indicating that TCD would underestimate the increase in flow of the MCA during hypercapnia (+9mmHgP\textsubscript{ET}CO\textsubscript{2} from baseline) by >20%. However, if the percent difference is quantified via the magnitude of change in flow and velocity during hypercapnia, the increase in flow is ~50% greater than that of velocity! This can be calculated as %difference = [(%increase in flow - %increase in velocity)/%increase in velocity] * 100 and therefore, [(62.5-40)/40]*100 = 56.25%. As such, we have conservatively represented the effect of changes in diameter on flow vs. velocity discrepancies. For hypocapnia we again assumed a baseline MCA velocity of 60cm/s and used the pre-hypocapnia CSA reported (Coverdale \textit{et al.}, 2014) to calculate baseline flow: 5.8mm\textsuperscript{2} * 60cm/s * 60s = Flow; therefore, 0.058cm\textsuperscript{2} * 60cm/s * 60s = 208.8 mL/min. Incorporating a 2% change in MCAv per mmHg reduction in P\textsubscript{ET}CO\textsubscript{2} (Willie \textit{et al.}, 2012) and the associated change in CSA, we estimated volumetric MCA flow during hypocapnia: 5.3mm\textsuperscript{2} *46.8cm/s *60s = Flow; therefore, 0.053cm\textsuperscript{2}46.7cm/s*60s = 148.8mL/min. As such, the percent change in flow between baseline and hypocapnia is [(148.8-208.8)/208.8]*100 = -28.7%. The percent difference in velocity between baseline and hypocapnia is [(46.8-60)/60]*100 = -22%, indicating that TCD would underestimate the decrease in flow of the MCA during hypocapnia (-13mmHgP\textsubscript{ET}CO\textsubscript{2} from baseline) by ~7%. Thus, it is evident by these calculations and those seen above that small changes in MCA diameter are responsible for large discrepancies between flow and velocity measures. Data are collated from (Valdueza \textit{et al.}, 1997; Serrador \textit{et al.}, 2000; Verbree \textit{et al.}, 2014; Coverdale \textit{et al.}, 2014). As noted in the hypocapnic calculations, this graph represents the most conservative way to quantify the percent difference in flow and velocity changes, highlighting the the large impact that changes in MCA have in quantifying CBF. Figure and text from (Ainslie \& Hoiland, 2014). ©Permission not required.
1.4.1.1. Doppler physics.

Doppler ultrasound functions on the principle that the velocity of red blood cells is directly proportional to the magnitude of the Doppler shift. This principle means that as pulses are sent out from the ultrasound probe, the difference in time between the transmitted signal and the returning signal between two separate pulses is indicative of red blood cell velocity. For example, in the instance that blood is flowing towards the ultrasound probe, a second of two pulses will have a shorter signal transmission and signal return time than that of the original first pulse. The term “Doppler shift” signifies the difference between pulses.

Acquisition of correct velocity measures is dependent on the angle of insonation. Specifically, measures must be taken with an angle of 0-60° between the incident beam and direction of blood flow. A perpendicular beam in Doppler mode would result in Doppler shifts that are too low, poorly formed velocity waveforms, and/or incorrect determination of velocity. Therefore, it is important to approach a blood vessel so that the angle between blood flow and the incident beam is minimized. It is in this respect that proper technique for locating cerebral blood vessels is paramount in accurately measuring cerebral blood velocity with TCD.

1.4.1.2. Vessel location and insonation.

The large intra-cranial cerebral vessels can be insonated using three primary techniques: (1) the trans-temporal technique, where the probe is placed superior to the zygomatic arch; (2) the trans-occular technique, where the probe is placed over the closed eye; and (3) the foramen magnum approach, in which the probe is placed on the posterior portion of the head immediately inferior to external occipital protuberance (Willie et al., 2011a). These varying probe locations allow for proper signal optimization dependent upon the vessel of interest. To locate the MCA, the intracranial vessel pertaining to this thesis study, the trans-temporal approach is used.
1.4.1.3. The trans-temporal approach.

Proper determination of absolute MCAv is dependent upon correct insonation angle (Willie et al., 2011a). The transtemporal technique includes three different approaches for the insonation of the MCA: 1) through the posterior window, where the probe location is directly anterior to the zygomatic arch and immediately rostral of the pinna, 2) through the anterior window, where the probe is placed above the anterior process of the zygomatic arch, and 3) the middle window, which lies between the posterior and anterior window. Ideally the MCA is insonated through the middle window as in the absence of anatomical abnormalities this approach provides the lowest insonation angle and thus, most accurate measure of absolute blood velocity.

1.4.1.4. Quantification of cerebral blood velocity from transcranial Doppler ultrasound.

Velocity indices from TCD represent the entire range of blood velocity values throughout a vessel lumen. If blood flow is laminar the resultant blood velocity across the vessel lumen is roughly parabolic in shape, with the highest velocity occurring in the center of the vessel. A parabolic velocity profile allows for simple and accurate calculation of mean blood through a vessel as one half the peak velocity waveform envelope, or one half the time averaged peak velocity (Evans, 1985). Analysis error may be present in cases where blood velocity through the center of the vessel (as would be present with a parabolic velocity profile) is not the maximum velocity in the vessel, although it is rare that maximum velocity does not occur in the center of the vessel (Evans, 1985).

1.4.2. Duplex ultrasound of extra-cranial cerebral blood Vessels

Peripheral duplex ultrasound allows for the assessment of cerebral blood velocity using the same principles described above (see “1.4.1.1. Doppler Physics”) whilst simultaneously assessing arterial diameter. Simultaneous measurement of arterial diameter and blood velocity, in contrast to TCD, allows for the calculation of volumetric CBF. The primary advantage of this technique is that interpretation of duplex ultrasound data is not dependent
upon the assumption that arterial diameter is constant through perturbations in $P_a CO_2$. Peripheral duplex ultrasound can be effectively utilized to measure CBF in the extra-cranial cerebral vessels of the neck, the ICA and VA (Willie et al., 2012).

In previous studies (Willie et al., 2012; Lewis et al., 2014a, 2014b), global CBF has been calculated as double the sum of unilateral VA and ICA flow measures. When calculating CBF from unilateral ICA and / or VA measures it is important to be aware of bilateral differences as well as sex differences. For example, Seidel et al., 1999 performed a study on patients hospitalized in the neurological department and reported a significantly higher flow volume in the VA of males compared with females (Seidel et al., 1999). However, their sample size was relatively small to establish normative values (n = 50) and the authors also reported an angle of insonation of $62 \pm 6^\circ$ (range 45° to 75°) which is suboptimal for accurate velocity measurement. In contrast, Schoning et al., 1994 reported no difference in VA flow between men and women in healthy volunteers, indicating that differences are not present in healthy populations (Schöning et al., 1994). This has recently been corroborated by MRI measures (Zarrinkoob et al., 2015). It has also been reported that the left VA has higher flow than the right VA (Schöning et al., 1994; Seidel et al., 1999). These studies did not report any bilateral differences in flow through the ICA (Zarrinkoob et al., 2015).

1.4.2.1. Location and insonation of the internal carotid artery.

When aiming to locate the ICA, it is typical to first obtain a cross-sectional view of the common carotid artery. While maintaining both the same probe position and the cross sectional image in the center of the B-mode image, a 90° clockwise turn of the transducer will bring the common carotid artery into a longitudinal view (Figure 1.8-A). From here, by tracking the common carotid artery caudally the common carotid bifurcation will come into view. At this stage of the location process, several steps are necessary to differentiate between the external carotid artery and the ICA:

First, when viewing the common carotid bifurcation in the frontal plane it is typically observed that the ICA branches upwards and then curves downwards, while the external
carotid artery branches off downwards. In some cases, both vessels can be seen from this point of view; if not, an upwards or downwards “sweeping” motion with the transducer will alternate visualization of each vessel. Second, at the common carotid bifurcation the diameter of the external carotid artery is typically smaller than that of the ICA. As such, the ICA tends to visually appear larger. Third, the ICA has no extracranial branches. Therefore, the ability to visualize branching off from a vessel distal of the carotid bifurcation is indicative that the insonated vessel is the external carotid artery. Fourth, the velocity waveform of the ECA and ICA are distinctly different. The external carotid artery typically has a narrower systolic peak than the ICA and overall greater pulsatility index. Collectively, combining these steps will ensure that the correct vessel (in this case the ICA) is being insonated.

1.4.2.2. Quantification of CBF from duplex ultrasound.

By simultaneously collecting diameter and velocity measures via B-mode and Doppler signal acquisition, respectively, one can calculate volumetric flow by multiplying the velocity of blood flowing through the insonated vessel and the cross sectional area of the vessel. Vessel cross sectional area is calculated using the equation:

\[
\text{Cross sectional area} = \pi r^2
\]

Whereby ‘r’ represents the vessel radius. In the case of duplex ultrasound, the following formula is used:

\[
\text{Cross sectional area} = \pi (0.5 \cdot d)^2
\]

Where ‘d’ represents the vessel diameter as measured by duplex ultrasound B-mode imaging. As in a vessel which flow is laminar, blood velocity is parabolic in nature across the vessel lumen. Thus, mean velocity can be simply and accurately calculated as one half of the peak velocity blood velocity through the vessel (see “1.4.1.4. Quantification of Cerebral Blood Velocity from Transcranial Doppler Ultrasound” for a more in depth explanation). Thus, volumetric blood flow can be calculated by the following equation (Evans, 1985):
Volumetric blood flow = \( (\pi (0.5 \cdot d)^2) \cdot ((1/2)(v_{\text{max}})) \)

Where ‘\( v_{\text{max}} \)’ represents the maximum blood velocity through the vessel. Thus, by simultaneously measuring vessel diameter, and blood velocity, calculation of beat-by-beat blood flow is possible with adequate analysis software (see “1.3.2.3. Analysis Software”). Other parameters pertinent to vascular function can also be determined with duplex ultrasound such as shear rate, flow mediated dilation, and vessel compliance. In summary, it is important to optimize both the B-mode image of the vessel and blood velocity waveform for the assessment of beat-by-beat blood flow. Insonation angle of the vessel is important for determining a true velocity (i.e., avoiding underestimation), while changes in steering (angle) of the ultrasound beam are used to assess blood velocity parallel to the vessel walls, and therefore, in line with the direction of blood flow using an automated correction angle of 60°. Further, angling of the transducer (i.e., heel / toe maneuver) provides another technique to ensure acquisition of a proper velocity measures. Optimization of the velocity signal as outlined here typically follows optimization of the B-mode image.
Figure 1.8. Example duplex ultrasound image of the ICA. A. A standard image of the ICA as noted in the red box, which corresponds to the anatomical area of insonation depicted on the human figure. The concurrent velocity measure is directly below the B-mode image. B. The same ultrasound image as panel A, as it would appear in the blood flow analysis software. The yellow rectangles represent example regions of interest for sampling ICA diameter and velocity, with the dotted lines representing example edge detection tracking by the software.

1.4.2.3. Analysis software.

To analyze both diameter and velocity changes during CO$_2$ perturbations, while reducing/eliminating observer bias and/or error, it is necessary to use automated edge detection software. The analysis software “FMD/BloodFlow Software Version 4.0” (LabView 10.0) has been previously shown as highly effective in both validly (as assessed through measurement of phantom models) and reliably determining arterial diameter (Woodman et al., 2001) and blood flow responses (Black et al., 2008). Using this specific analysis software is simple and requires few steps making it a viable tool for the quantification of CBF.
During the experimental procedure, ultrasound images must be screen captured and saved for offline analysis. Saved files can then later be loaded into the FMD/BloodFlow Software Version 4.0 program for analysis. Regions of interest (ROI) must be made to calibrate for both vessel diameter and blood velocity. Placing the ROI over a segment of the ultrasound image with a known length (i.e., B-mode depth scale or pulsewave mode velocity scale) allow for the calibration of both diameter and velocity values. Subsequently, an ROI is placed over the entire waveform. It is imperative to watch the ultrasound video through once prior to selecting the diameter ROI as it is important to select the most stable section. These procedures are adapted from Woodman *et al.*, 2001.

The FMD/BloodFlow Software Version 4.0 program edge detection varies for the assessment of velocity and diameter. First, the peak envelope velocity waveform is it automatically detected due to the contrast in the velocity ROI. From the top down, the software detects the first pixel of contrast and uses this as a velocity value. For diameter, two signals are sent out from the middle of the ROI, one signal up and one down, detecting the first pixel of contrast. The resulting distance between the two points is calculated as a diameter value. Of note, many points are calculated at the same time with their average used as the diameter value at a rate of 30Hz (Figure 1.8-B). A representation of the software data output can be seen in Figure 1.9.
1.4.3. Variability in assessing cerebrovascular CO$_2$ reactivity

Measurement of cerebrovascular CO$_2$ reactivity is subject to observer error stemming from both measurement error and technical limitations, as well as physiological variability either between trials or between days. Combined, these two factors can potentially make assessment of small differences difficult necessitating a complete understanding of potential sources of error when interpreting data. For example, as blood pressure changes influence the magnitude of cerebrovascular reactivity during CO$_2$ perturbations (Willie et al., 2012; Regan et al., 2014), variations in the blood pressure response to CO$_2$ may contribute to between trial or between day differences in cerebrovascular reactivity. Moreover, reactivity will change throughout the day in relation to changes in endothelial function, whereby reactivity is higher in the evening than in the morning (Ainslie et al., 2007). Through understanding of the potential factors implicated in measurement variability, it is possible to design studies with
adequate controls in place to mitigate the risk of artificial variability unrelated to study interventions (i.e., testing all subjects at the same time of day).

1.4.3.1. Measurement variability.

Pertaining to this thesis, within day and between day variation are of utmost interest. Typically for biological comparisons a coefficient of variation (CoV) of <10% signifies good reproducibility of a measure, while a CoV of 10-20% represents moderate reproducibility and is still considered acceptable (Quan & Shih, 1996). Calculation of CoV is a viable method to assess reliability in studies with as small of a sample size as five, indicating feasible use in human physiology experiments (Tian, 2006). Thus, ultrasound measurements for the assessment of reliability were collected as part of this thesis, and the reliability of assessing ICA blood flow (Q_{ICA}) and MCAv will now be summarized.

Within and between day variability of resting Q_{ICA} measures are illustrated in the Bland Altman plots of figure 1.10. For within day measures of Q_{ICA} the test-retest (measures separated by ~3.5hours) CoV was 8.5%, while as expected the between day CoV was slightly higher at 12.5%. Importantly, there is no appreciable mean shift in resting Q_{ICA} (<3mL/min) for both within and between day test-retest differences.

![Bland-Altman plots of duplex ultrasound measurements of volumetric flow through the ICA. A. Within day reliability of measuring Q_{ICA} at rest. B. Between day reliability of measuring Q_{ICA} at rest. The red line represent the mean difference between tests for both figure A & B, while the dotted blue lines represent the upper and lower limits of agreement.](image-url)
Figure 1.11 shows Bland Altman plots of within day and between day test-retest measures of resting MCAv. The within day test-retest CoV was 7.6% with the between day CoV at 9.8%. Again there is no appreciable shift in the mean differences, which are both <3.5cm/s from zero. It is important to note that some of the observed variation will be due to differences in resting P_{ET}CO_2 as normal resting P_{ET}CO_2 can change within and between days. Collectively, these data are similar to that previously reported using duplex ultrasound (Lewis et al., 2015), and fall within the acceptable range for biological variability (i.e., <20%; Quan & Shih, 1996).

![Figure 1.11. Bland-Altman plots of transcranial Doppler ultrasound measurements of blood velocity through the MCA. A. Within day reliability of measuring MCAv at rest. B. Between day reliability of measuring MCAv at rest. The red line represent the mean difference between tests for both figure A & B, while the dotted blue lines represent the upper and lower limits of agreement.](image)

1.5. Experimental models to assess cerebrovascular CO_2 reactivity

Three primary methods are used to assess cerebrovascular CO_2 reactivity: Changes in fractional inspired CO_2 (FiCO_2); end-tidal forcing; and rebreathing methods. Each of these methods differs in their fundamental approach to alter P_{a}CO_2 and thus differ in their ability to assess a specific research question (Ainslie & Duffin, 2009; Fierstra et al., 2013). Moreover, the fashion in which these three methods are used varies between laboratories with specific variations being developed to further the utility of these tests. The specifics of each method pertinent to assessing cerebrovascular CO_2 reactivity will be briefly reviewed.
1.5.1. Changes in fractional inspired CO₂

Elevations in fractional inspired CO₂ (FiCO₂) are commonly used to induce hypercapnia. This technique is technically simple in nature and only requires the use of a Douglas bag containing above ambient concentrations of CO₂ (i.e., 6%) in air. However, there are several limitations associated with this technique. Variation in the ventilatory response to CO₂ will directly effect the resultant magnitude of hypercapnia. For example, a higher ventilatory response will effectively lower PₐCO₂, while a low ventilatory response will result in a higher PₐCO₂; effectively the cerebrovascular response, which is driven by PₐCO₂, using steady state changes in FiCO₂ is ventilatory dependent (Ainslie & Duffin, 2009). Thus it is difficult to precisely control the stimulus magnitude when assessing cerebrovascular CO₂ reactivity by this method due to highly variable between subject ventilatory sensitivity (Hirshman et al., 1975). Implicated with this methodological limitation is the fact that the degree of ventilation will effect the end-tidal to arterial CO₂ gradient, indicating that the accuracy of P_ETCO₂ in reflecting PₐCO₂ may also be different between subjects. Specifically, during elevations in FiCO₂, P_ETCO₂ over estimates PₐCO₂ (Peebles et al., 2007), leading to an underestimation of reactivity.

1.5.2. The rebreathe method

Rebreathing is a simple, but specialized technique used to “decompartmentalize” CO₂ in the body, or in other words eliminate the gradient between alveolar, arterial, brain tissue, and venous CO₂. This is beneficial when assessing cerebrovascular CO₂ reactivity as it eliminates the gradient between P_ETCO₂ and PₐCO₂ and thus the cerebrovascular CO₂ stimulus is known. However, the rebreathing technique precludes achievement of steady state measurements. As the full extent of CBF increases in response to CO₂ are not immediate (Shapiro et al., 1966), it has been commonly demonstrated that rebreathing reactivity underestimates that of both end-tidal forcing (Pandit et al., 2003, 2007) and FiCO₂ changes (Fan et al., 2010), with little data to the contrary (Brothers et al., 2014). Therefore, despite the utility of this technique for assessing other physiological parameters (i.e., ventilatory sensitivity), it is not an ideal approach to assess the full cerebrovascular response to CO₂.
1.5.3. End-tidal forcing

End-tidal forcing is a technique whereby end-tidal gases (\( P_{\text{ET}}O_2 \) & \( P_{\text{ET}}CO_2 \)) are controlled independently of ventilation. End-tidal forcing functions by prospectively calculating the required volume of \( O_2 \), \( CO_2 \), and \( N_2 \) in the inspirate to achieve desired end-tidal values. This is achieved through breath-by-breath determination of inspiratory and expiratory tidal volume, \( P_{\text{ET}}O_2 \) and \( P_{\text{ET}}CO_2 \), and the subsequent use of these values to determine the required inspirate via an error reduction algorithm (Tymko et al., 2015). As this test is ventilatory independent it allows for standardization of stimulus magnitude across subjects irrespective of their inherent variability in ventilatory sensitivity. These systems also minimize the gradient between \( P_{\text{ET}}CO_2 \) and \( P_aCO_2 \), which greatly reduces the associated risk for underestimation of cerebrovascular \( CO_2 \) reactivity that is characteristic of implementing changes in \( FiCO_2 \) (see “1.5.1. Changes in fractional inspired \( CO_2 \)”). Due to both the relatively minimal \( P_{\text{ET}}CO_2 \) to \( P_aCO_2 \) gradient, and ventilatory independent nature of end-tidal forcing it is an ideal scientific model to manipulate \( CO_2 \) for the assessment of cerebrovascular reactivity.

1.6. Purpose and hypotheses.

As the regulation of CBF in response to changes in \( P_aCO_2 \) remains incompletely understood, the purpose of this thesis study was to further investigate the role of PG’s in mediating cerebrovascular responses to hypercapnia and hypocapnia. More specifically, to determine the role of PG’s in mediating vasomotion of the large extra-cranial cerebral vessels through ultrasound examination of the ICA during \( P_aCO_2 \) perturbations.

It was hypothesized that:

1) The ICA would dilate and constrict in response to hyper and hypocapnia, respectively;

2) Orally administering INDO would markedly reduce the cerebral vasomotor (i.e., dilation / constriction) response to both hyper and hypocapnia in the ICA.
It was further reasoned, based upon earlier reports (Eriksson et al., 1983; Markus et al., 1994), that another non-selective COX inhibitor (Ketorolac) would have no effect on the vasomotor response of the ICA to changes in CO₂.
Chapter 2: Carbon dioxide mediated vasomotion of extra-cranial cerebral arteries: a role for prostaglandins?

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2.1. Overview.

Cerebrovascular regulation during perturbations in arterial CO₂ is thought to occur solely at the level of the pial vessels. However, recent evidence implicates large extra-cranial cerebral blood vessels in this regulatory process. Although the mechanisms governing CO₂ mediated vasomotion remain unclear, animal and human studies support a large role of prostaglandins. Thus, we examined two hypotheses: 1) vasomotion of the internal carotid artery (ICA) would occur in response to both hyper and hypocapnia; and 2) pharmacological inhibition of prostaglandin synthesis with Indomethacin (INDO; a non-selective cyclooxygenase inhibitor) would reduce the vasomotor response of the ICA to changes in end-tidal PCO₂ (P_{ET}CO₂). Using a randomized single-blind placebo controlled study, subjects (n=10) were tested on two occasions. Before and 90-minutes following either oral INDO (1.2mg/kg) or placebo capsule, concurrent measures of beat-by-beat blood flow, velocity and diameter of the ICA were made at rest and during steady state stages (4 min) of iso-oxic hypercapnia (+3, +6, +9mmHg above baseline) and hypocapnia (-3, -6, -9mmHg below baseline). End-tidal forcing was employed for the control of blood gases. To examine if INDO affected ICA vasomotion in a cyclooxygenase inhibition independent manner, a subset of subjects (n=5) were tested before and 45-minutes following oral Ketorolac (0.25mg/kg). During pre-drug testing in the INDO trial, the ICA dilated during hypercapnia at +6mmHg (4.72±0.45 vs. 4.95±0.51mm; P<0.001) and +9mmHg (4.72±0.45 vs. 5.12±0.47mm; P<0.001), and constricted during hypocapnia at -6mmHg (4.95±0.33 vs. 4.88±0.27mm; P<0.05) and -9mmHg (4.95±0.33 vs. 4.82±0.27mm; P<0.001). Following INDO, dilation of the ICA was still observed at +6mmHg (4.50±0.54 vs. 4.57±0.52mm; P<0.05) and +9mmHg (4.50±0.54 vs. 4.61±0.50mm; P<0.01); however, INDO reduced the vasomotor responsiveness by
67±28\% (0.045±0.015 vs. 0.015±0.012\text{mm} \cdot \text{mmHg} P_{ET} CO_2^{-1}). In the Ketorolac condition, there was no effect of the drug on the vasomotor response to hyper or hypocapnia. We conclude that: 1) changes in $P_{ET} CO_2$ mediate vasomotion of the ICA, 2) inhibition of non-selective prostaglandin synthesis via INDO markedly reduces the vasomotor response to changes in $P_{ET} CO_2$; and 3) INDO may be acting via a mechanism(s) independent of cyclooxygenase inhibition to reduce CO$_2$ mediated vasomotion.

2.2. Background.

The cerebral vasculature is highly sensitive to alterations in $P_a$ CO$_2$. Elevations in $P_a$ CO$_2$ (hypercapnia) cause a reduction in cerebrovascular resistance and consequent increases in CBF, while reductions in $P_a$ CO$_2$ (hypocapnia) cause an increase in cerebrovascular resistance and decrease in CBF (Kety & Schmidt, 1948) – this response is termed cerebrovascular CO$_2$ reactivity. Cerebrovascular CO$_2$ reactivity acts to attenuate fluctuations in central pH and maintain homeostatic function (Ainslie & Duffin, 2009). Until recently, dogma posited that hypercapnia exclusively induces small pial vessel dilation, with little to no vasomotion occurring in the larger cerebral arteries (Wolff & Lennox, 1930; Serrador et al., 2000). This assumption persisted despite human (Giller et al., 1993) and animal studies (Heistad et al., 1978) providing evidence to the contrary. Recently, however, new insight from high resolution magnetic resonance imaging has revealed that vasomotion of the MCA occurs in response to both hyper- and hypo-capnia (Verbree et al., 2014; Coverdale et al., 2014). Furthermore, using vascular ultrasound combined with automated edge detection analysis software, dilation of large extra-cranial cerebral arteries (e.g., VA & ICA) has also been reported (Willie et al., 2012); however, these latter findings have not been consistently demonstrated when caliper based manual diameter analysis methods are used (Sato et al., 2012; Coverdale et al., 2015).

The potential mechanism(s) whereby changes in $P_a$ CO$_2$ result in vasomotion of larger cerebral arteries include adenosine (Phillis & DeLong, 1987), NO (Parfenova et al., 1994; Smith et al., 1997), and PG’s (Wennmalm et al., 1981; Bruhn et al., 2001; St Lawrence et al., 2002). The latter mechanism is clearly highlighted in that administration of INDO - a
non-selective COX inhibitor - reduces basal CBF by ~20-30% and cerebrovascular CO\(_2\) reactivity by ~50-60% (Eriksson et al., 1983; Xie et al., 2005; Fan et al., 2010; Hoiland et al., 2015). While animal studies report prostaglandin mediated hypercapnic vasodilation at the level of the small pial vessels and arterioles (Pickard et al., 1980; Busija & Heistad, 1983; Leffler et al., 1991), some data collected in post-mortem humans indicate that prostaglandin-mediated vasomotion may also take place within the larger cerebral arteries (i.e., MCA; Davis et al., 2004). As cerebrovascular CO\(_2\) reactivity is important in stabilizing central pH, and predictive of health outcome (Portegies et al., 2014), it is imperative to understand the underlying mechanisms that regulate this response. Although COX inhibition reduces CBF (Xie et al., 2006; Fan et al., 2010; Hoiland et al., 2015) and blunts cerebrovascular CO\(_2\) reactivity (Xie et al., 2006; Fan et al., 2010; Hoiland et al., 2015), it is unknown if this is due in part to a reduction in vasomotion of the larger extra-cranial cerebral arteries. Therefore, using a single blinded placebo-controlled and randomized design, the purpose of this study was two-fold: 1) resolve the conflicting data surrounding hypercapnic dilation and hypocapnic vasoconstriction of the ICA, and 2) determine if PG’s are implicated in CO\(_2\) mediated large cerebral artery vasomotion. We hypothesized that the ICA would dilate in response to hypercapnia, and constrict in response to hypocapnia. Moreover, it was hypothesized that COX inhibition with INDO would markedly attenuate the dilatory response to hypercapnia, and the constrictive response to hypocapnia in the ICA. Since INDO has demonstrated a unique ability to inhibit CO\(_2\) mediated cerebrovascular responses when compared to other COX inhibitors (i.e., Aspirin and Naproxen; Eriksson et al., 1983; Markus et al., 1994), in follow up experiments, we sought to assess the effects of Ketorolac - also a potent non-selective COX inhibitor - on CO\(_2\) mediated vasomotion to determine if there is a unique influence of INDO under the conditions of our experimental approach. In this respect, we reasoned that Ketorolac would not affect the vasomotor response of the ICA to hypercapnia and hypocapnia.
2.3. Methods.

2.3.1. Subjects.

In total, fifteen healthy young volunteers were recruited to participate in this study. The main study, (INDO investigations) included 10 subjects (1 female) with a mean age of 23±7 years, and body mass index of 22±2 Kg/m². In the follow-up study (Ketorolac investigations), a subgroup of five subjects (all male) were examined (30±7 years; body mass index of 24±2 Kg/m²). All subjects first completed written informed consent followed by a familiarization session. During familiarization, subjects were screened to ensure reliable neck artery (VA & ICA) ultrasound images could be attained along with intracranial (MCA & PCA) signals. Subjects were familiarized with the remaining experimental equipment and procedures during this session. All subjects were free of any past or present cardiorespiratory and cerebrovascular disease and were not taking any prescription drugs (other than oral contraceptives; n=1) at their time of participation, as determined by a screening questionnaire. This study was approved by the University of British Columbia Clinical Research Ethics Board and conformed to the Declaration of Helsinki.

2.3.2. Experimental protocol

On the day of experimental sessions subjects arrived to the laboratory at the same time of day having refrained from alcohol, exercise and caffeine for the previous 24 hours. Subjects were instructed to lie supine for at least 15 minutes prior to beginning the study protocol and were instrumented with the experimental equipment.

2.3.2.1. Study 1.

To investigate the role of non-selective COX inhibition via oral INDO we used a single blinded, randomized, and counter balanced placebo controlled trial requiring two laboratory visits. On each day, following baseline measurements while breathing room air, the subjects performed two CO₂ reactivity tests (a hypercapnic test followed by a hypcapnic test).
Thereafter, they were orally administered 1.2mg/Kg of INDO or placebo (sugar pill matched for weight and capsule size), and repeated the baseline measures and the CO\textsubscript{2} tests 90-minutes later (Xie et al., 2006). This dose of INDO used has been previously shown to effectively inhibit COX activity (Eriksson et al., 1983). Test days were separated by 10±9 days. The CO\textsubscript{2} reactivity tests are as follows:

**Test 1: Progressive steady state iso-oxic elevations of P\textsubscript{ET}CO\textsubscript{2}**. End-tidal forcing was utilized to maintain P\textsubscript{ET}CO\textsubscript{2} and P\textsubscript{ET}O\textsubscript{2} at baseline (resting) values on an individual basis for four minutes. Upon completion of this baseline stage, P\textsubscript{ET}O\textsubscript{2} remained unchanged while P\textsubscript{ET}CO\textsubscript{2} was sequentially elevated to +3, +6, and +9mmHg above baseline, with each stage lasting four minutes. Upon completion of the +9mmHg P\textsubscript{ET}CO\textsubscript{2} stage the subject breathed room air. A end-tidal forcing system was used to manipulate P\textsubscript{ET}CO\textsubscript{2} and maintain P\textsubscript{ET}O\textsubscript{2} during the hypercapnic CO\textsubscript{2} reactivity test as previously described (Tymko et al., 2015).

**Test 2: Progressive steady state iso-oxic reductions in P\textsubscript{ET}CO\textsubscript{2}**. End-tidal forcing was utilized to maintain P\textsubscript{ET}CO\textsubscript{2} and P\textsubscript{ET}O\textsubscript{2} at baseline (resting) values on an individual basis for four minutes. Upon completion of this baseline stage, subjects were instructed to hyperventilate to sequentially lower their P\textsubscript{ET}CO\textsubscript{2} to -3, -6, -9mmHg below baseline values while P\textsubscript{ET}O\textsubscript{2} remained unchanged. Once adequate hyperventilation was achieved, subjects were instructed to maintain constant breathing for the remainder of the test (i.e., for -3, -6, & -9mmHg stages). To achieve precise reductions of P\textsubscript{ET}CO\textsubscript{2}, the CO\textsubscript{2} concentration of the inspirate was altered on a breath-by-breath basis to compensate for variability in ventilation. A end-tidal forcing system was used to control P\textsubscript{ET}CO\textsubscript{2} and maintain P\textsubscript{ET}O\textsubscript{2} during the hypocapnic CO\textsubscript{2} reactivity test as previously described (Tymko et al., 2015).

2.3.2.2. Study 2.

To investigate the role of non-selective COX inhibition, using orally administered Ketorolac (Toradol), subjects attended the lab on one occasion. Following baseline measurements while breathing room air, the subjects performed the identical two CO\textsubscript{2} reactivity tests as in *Study 1* (a hypercapnic test followed by a hypocapnic test). These tests were repeated 45 min later.
following orally administered Ketorolac (0.25mg/kg). Previous studies have confirmed the effectiveness on COX inhibition of this dose of Ketorolac at 45 min (peak plasma concentration) with an associated half-life of ~5-6 hours (Jung et al., 1989; Jallad et al., 1990).

2.3.3. Experimental measures.

2.3.3.1. Cardiorespiratory measures.

All cardiorespiratory variables were sampled continuously throughout the protocol at 1000Hz via an analogue-to-digital data acquisition system (Powerlab, 16/30; ADInstruments, Colorado Springs, CO). Heart rate (HR) was measured by a 3-lead electrocardiogram (ECG), and beat-to-beat blood pressure by finger photoplethysmography (Finometer PRO, Finapres Medical Systems, Amsterdam, Netherlands). Both $P_{ET}CO_2$ and $P_{ET}O_2$ were sampled at the mouth and recorded by a calibrated gas analyzer (model ML206, ADInstruments) while respiratory flow was measured by a pneumotachograph (model HR 800L, HansRudolph, Shawnee, KS). Subjects’ MAP was calculated as the mean of the reconstructed brachial waveform from the Finometer. All data was interfaced with LabChart (Version 7), and analyzed offline. Average values for the last minute of each stage were recorded.

2.3.3.2. Cerebrovascular measures.

Blood velocity through the right MCA was measured using a 2MHz TCD (Spencer Technologies, Seattle, WA). The TCD probe was attached to a specialized headband fixation device (model M600 bilateral head frame, Spencer Technologies), and then secured into place. The MCA was insonated through the middle trans-temporal window, using previously described location and standardization techniques (Willie et al., 2011a; see “1.4.1. Transcranial Doppler Ultrasound”).
Blood velocity and vessel diameter of the ICA was measured using a 10MHz multi-frequency linear array vascular ultrasound (Terason T3200, Teratech, Burlington, MA). Specifically, B-mode imaging was used to measure arterial diameter, while pulse-wave mode was used to simultaneously measure peak blood velocity. Measures of $Q_{ICA}$ were made ipsilateral to the MCA. The ICA diameter and velocity were measured at least 1.5 cm distal to the common carotid bifurcation to eliminate recordings of turbulent and retrograde flow. Great care was made to ensure that the insonation angle (60°) was unchanged throughout each test. Further, for all experimental sessions, upon acquisition of the first ultrasound image there was no alteration of B-mode gain to avoid any artificial changes in arterial wall brightness / thickness.

All of the ICA recordings were screen captured and stored as AVI files for offline analysis. This analysis involved concurrent determination of arterial diameter and peak blood velocity at 30Hz, using customized edge detection and wall tracking software designed to eliminate observer bias (Woodman et al., 2001). No less than 12 consecutive cardiac cycles were used to determine $Q_{ICA}$. Volumetric blood flow was subsequently calculated using the following formula:

$$Q_{ICA} = \frac{\text{Peak Envelope Velocity}}{2} \times \left[ \pi \left( \frac{1}{2} \right) \text{Diameter} \right]^2$$

Volumetric blood flow (ICA) and velocity (MCA) values were calculated within the final minute of each four-minute steady state stage. Cerebrovascular responses were calculated separately for the hypercapnic and hypocapnic reactivity tests. All response slopes (i.e., mm · mmHg$\cdot$P$_{ET}$CO$_2^{-1}$) were calculated using linear regression.

2.3.4. Statistical analysis.

All resting data were compared between conditions using a one-way repeated measures ANOVA and Tukey post-hoc tests where applicable. Changes in diameter during hyper and hypocapnia were analyzed by a one-way repeated measures ANOVA within each experimental trial with Tukey post-hoc tests. Between-condition differences in diameter for a
specific P\textsubscript{ET}CO\textsubscript{2} manipulation (i.e., pre vs. post-INDO at +9mmHgP\textsubscript{ET}CO\textsubscript{2}) were analyzed using a two-tailed paired t-test. Between-condition reactivities (i.e., flow and vasomotor reactivities) were compared using a one-way repeated measures ANOVA with Tukey post-hoc tests. Comparisons between pre- and post-Ketorolac variables were made with two-tailed paired t-tests. All data are expressed as means ± SD with \textit{a priori} statistical significance set at \( P < 0.05 \).

\textbf{2.4. Results.}

\textbf{2.4.1. Resting cerebral blood flow and cardiorespiratory variables.}

Resting \( Q\textsubscript{ICA} \) was reduced by 40±12\% following INDO (257.3±57.2 vs. 151.9±36.6 mL \cdot min\textsuperscript{-1}; \( P<0.001 \)), while placebo treatment had no effect (257.8±60.2 vs. 252.3±62.4 mL \cdot min\textsuperscript{-1}; \( P=0.55 \)). Similarly, INDO reduced resting MCAv by 36±11\% (65.5±8.6 vs. 42.0±8.8 cm \cdot s\textsuperscript{-1}; \( P<0.001 \)), while placebo had no effect (62.4±12.4 vs. 60.7±14.3 cm \cdot s\textsuperscript{-1}; \( P=0.43 \)). Pre-INDO resting \( Q\textsubscript{ICA} \) and MCAv were not different from the pre-placebo and placebo trials. Resting ventilation was unaffected by INDO; however, compared to the placebo, INDO caused a modest increase in MAP (77.0±5.6 vs. 83.8±8.7 mmHg; \( P<0.05 \)) and decreased HR (57±11 vs. 50±9 bpm; \( P<0.01 \)).

\textbf{2.4.2. Cerebrovascular CO\textsubscript{2} reactivity (Table 2.0).}

Absolute hypercapnic \( Q\textsubscript{ICA} \) and MCAv reactivity were reduced by 69±20\% and 59±28\% following INDO, respectively, but were unaltered by placebo treatment (Figure 2.0). For both \( Q\textsubscript{ICA} \) and MCAv, the pre-INDO, pre-placebo, and placebo trials did not differ in their respective absolute hypercapnic reactivities. Following INDO, relative hypercapnic \( Q\textsubscript{ICA} \) and MCAv reactivity were reduced by 50±33\% and 38±36\%, respectively, but unaltered by placebo treatment. For both \( Q\textsubscript{ICA} \) and MCAv, the pre-INDO, pre-placebo, and placebo trials did not differ in their respective relative hypercapnic reactivities.
Table 2.0. Cerebrovascular and blood pressure responses to CO$_2$ before and following INDO or placebo.

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<th>Absolute Reactivity</th>
<th>Relative Reactivity</th>
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<tr>
<td></td>
<td>Units</td>
<td>Pre-INDO INDOPre-Placebo Pre-INDO Pre-Placebo Pre-INDO Pre-Placebo Pre-INDO Pre-Placebo Pre-INDO Pre-Placebo</td>
</tr>
<tr>
<td>Q$<em>{ICA}$ (mL·min$^{-1}$·mmHgP$</em>{ET}$C O$_2^{-1}$)</td>
<td>18.9±7.3 ‡ 5.3±3.0** † 17.1±5.3 ‡ 15.9±3.3 ‡ 7.3±2.0‡ 3.5±2.2** † 6.7±1.9‡ 6.2±1.2‡</td>
<td></td>
</tr>
<tr>
<td>MCAv (cm·s$^{-1}$·mmHgP$_{ET}$C O$_2^{-1}$)</td>
<td>3.2±0.7‡ 1.3±1.0** † 2.8±0.7‡ 2.9±0.7‡ 4.8±0.9‡ 3.0±1.9** † 4.5±1.3‡ 5.0±1.8‡</td>
<td></td>
</tr>
<tr>
<td>VE (L·min$^{-1}$)</td>
<td>2.7±0.8 3.1±1.0 3.0±1.2 2.9±1.3 19.8±6.1 19.9±8.1 22.1±10.6 19.7±9.7</td>
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<tr>
<td>MAP (mmHg·mmHgP$_{ET}$C O$_2^{-1}$)</td>
<td>0.9±0.6 0.8±0.5 0.9±0.4 1.2±0.6 1.2±0.8 1.0±0.7 1.0±0.4 1.5±0.4</td>
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<th>Absolute Reactivity</th>
<th>Relative Reactivity</th>
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<tr>
<td></td>
<td>Units</td>
<td>Pre-INDO INDOPre-Placebo Pre-INDO Pre-Placebo Pre-INDO Pre-Placebo Pre-INDO Pre-Placebo Pre-INDO Pre-Placebo</td>
</tr>
<tr>
<td>Q$<em>{ICA}$ (mL·min$^{-1}$·mmHgP$</em>{ET}$C O$_2^{-1}$)</td>
<td>8.2±4.2 2.2±2.0** 7.9±2.2 6.8±2.2 3.0±1.0 1.2±0.9** 3.0±0.6 2.6±0.4</td>
<td></td>
</tr>
<tr>
<td>MCAv (cm·s$^{-1}$·mmHgP$_{ET}$C O$_2^{-1}$)</td>
<td>1.6±0.4 0.4±0.5** 1.6±0.4 1.5±0.5 2.4±0.5 0.7±0.8** 2.5±0.4 2.4±0.5</td>
<td></td>
</tr>
<tr>
<td>MAP (mmHg·mmHgP$_{ET}$C O$_2^{-1}$)</td>
<td>-0.4±0.5 -0.08±0.6 -0.3±0.6 -0.04±0.8 -0.5±0.7 -0.07±0.7 -0.4±0.8 -0.002±0.9</td>
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* significantly lower than pre-INDO, P<0.01; ** significantly lower than Pre-INDO, P<0.001; † significant difference between hypercapnic and hypocapnic reactivities within an experimental trial, P<0.01; ‡ significant difference between hypercapnic and hypocapnic reactivities within an experimental trial, P<0.001
Although unchanged in the placebo trial, absolute hypocapnic $Q_{ICA}$ and MCAv reactivity were significantly reduced by $72\pm25\%$ and $79\pm25\%$, respectively following INDO (Figure 2.1). For both $Q_{ICA}$ and MCAv, the pre-INDO, pre-placebo, and placebo trials did not differ in their respective absolute hypocapnic reactivities. Relative hypocapnic $Q_{ICA}$ and MCAv reactivity were reduced by $60\pm32\%$ (P<0.001) and $76\pm32\%$ (P<0.001), respectively, following INDO, but were unaltered following the placebo treatment. For both $Q_{ICA}$ and MCAv, the pre-INDO, pre-placebo, and placebo trials did not differ in their respective relative hypocapnic reactivities.

**Figure 2.0. Volumetric flow and velocity cerebrovascular reactivity to hypercapnia.** A. Hypercapnic $Q_{ICA}$ reactivity prior to and following INDO and placebo treatments. B. Hypercapnic MCAv reactivity prior to and following INDO and placebo treatments. Horizontal lines denote significant differences between trials, P<0.001.

Hypercapnic reactivity was greater than that during hypocapnia for $Q_{ICA}$ and MCAv during all trials. During hypercapnia, $Q_{ICA}$ reactivity was greater than that of both ICA velocity (ICAv; $7.3\pm2.0$ vs. $3.5\pm2.2 \% \cdot \text{mmHg} P_{ET} CO_2$; P<0.001) and MCAv ($7.3\pm2.0$ vs. $4.8\pm0.9 \% \cdot \text{mmHg} P_{ET} CO_2$; P<0.001) reactivity, which could be explained by progressive dilation of the ICA (see “2.4.3. Vasomotor response to CO$_2$”). There was no difference in hypocapnic $Q_{ICA}$, ICAv, and MCAv reactivity, likely due to a modest vasomotor reactivity to hypocapnia.
Figure 2.1. Volumetric flow and velocity cerebrovascular reactivity to hypocapnia. A. Hypocapnic \( Q_{\text{ICA}} \) reactivity prior to and following INDO and placebo treatments. B. Hypocapnic MCAv reactivity prior to and following INDO and placebo treatments. Horizontal lines denote significant differences between trials, \( P<0.001 \).

2.4.3. Vasomotor response of the internal carotid artery to CO\(_2\).

Prior to INDO, the ICA dilated significantly at +6 & +9 mmHg \( P_{\text{ET}}\text{CO}_2 \); although to a lesser extent, dilation still occurred at +6 & +9 mmHg \( P_{\text{ET}}\text{CO}_2 \) following INDO (Figure 2.2-A). The ICA dilated at every stage of hypercapnia pre and post placebo treatment (Figure 2.2-B). During hypocapnia the ICA constricted at -9 mmHg \( P_{\text{ET}}\text{CO}_2 \) prior to INDO, with no constriction post-INDO (Figure 2.3-A). During both the pre-placebo and placebo trial, the ICA constricted at -6 & -9 mmHg \( P_{\text{ET}}\text{CO}_2 \) (Figure 2.3-B).
Figure 2.2. The vasomotor response to hypercapnia. A. The vasomotor response to hypercapnia pre (●) and post (○) INDO. B. The vasomotor response to hypercapnia pre (■) and post (□) placebo. * difference from baseline P<0.05; ** difference from baseline P<0.01; *** difference from baseline P<0.001; # difference from previous stage P<0.05; ## difference from previous stage P<0.01; ### difference from previous stage P<0.001; † within day difference in diameter between baseline and intervention P<0.05; †† within day difference in diameter between baseline and intervention P<0.01; ††† within day difference in diameter between baseline and intervention P<0.001.

The slope of the vasomotor response to hypercapnia was reduced by 67±28% following INDO (0.045±0.015 vs. 0.015±0.012 mm · mmHgP_{ETCO2}^{-1}; P<0.001) but was unaffected by placebo (0.036±0.006 vs. 0.033±0.006 mm · mmHgP_{ETCO2}^{-1}; P=0.25). No change in the slope of the response to hypocapnia occurred following INDO (0.019±0.015 vs. 0.006±0.008 mm · mmHgP_{ETCO2}^{-1}; P=0.08). Vasomotor reactivity was greater during hypercapnia than during hypocapnia (0.045±0.015 vs. 0.019±0.015 mm · mmHgP_{ETCO2}^{-1}; P<0.01).
2.4.4. Effect of Ketorolac on CO₂ mediated responses (Table 2.1).

Administration of Ketorolac had no effect on resting ventilation (11.3±1.5 vs. 11.8±1.9 L · min⁻¹; P=0.65), MAP (79.2±8.6 vs. 80.0±5.9 mmHg; P=0.79), HR (55.1±12.5 VS. 53.9±14.6 bpm; P=0.54), QICA (282.8±66.7 vs. 295.6±80.3 mL · min⁻¹; P=0.38), or MCAv (56.5± 13.3 vs. 58.3±8.1 cm · s⁻¹; P=0.54). Following Ketorolac there was no change in absolute QICA or MCAv reactivity during both hypercapnia and hypocapnia. Relative reactivities for QICA and MCAv did not differ before and after Ketorolac either. The vasomotor response of the ICA to both hypercapnia (0.026±0.015 vs. 0.026±0.022 mm · mmHgPₑ₅CO₂⁻¹; P=0.99) and hypocapnia (0.020±0.024 vs. 0.017±0.014 mm · mmHgPₑ₅CO₂⁻¹; P=0.73) were unchanged following Ketorolac.
Table 2.1. Cerebrovascular and blood pressure responses to CO₂ before and following Ketorolac.

<table>
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<tr>
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<th>Hypercapnia</th>
<th>Relative Reactivity</th>
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<tr>
<td></td>
<td>Absolute Reactivity</td>
<td></td>
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<tr>
<td></td>
<td>Units</td>
<td>Pre-Ketorolac</td>
</tr>
<tr>
<td>QICA (mL · min⁻¹ · mmHgPETCO₂⁻¹)</td>
<td>16.6±8.6+</td>
<td>16.2±8.5+</td>
</tr>
<tr>
<td></td>
<td>(%) · mmHgPETCO₂⁻¹</td>
<td></td>
</tr>
<tr>
<td>MCA (cm · s⁻¹ · mmHgPETCO₂⁻¹)</td>
<td>2.6±0.6‡</td>
<td>2.7±0.6‡</td>
</tr>
<tr>
<td></td>
<td>(%) · mmHgPETCO₂⁻¹</td>
<td></td>
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<tr>
<td>VE (L · min⁻¹)</td>
<td>3.0±2.0</td>
<td>2.8±1.74</td>
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<tr>
<td></td>
<td>(%) · min⁻¹</td>
<td></td>
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<tr>
<td>MAP (mmHg · mmHgPETCO₂⁻¹)</td>
<td>1.1±0.7†</td>
<td>0.8±0.5+</td>
</tr>
<tr>
<td></td>
<td>(%) · mmHgPETCO₂⁻¹</td>
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</table>

|                      | Hypocapnia                   |                     |
|                      | Absolute Reactivity          |                     |
|                      | Units                        | Pre-Ketorolac       | Ketorolac |
| QICA (mL · min⁻¹ · mmHgPETCO₂⁻¹) | 8.0±2.1                      | 6.5±4.0             | 2.8±0.7   |
|                       | (%) · mmHgPETCO₂⁻¹           |                     |
| MCA (cm · s⁻¹ · mmHgPETCO₂⁻¹) | 1.2±0.6                      | 1.7±0.7             | 2.1±0.8   |
|                       | (%) · mmHgPETCO₂⁻¹           |                     |
| MAP (mmHg · mmHgPETCO₂⁻¹) | -0.4±0.3                     | -0.2±0.4            | -0.5±0.3 |
|                       | (%) · mmHgPETCO₂⁻¹           |                     |

+ significant difference between hypercapnic and hypocapnic reactivities within an experimental trial, P<0.05; † significant difference between hypercapnic and hypocapnic reactivities within an experimental trial, P<0.01; ‡ significant difference between hypercapnic and hypocapnic reactivities within an experimental trial, P<0.001.

2.5. Discussion.

The main novel findings of this study were: 1) The ICA dilates in response to hypercapnia and modestly constricts in response to hypocapnia; 2) Vasomotion of the ICA in response to hypercapnia is markedly blunted (-67%) following INDO administration; and 3) INDO, but not Ketorolac, inhibits cerebrovascular responses to CO₂, indicating a permissive (i.e., non-COX inhibition mediated) effect(s) of INDO.

2.5.1. Cerebrovascular responses to CO₂.

Our volumetric data have revealed similar hypercapnic reactivity values to that first collected using the Kety & Schmidt technique (Kety & Schmidt, 1948b) of ~7-8 % · mmHgPETCO₂⁻¹; such values are consistent with recent studies (Willie et al., 2012; Hoiland et al., 2015). This
volumetric reactivity, during hypercapnia, greatly exceeds that recorded using TCD measures of MCAv in the current and previous studies [e.g., (Ide et al., 2003, 2007; Willie et al., 2012; Regan et al., 2014; Brothers et al., 2014; Hoiland et al., 2015)]. Since MCAv and ICAv reactivity to hypercapnia did not differ, this indicates that vasomotion is responsible for the difference between volumetric flow and velocity indices of cerebrovascular reactivity. Consistent with this finding, are recent reports that the MCA dilates in response to hypercapnia (Verbree et al., 2014; Coverdale et al., 2014, 2015) leading to consequent underestimation of cerebrovascular reactivity with velocity measures (Ainslie & Hoiland, 2014).

The vasomotor response of the ICA to hypercapnia was markedly greater than that during hypocapnia; thus, there was no appreciable difference in volumetric and velocity indices of reactivity in the hypocapnic range. Upon close examination of the vasomotor profile of the ICA during changes in $P_{ET}CO_2$, it is quite similar to the expected vasomotor profile of the MCA diameter (see Figure 1 in: Ainslie & Hoiland, 2014), wherein small changes in $P_{ET}CO_2$ do not elicit measurable changes in diameter. It seems that a larger hypocapnic stimulus (e.g., ~9mmHg below baseline) is needed to elicit constriction than the necessary hypercapnic stimulus for dilation, likely a function of the overall lower cerebrovascular reactivity during hypocapnia. A mechanistic basis for these differences is not currently known. However, using our experimental approach (see below “2.5.2. comparison between studies” for explanation) it appears that the ICA may be more sensitive to changes in $CO_2$ in the hypercapnic range as dilation at $+3mmHgP_{ET}CO_2$ was observed in both the pre- and post-placebo trials, while the MCA does not appear to dilate (as measured using 3-T MRI) until approximately $+9mmHgP_{ET}CO_2$ (Coverdale et al., 2014).

2.5.2. Comparisons between studies.

Conflicting data exists as to whether $CO_2$ mediated vasomotion of the ICA does (Willie et al., 2012; Hoiland et al., 2014) or does not occur (Coverdale et al., 2015). The present study aimed to resolve these conflicting data, and corroborates the data exemplifying that vasomotion of the ICA does occur. Differences between studies are likely due to several
methodological and analytical differences. These differences include the method of manipulating $P_aCO_2$, and determination of vessel diameter. For example, Coverdale et al., 2015 utilized elevations in $F_1CO_2$ (0.06) to elicit a ~10mmHg increase in $P_{ET}CO_2$. This technique is limited in that $P_{ET}CO_2$ tends to overestimate $P_aCO_2$ by ~4-6mmHg at this level of hypercapnia (Peebles et al., 2007). In contrast, our approach of end-tidal forcing, only results in $P_{ET}CO_2$ over estimating $P_aCO_2$ by ~1mmHg (Tymko et al., 2015). Therefore, it is likely that the hypercapnic $P_aCO_2$ (not $P_{ET}CO_2$) stimulus applied by Coverdale et al., 2015 was only around 5mmHg above baseline whereas the $P_{ET}CO_2$ stimulus in the current study is representative of the true $P_aCO_2$ stimulus. As the current study shows very modest changes in diameter with mild hypercapnia, this provides an explanation, in part, for the lack of ICA dilation observed by Coverdale et al., 2015. Second, and importantly, discrepancies between studies may be a reflection of the use of caliper based manual diameter measures (quantified over three cardiac cycles) rather than our automated approach (quantified over a minimum of 12 consecutive cardiac cycles). The latter approach is affected less by artifacts, and the potential influences of respiration and blood pressure variability. As such, using edge-detection software not only provides a more robust and sensitive assessment of vessels diameter and velocity (Woodman et al., 2001), it also limits subjectivity and bias during data analysis.

2.5.3. Cyclooxygenase inhibition.

It has been previously established that the dose of INDO in the current study is sufficient to effectively inhibit prostaglandin synthesis (Eriksson et al., 1983). Inhibition of COX with INDO resulted in a significant reduction in both resting CBF and cerebrovascular $CO_2$ reactivity in both the hypo- and hyper-capnic range. This result is in concordance with previous studies using various measurement techniques to quantify CBF (Wennmalm et al., 1981; Bruhn et al., 2001; St Lawrence et al., 2002; Xie et al., 2006; Hooland et al., 2015). However, COX inhibition with Ketorolac did not affect resting CBF, cerebrovascular $CO_2$ reactivity, or the vasomotor response of the ICA. Some previous data, comparing INDO to other COX inhibitors, has indicated that INDO reduces CBF and cerebrovascular $CO_2$ reactivity via a ‘COX inhibition independent’ mechanism (Eriksson et al., 1983; Markus et
al., 1994); however, these studies provided limited suggestion of alternative mechanisms. On the basis of previous studies in vitro and in highly controlled animal models, we reason here that the selectivity of INDO is related to either the inhibition of cAMP-dependent protein kinase (Kantor & Hampton, 1978), inhibition of prostaglandin receptor binding mediated increases in vascular smooth muscle cAMP (Parfenova et al., 1995b), or both (see “2.5.4. Pharmacological interventions” below).

2.5.4. Pharmacological interventions.

Several factors have supported INDO as a useful pharmacological agent to assess the effects of COX inhibition on CBF: cardiac output is minimally affected by the administration of INDO (Nowak & Wennmalm, 1978; Wennmalm et al., 1984) and INDO does not appear to alter cerebral metabolism (Kraaier et al., 1992), resting ventilation (Hoiland et al., 2015), peripheral chemosensitivity (Xie et al., 2006), or plasma catecholamines (Staessen et al., 1984; Green et al., 1987). Thus, INDO has been extensively used to assess the influence of prostaglandins on cerebrovascular CO₂ reactivity (Wennmalm et al., 1981; Eriksson et al., 1983; Bruhn et al., 2001; St Lawrence et al., 2002) in addition to many other experimental paradigms. The assumption was made that INDO reduces cerebrovascular CO₂ reactivity through a prostaglandin independent mechanism, a conclusion drawn from divergent findings using other COX inhibiting drugs such as Aspirin and Naproxen (Eriksson et al., 1983; Markus et al., 1994). Yet, it is still continually used to assess prostaglandin-mediated responses. For example, extremely low doses of INDO have been reported to inhibit cAMP – dependent protein kinase activity (Kantor & Hampton, 1978). This inhibition will directly effect vascular smooth muscle cell calcium sensitivity (Adelstein & Conti, 1978), and thus vasomotor tone (Kerrick & Hoar, 1981). Moreover, in newborn pigs, which also demonstrate reductions in CBF/CO₂ reactivity to INDO but not other COX inhibitors (e.g., Aspirin, Ibuprofen, Naproxen; Chemtob et al., 1991), INDO blocks prostaglandin receptor mediated signal transduction during hypercapnia (Parfenova et al., 1995b), specifically downstream increases in cAMP. However, Aspirin does not inhibit prostaglandin receptor mediated signalling and subsequent increases in smooth muscle cell cAMP (Parfenova et al., 1995b), highlighting that this is a unique effect of INDO. Therefore, although the primary purpose of
this study was to characterize the effect of INDO on CO\textsubscript{2} mediated vasomotor responses of the ICA, we also aimed to test the effect of Ketorolac on such vasomotor responses. This agent was to determine if INDO acts differently from other COX inhibitors in its ability to blunt CO\textsubscript{2} mediated vasomotion (in addition to flow reactivity) of large extra-cranial cerebral arteries. With Ketorolac we showed no difference in the vasomotor response, consistent with these aforementioned studies (Eriksson et al., 1983; Markus et al., 1994), indicating that INDO is also affecting the vasomotor tone of larger cerebral arteries in a permissive (i.e., independent of COX inhibition) manner.

The possibility remains that only low levels of prostaglandins are required to induce vasomotion (through downstream increases in cAMP) during CO\textsubscript{2} perturbations. This possibility provides an explanation for the lack of effect of COX inhibitors - with the exception of INDO - on cerebrovascular CO\textsubscript{2} reactivity, consistent with the lack of detectable levels of prostaglandins during hypercapnia (Eriksson et al., 1983). For example, near complete inhibition of prostaglandin synthesis with Aspirin does not inhibit prostaglandin receptor agonism (i.e., via Iloprost) or hypercapnia mediated increases in cAMP and vascular diameter in newborn pigs (Parfenova et al., 1995b). If large doses of prostaglandins were necessary to produce dilation one may expect a dose-dependent relationship between prostaglandin receptor agonism and vasodilation; however, this is not the case during hypercapnia (Leffler et al., 1999). Collectively, these data provide a possible explanation (i.e., inhibition of prostaglandin receptor mediated signal transduction) as to how INDO inhibits cerebrovascular CO\textsubscript{2} reactivity both independent of COX inhibition and in a manner unique from other COX inhibitors.

2.5.5. Implications.

For the last 30 years, assessment of cerebrovascular responses has been dominated by the use of TCD. However, assessment of CO\textsubscript{2} reactivity with TCD operates on the assumption (also its primary limitation) that the diameter of the MCA does not change in response to CO\textsubscript{2} - an assumption previously thought to be true (Serrador et al., 2000). Recently, studies using higher resolution magnetic resonance imaging (Verbree et al., 2014; Coverdale et al., 2014)
have reported MCA vasomotion in response to changes in CO$_2$ and consequent underestimation of reactivity by TCD measures of velocity. Other studies assessing volumetric flow reactivity through the extra-cranial cerebral arteries (Willie et al., 2012; Hoiland et al., 2015) provide further evidence that TCD is limited in its ability to accurately measure cerebrovascular CO$_2$ reactivity.

Reduced cerebrovascular CO$_2$ reactivity is indicative of an increased risk for all cause and cardiovascular (inclusive of stroke) mortality (Portegies et al., 2014). Since it seems that changes in diameter can contribute to nearly half of the increase in flow observed during elevations in P$_{ET}$CO$_2$, we speculate that the magnitude of the vasomotor response (i.e., diameter change) in response to P$_{ET}$CO$_2$ perturbations may be indicative of cerebrovascular health (i.e., endothelial function), much like peripheral flow mediated dilation is indicative of cardiovascular risk (Inaba et al., 2010). The incorporation of diameter measures into the prediction of cerebrovascular events and related mortality is now needed.

While INDO is exemplary in its ability to reduce cerebrovascular reactivity, and is thus an attractive tool for the assessment of physiological function associated with cerebrovascular reactivity (i.e., control of breathing; Xie et al., 2006; Hoiland et al., 2015), its utility for investigating the role of prostaglandins in mediating cerebrovascular responses should be cautioned. As we and others (Eriksson et al., 1983; Markus et al., 1994) have identified, INDO reduces cerebrovascular CO$_2$ reactivity and CO$_2$ mediated vasomotion in a manner that is unique from other COX inhibitors. It is clear that INDO is acting in a prostaglandin independent manner, likely through the inhibition of cAMP-dependent protein kinase (Kantor & Hampton, 1978), and reductions in prostaglandin receptor mediated increases in smooth muscle cAMP (Parfenova et al., 1995b) to affect cerebrovascular reactivity to CO$_2$.

2.6. Synopsis.

We have demonstrated for the first time that INDO reduces the vasomotor response of the ICA to changes in P$_{ET}$CO$_2$ and provided evidence that it is independent of inhibiting COX. Future studies should aim to determine the mechanism(s) underlying INDO’s unique ability
to reduce CO$_2$ mediated cerebrovascular vasomotion and to determine other regulatory mechanisms governing cerebrovascular vasomotion in healthy humans.

2.7. Author Contributions.

Conception and design of experiments: RLH, PNA. Data Collection: RLH, MMT, KWW, ARB, BM, PNA. Data analysis and interpretation: RLH, PNA. Manuscript first draft: RLH, PNA. Critical revisions of manuscript for important intellectual content: RLH, MMT, KWW, ARB, BM, PNA. Approval of final draft: RLH, PNA.

2.8. Funding.

This research was supported by an NSERC Discovery Grant and Canadian Research Chair in Cerebrovascular Physiology (PNA).

2.9. Special Recognition.

Special thanks to Dr. Glen Foster for aiding in the optimization and the functioning of the end-tidal forcing system and technical support.
Chapter 3. Conclusion.

3.1. Indomethacin induced impairments of cerebrovascular reactivity.

It has been well documented that INDO reduces cerebrovascular CO$_2$ reactivity by approximately 40-60% (Eriksson et al., 1983; Xie et al., 2006; Hoiland et al., 2015). However, it is not as well documented if INDO inhibits cerebrovascular reactivity in a regional or sex dependent manner, and if chronic treatment has a differential effect on reactivity from that of acute treatment. There is some evidence that INDO inhibits the reactivity of grey matter to a greater extent than white matter (St Lawrence et al., 2002); however, this may simply be a product of grey matter possessing a much higher reactivity than white matter in normal conditions (Ramsay et al., 1993), and thus a greater potential for reductions in reactivity with INDO administration.

For the influence of sex on INDO mediated inhibition of cerebrovascular reactivity, there is evidence suggesting that INDO impairs cerebrovascular CO$_2$ reactivity to a greater extent in females than in males (Kastrup et al., 1999). Of note, this study utilized TCD impairing our ability to firmly conclude sex differences are present (see “1.4.1. Transcranial Doppler ultrasound” for limitations of TCD). Further, as it has been clearly outlined already, due to INDO’s unique efficacy for reducing cerebrovascular CO$_2$ reactivity (Eriksson et al., 1983), the reported sex differences are likely unrelated to COX. As noted by Kastrup et al., 1999, the larger reduction seen in females is likely due to a higher reactivity prior to INDO and resultant greater potential for larger reductions in reactivity to occur. Indeed, the authors noted that the magnitude of reductions in cerebrovascular CO$_2$ reactivity were positively correlated with resting MCAv (r=0.74). However, data collected in rats support an interaction between estrogen levels and the vasodilatory and constrictive balance of prostanoids. For example, estrogen increases cerebral prostacyclin production in rats (Ospina et al., 2003) shifting the balance towards more vasodilatory prostanoids inducting sex differences may be important. This raises the possibility that inhibition of COX with INDO likely produces larger reductions in cerebrovascular CO$_2$ reactivity in biological systems with higher estrogen levels. Further study is required to determine if the effects of
prostaglandin inhibition differ between males and females in humans. In the present study, the single female subject was tested on days 1 and 3 of her follicular phase, when estrogen levels are the lowest relative to the rest of the menstrual cycle (Marsh et al., 2011).

Related to the chronic use of INDO, there is little and conflicting data as to the effects on cerebrovascular CO₂ reactivity. A study by Eriksson et al., 1983, using the Kety & Schmidt technique (Kety & Schmidt, 1948a) to assess CBF, reported that after 1-week of chronic INDO (0.8mg/Kg three times daily), cerebrovascular CO₂ reactivity was reduced to the same extent as that observed after acute administration of INDO. Reactivity after chronic INDO administration was tested 2-3 hours after the final dose of INDO (Eriksson et al., 1983). In contrast to these findings, Pickles et al., (1984) reported that after three days of treatment with INDO (100mg/day in divided doses), cerebrovascular CO₂ reactivity was not different from that assessed prior to INDO (Pickles et al., 1984). In this study, using the Xenon¹³³ clearance technique to assess CBF it was not reported how long after the final dose of INDO reactivity was assessed. These contrasting studies make difficult the interpretation of the effect of chronic INDO treatment on cerebrovascular CO₂ reactivity and, therefore, warrant further study of this topic. If chronic INDO dosing does reduce cerebrovascular CO₂ reactivity, especially in the elderly, this reduction in reactivity may be pre-disposing people to the risk of cardiovascular/cerebrovascular events to an extent greater than that assumed as a result of drug induced changes in the pro- versus anti-thrombotic prostanoid balance.

3.2. Cerebrovascular CO₂ reactivity: Implications for disease.

3.2.1. Sleep apnea.

Cerebrovascular reactivity is an integral component in the control of breathing both in an awake (Fan et al., 2010) and sleeping (Xie et al., 2009) state, with pharmacological reductions in reactivity leading to breathing instability. Accordingly, a blunted cerebrovascular CO₂ reactivity is fundamental to the pathogenesis of central (Javaheri & Dempsey, 2013) and obstructive (Dempsey et al., 2010) sleep apnea, diseases both characterized by ventilatory dysregulation. For example, INDO induced reductions in
cerebrovascular CO\textsubscript{2} reactivity have been reported to worsen obstructive sleep apnea at sea level (Burgess \textit{et al.}, 2010), and central sleep apnea at high-altitude (Burgess \textit{et al.}, 2014). Blunted reactivity is also implicated in the pathogenesis of central sleep apnea in heart failure patients (Xie \textit{et al.}, 2005). Therefore, treatment of central and obstructive sleep apnea may benefit from an improved understanding of the underlying mechanisms regulating cerebrovascular CO\textsubscript{2} reactivity and the development of therapeutic strategies to increase cerebrovascular CO\textsubscript{2} reactivity (i.e., exercise training in the elderly; Ainslie \textit{et al.}, 2008).

3.2.2. Prediction of mortality.

Assessment of cerebrovascular CO\textsubscript{2} reactivity in a general population has been shown as an effective modality for the prediction of all cause and cardiovascular mortality (Portegies \textit{et al.}, 2014). The study by Portegies \textit{et al.}, 2014 reported that reduced cerebrovascular CO\textsubscript{2} reactivity was not associated with incidence of stroke despite its relation to both cardiovascular and all cause mortality. While these findings may be interpreted as discrediting the value of cerebrovascular CO\textsubscript{2} reactivity as a predictor of cerebrovascular disease, they may simply be limited in that impaired vascular function can manifest in a variety of pathological conditions in addition to stroke, especially if vascular function is systemically impaired. Moreover, as this study utilized TCD, and cerebral vasomotion contributes largely to reactivity, between-individual variations in the dilatory response to hypercapnia will likely have affected the velocity reactivity profiles in an undetectable manner and potentially led to miss-classification of subjects. It is plausible that incorporation of the dilatory response to hypercapnia into such a study would improve the ability to predict cerebrovascular and cardiovascular events. Whilst the efficacy of cerebrovascular CO\textsubscript{2} reactivity in the prediction of cerebrovascular events in otherwise healthy subject requires much further study, the link between reduced reactivity and risk of stroke is much more robust in patients already suffering from cerebrovascular disease such as carotid stenosis [e.g. (Gupta \textit{et al.}, 2012)]. Therefore, while pre-existing cerebrovascular disease clearly predisposes individuals to an increased risk of stroke with reduced cerebrovascular CO\textsubscript{2} reactivity, furthering the understanding of the link between cerebrovascular reactivity and risk for stroke remains an important task (Bos \textit{et al.}, 2007).
Currently, the mechanisms underlying reduced cerebrovascular reactivity relative to a normal healthy aging population, as was used by Portegies et al., 2014 (~70 years of age), are relatively unknown. There are data, however, attributing reduced prostaglandin-mediated dilation to the overall reduction of cerebrovascular CO$_2$ reactivity associated with aging (Barnes et al., 2012). This indicates a potential role for a loss of vasodilatory prostaglandins (i.e., prostacyclin) being responsible, at least in part, for reductions in cerebrovascular CO$_2$ reactivity associated with vascular disease and increased risk of mortality. Moreover, a shift in the balance between anti-thrombotic (i.e., prostacyclin) and pro-thrombotic (i.e., thromboxane) prostanoids is likely also responsible (via shifting to a pro-thrombotic state). It is important to note that the effect of INDO observed by Barnes et al., 2012 is likely downstream of COX inhibition and manifesting through inhibition of PGI$_2$ and PGE$_2$ receptor mediated signal transduction and related modulation of smooth muscle cell cAMP (Parfenova et al., 1995b).

3.3. Methodological limitations.

Two different non-selective COX inhibitors were used in this study to investigate the role of PG’s in mediating cerebrovascular CO$_2$ reactivity and the vasomotor response of the ICA to changes in P$_a$CO$_2$. For this purpose, end-tidal forcing of P$_{ET}$CO$_2$ was used as a modality for manipulating P$_a$CO$_2$ and driving changes in CBF. However, both of these methods, the pharmacological inhibition of COX and the use of P$_{ET}$CO$_2$ as a non-invasive surrogate of P$_a$CO$_2$, come with inherent limitations. These limitations will be discussed below.

3.3.1. Pharmacological inhibition of cyclooxygenase.

In the present study we used either 1.2mg/Kg INDO or 0.25mg/Kg Ketorolac to inhibit COX activity and consequently the conversion of arachidonic acid to PGH$_2$. Such measurement of the efficacy of COX inhibition requires the sampling of venous blood, centrifuging of the blood to extract platelet rich plasma samples, and subsequent addition of Na-arachidonate to the plasma sample to assess platelet aggregation. For example, Eriksson et al., 1983 showed
that prior to Aspirin, addition of Na-arachidonate to a platelet rich plasma sample lead to platelet aggregation, whereas, following treatment with INDO, platelet aggregation in response to Na-arachidonate was completely inhibited signifying prostaglandin synthesis was abolished (Eriksson et al., 1983). While we did not assess the efficacy of our INDO administration in inhibiting prostaglandin synthesis, we used a similar dosage as that shown to be effective by Eriksson et al., 1983.

As INDO possesses effects additional to the inhibition of COX (i.e., inhibition of cAMP-dependent protein kinase) we chose to also test an alternate non-selective COX inhibitor, Ketorolac. By using Ketorolac we were able to assess the role of COX activity in mediating cerebral vasomotor responses to changes in $P_{ET CO_2}$ without the confounder of simultaneous cAMP-dependent protein kinase inhibition. As no effect of Ketorolac was observed on the vasomotor response to hypercapnia and hypocapnia, the obvious conclusion would be that prostaglandins are not an obligatory mediator of cerebral vasomotor responses to changes in $P_{ET CO_2}$. However, there is evidence in animals, that downstream from COX inhibition, prostaglandin receptor mediated increases in cAMP are responsible for dilation of cerebral vessels during hypercapnia (Parfenova et al., 1995b). Specifically, inhibition of COX with Aspirin has no effect on IP receptor (prostacyclin receptor) agonism or hypercapnia mediated increases in cAMP and subsequent vessel dilation, but INDO reduces both IP receptor agonism and hypercapnia mediated increases in cAMP and subsequent vessel dilation (Parfenova et al., 1995b). These findings by Parfenova et al., 1995 would indicate that despite COX inhibition in the Aspirin trial, prostaglandins are still mediating dilation, whereas, INDO inhibits IP receptor specific increases in smooth muscle cell cAMP that are essential to cerebral vessel dilation during hypercapnia. This provides evidence for the theory that only low levels of prostaglandins (too low to be affected by COX inhibition) are required for cerebral vessel dilation to hypercapnia – in support of this, prostacyclin does not have a dose dependent effect on facilitating hypercapnic dilation (Parfenova & Leffler, 1996), and CBF increases in response to hypercapnia despite no observed increases in prostaglandin production in humans (Eriksson et al., 1983). Exactly how INDO inhibits IP receptor mediated increases in smooth muscle cell cAMP and vessel dilation is not currently
known, and requires further investigation in highly controlled animal models prior to translation into human studies.

3.3.2. End-tidal versus arterial CO₂.

In the current study we used end-tidal forcing to control \( P_{ET}CO_2 \), a commonly used surrogate for \( P_aCO_2 \). While various methods can be used to manipulate \( P_{ET}CO_2 \) for the purpose of measuring cerebrovascular responses (see “1.5. Experimental models to assess cerebrovascular CO₂ reactivity”), we chose to use end-tidal forcing as it minimizes the gradient between \( P_{ET}CO_2 \) and \( P_aCO_2 \) (~2mmHg; Tymko et al., 2015), while allowing for the assessment of CBF during steady state conditions. Previously collected data shows that during end-tidal forcing the \( P_{ET}CO_2-P_aCO_2 \) gradient does not change from baseline to hypercapnia or hypocapnia (Tymko et al., 2015); therefore, it is evident that our \( P_{ET}CO_2 \) stimulus magnitude is representative of the \( P_aCO_2 \) stimulus magnitude. Moreover, while ventilatory sensitivity will affect the necessary FiCO₂ of the inspirate and subsequently the level of CO₂ rebreathed from apparatus deadspace (Tymko et al., 2015), ventilatory sensitivity to CO₂ was not different between trials for either drug intervention. As such, ventilation will not have impacted on the \( P_{ET}CO_2 \) and \( P_aCO_2 \) gradient between trials.

3.4. Future studies.

This study has indicated that vasodilation of the ICA is an integral component to the increases in flow observed during hypercapnia. Moreover, these data support an effect of INDO on cerebrovascular CO₂ reactivity that is independent of COX inhibition. Thus, future studies should endeavor to further the understanding of the cellular mechanisms regulating cerebrovascular CO₂ reactivity, the mechanisms behind INDO’s unique ability to blunt cerebrovascular CO₂ reactivity compared to other COX inhibitors, and the implications of large extra-cranial cerebral vasomotion in the context of aging and disease.
3.4.1. Determining the role of cyclic adenosine monophosphate inhibition on cerebrovascular vasomotor responses in humans.

As INDO inhibits cAMP-dependent protein kinase at submicromolar doses, and is ~80-220 times more potent an inhibitor of cAMP-dependent kinase than it is of either COX isoform, one could theoretically use low doses of this drug to determine the role of cAMP in regulating cerebrovascular responses independent of COX. The potential use of this approach would require pilot work to assess the necessary oral dose of INDO needed to reduce cerebrovascular vasomotor response to CO\(_2\), while prostaglandin synthesis remains intact. Using previously established methods (Eriksson et al., 1983), the extent of prostaglandin synthesis inhibition for various doses of INDO could then be established. Following establishment of this effect in several subjects, the desired dose could be administered to a larger group of subjects (i.e., n≈10) to assess if cerebrovascular vasomotor responsiveness is reduced despite prostaglandin synthesis remaining intact.

3.4.2. The assessment of cerebral endothelial function.

Endothelial function of the peripheral vasculature, assessed by flow mediated dilation, is predictive of cardiovascular events (Inaba et al., 2010). If an assessment of cerebral endothelial function were available it would have the potential to be an effective predictor of cerebrovascular events. Thus, a test to determine the flow-mediated dilation of cerebral vessels is needed. As the ICA is bilaterally responsible for supplying ~70% of the brains blood supply and can be insonated in most individuals, it provides an attractive avenue to assess cerebrovascular responses. Future study should assess the applicability of a shear stress test of the ICA, similar to that done in the brachial artery to assess peripheral endothelial function. If highly controlled and short term increases in P\(_{ET}\)CO\(_2\) are achievable with end-tidal forcing, P\(_{ET}\)CO\(_2\) may provide an avenue to manipulate shear stress in a time course similar to that induced by cuff release in brachial tests. For example, abrupt (30 second) increases in CO\(_2\), with a rapid return to baseline will result in a large (~3-fold) increase in shear rate (Hoiland et al., unpublished findings) mimicking the shear profile following brachial cuff release. By rapidly returning P\(_{ET}\)CO\(_2\) to baseline it is likely that any
any vascular changes are occurring as a result of the shear stimulus. The extent to which these changes in shear patterns may provide insight into the endothelial function of the cerebral vasculature remains to be fully explored.

3.4.3. Vasomotor responsiveness of the internal carotid artery with aging.

It has been previously established that CO₂ reactivity is reduced with aging, likely due to a reduction in vasodilatory prostaglandin production (Barnes et al., 2012). Thus, future studies should seek to determine whether or not the vasodilatory response of the ICA is impaired with age. Such a study would require replication of the CO₂ reactivity tests used in this thesis and concurrent measuring of $Q_{ICA}$ and vasomotor reactivity to CO₂ in young and old subjects. Further, it should be determined if COX inhibition affects CO₂ reactivity in older individuals following chronic usage of COX inhibitors. Such chronic use of COX inhibitors (typically 7-10 days) is common in elderly populations despite the well-known increased risk of cardiovascular events from such medications (Brune & Patrignani, 2015).

3.4.4. Other regulatory factors in the cerebral vasomotor response to CO₂ perturbations.

As outlined in the introduction of this thesis (see “1.2.3 Potential cellular mechanisms mediating cerebrovascular CO₂ reactivity”), several vasoactive factors have been postulated to contribute to the cerebral vasomotor response to CO₂. However, the vast majority of these data have been collected in animal models, with related studies in human lacking. Thus, the present study provides a well-controlled model (i.e., ICA vasomotion) that can be used to explore the effects of other pharmacological interventions on the human cerebral vasomotor response to CO₂. For example, the current study could be repeated using theophylline (adenosine receptor antagonists), Nω-nitro-L-arginine methyl ester (NOS inhibitor), or glibenclamide (an $K_{ATP}$ channel inhibitor). Much research is needed to better understand the fundamental mechanisms of cerebrovascular regulation and potential interactions between vasoactive factors.
Bibliography


