CEREBROVASCULAR AND CARDIORESPIRATORY RESPONSES TO CONDITIONS OF LOCAL ISCHAEMIA AND SYSTEMIC HYPOXAEMIA

by

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ABSTRACT

Conditions of local ischaemia and systemic hypoxemia trigger necessary cardiorespiratory and cerebrovascular responses to ensure nutrients and oxygen (O_2) are delivered to vital organs such as the brain and the heart. The aim of this thesis is to determine cardiorespiratory and cerebrovascular responses to conditions of local ischaemia and systemic hypoxemia. Four experimental studies investigated cardiorespiratory and cerebrovascular responses during diving (simulated by facial cooling), the ventilatory response during hypoxia and the interaction between hypoxia and metaboreflex during exercise. The first experimental study aimed to uncouple the effects of trigeminal nerve stimulation on cerebral blood flow (CBF), from other modifiers associated with the diving response, such as apnoea and changes in arterial carbon dioxide tension. It was observed that the CBF responses to diving are attributable to factors related with breath-hold and not facial cooling (i.e., trigeminal nerve stimulation). The second experimental study determined whether moderate acute systemic hypoxia increases cerebral perfusion via the hypoxic ventilatory response, independently of changes in arterial oxygenation. Increases in cerebral perfusion during hypoxia were observed to be mainly influenced by reduced arterial oxygen saturation (SpO_2) and not the hypoxic ventilatory response. The final experimental studies investigated the influence of hypoxia on cardiorespiratory and cerebrovascular responses to metaboreflex activation. Normobaric poikilocapnic hypoxia (FiO2=13.5%) failed to augment the cardiorespiratory and cerebrovascular responses to metaboreflex activation. However, a more robust normobaric isocapnic hypoxia (FiO2=10.5%) caused augmented heart rate (HR) and minute ventilation (VE) to metaboreflex activation. Collectively, these studies reinforce the notion that conditions of local ischaemia and systemic hypoxaemia evoke a highly integrated cardiorespiratory and cerebrovascular response.

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ABBREVIATIONS

ANOVA – Analysis of variance

BH – Breath-hold

BH +FC – Breath-hold with facial cooling

BH -FC – Beath-hold without facial cooling

BP – Blood pressure

CBF – Cerebral artery flow

CO – Cardiac Output

CO_2 – Carbon dioxide

CV – Coefficient of variation

CVCi – Cerebrovascular conductance index

FC +BH – Facial cooling with breath-hold

FC -BH – Facial cooling without breath-hold

FiO_2 – fraction of inspired oxygen

HR – Heart rate

ICA – Internal Carotid Artery

ICAd – Internal carotid artery diameter

ICA_Q – Internal carotid artery flow

ICAv – Mean internal carotid artery velocity

ICC – Intraclass correlation coefficient

IHG – Static handgrip exercise

MAP – Mean arterial blood pressure

MCA – Middle cerebral artery

MCAv – Mean middle cerebral artery velocity

MVC – Maximum voluntary contraction

O_2 – Oxygen

P_{aCO2} – Arterial partial pressure of carbon dioxide

P_{aO2} – Arterial partial pressure of oxygen

PEI – Post exercise ischemia

$P_{ET}CO_2$ – end-tidal carbon dioxide

$P_{ET}O_2$ – Partial-pressure of end-tidal oxygen

PFR – Partial flow restriction

RB – Restricted breathing

RF – Respiratory frequency

SpO_2 – Arterial oxygen saturation

TCD – Transcranial doppler

TV – Tidal volume

VE – Minute ventilation

VO_2max – maximal oxygen uptake

CHAPTER 1: INTRODUCTION

The cerebral metabolic rate for oxygen is high, and as energy substrate storage is low in the brain, any significant disruption in oxygen delivery will result in irreversible cell damage or even death. To mitigate against this vulnerability, a continuous CBF is crucial for ensuring normal brain function. Indeed, conditions of local ischaemia and systemic hypoxia elicit compensatory responses which help preserve cerebral oxygen delivery and brain function. However, the underlying mechanisms remains incompletely understood. Therefore, the overall aim of this thesis is to investigate the effect of conditions of local and systemic hypoxemia on cerebrovascular and cardiorespiratory responses in humans.

The literature review (Chapter 2) includes a concise review of the literature regarding the anatomy of the cerebral vascular system and approaches used for the assessment of CBF. It also considers cerebrovascular regulation during rest, during conditions of systemic hypoxia and during diving, exercise and when local ischaemia occurs in exercising muscle. These common physiological stressors compromise cerebrovascular function equilibria, and the results of these stressors are explained and discussed. The General Methods are presented in Chapter 3.

The first experimental study (Chapter 4) aimed to uncouple the effects of trigeminal nerve stimulation on CBF from other potential modifiers (i.e., apnoea, carbon dioxide (CO_2)) during diving (simulated by facial cooling). Six experimental protocols were performed, and it was deduced that trigeminal nerve stimulation *per se* had no effect on CBF. Most compellingly, CBF increased similarly during facial cooling when accompanied either with or without an apnoea.

The second experimental chapter (Chapter 5) investigated the effect of ventilation on cerebrovascular responses to systemic hypoxia. To discern the effect of ventilation, 4 separate isocapnic trials of spontaneous normoxia, hypoxia, breathing in hypoxia with

ventilation matched to normoxia, and breathing in normoxia with ventilation matched to hypoxia. Cerebral perfusion indexed by velocity was similar in both hypoxic trials. Findings indicate that factors associated with hypoxaemia make the predominant role in CBF increases during moderate isocapnic acute hypoxia.

The final two experimental studies (Chapters 6 and 7) investigated the cerebrovascular and cardiovascular responses to exercise with local ischaemia in leg muscle and systemic reductions in oxygen availability. Systemic hypoxia was induced by decreasing the fraction of inspired oxygen (FiO2) (13.5% in Chapter 6 and 10.5% in Chapter 7). Local ischaemia in leg muscles was achieved by the partial inflation of cuffs around the upper thighs during (PFR) and after (PEI) leg cycling. This is known to accelerate anaerobic muscle metabolism and stimulate metabolically sensitive skeletal muscle afferents (i.e., muscle metaboreflex). The use of an environmental chamber to control the hypoxic exposure (Chapter 6) throughout the protocol had no effect of the magnitude of the cerebrovascular or cardiorespiratory responses to metaboreflex activation. There are several possible explanations for these results. Firstly, the intensity of hypoxia used may be insufficient to elicit any cerebrovascular or cardiorespiratory response. Secondly, it is difficult to rule out adaptation to hypoxia since hypoxia was applied for a long continuous duration (~3.5 hr). The other possibility is related to the confounding effect of increases in $P_{ET}CO_2$ in restricting CBF responses as a consequence of hypoxic induced hyperventilation. Therefore, Chapter 7 involved an intense hypoxic application (FiO_2=10.5%) and concurrent metaboreflex activation (PEI and PFR). VE and HR responses to PFR were augmented with hypoxia compared to normoxia. However, the sum of the HR and VE response during metaboreflex activation in hypoxia was not different than the sum of hypoxia alone and muscle metaboreflex activation in normoxia. The final chapter of this thesis (Chapter 8) summarises and synthesises the main findings of the thesis and provides some general conclusions and future directions.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

The human brain comprises ~2% of the total body weight, albeit its supplied with an astonishing ~15% of cardiac output (CO) and ~25% of resting O_2 consumption (Drake et al., 2009, Cipolla, 2010). With limited substrate energy stores, necessary and precise regulation of CBF ensures normal brain function. But any unexpected brief pause of blood to the brain or any interruption lasting up to 4 minutes may result in unconsciousness or permanent brain cells damage (Cipolla, 2010).

2.2 Cerebrovascular Anatomy

2.2.1 Extracranial Arteries

Extracranial arteries refer to those arteries that are outside the skull (i.e., vertebral artery and internal carotid artery (ICA)). The anterior brain blood supply originates at the aortic arch, which gives rise to the brachiocephalic and then to the right common carotid artery, the left common carotid artery and the left subclavian artery. Both common carotid arteries then branch into the ICA and the external carotid artery, with the former continuing to the cranium and the latter suppling blood to the face and scalp (Navarro-Orozco and Sanchez-Manso, 2018). The bilateral pair of ICA branches extensively to form the anterior circulation. The ICA arises from the bifurcation of the common carotid artery in the neck and ascends to the temporal bone where it enters the cranium through the carotid canal (Drake et al., 2009). The ICA main contribution is to the anterior cerebral circulation and has 3 distinctive parts, petrous, cavernous, and intracranial, with the latter being the end part of the ICA and is the only segment of the ICA found inside the cranium (Drake et al., 2009). Areas such as the frontal, temporal, and parietal lobes and parts of the diencephalon and the internal capsule are supplied by ICA branches. Some of the important cortical regions that the ICA supplies are the motor, speech, and cognitive

areas. A recent magnetic resonance imaging study explored the magnitude of CBF provided by the ICA and has indicated that 72% of the blood perfused in the brain is provided by ICA (Zarrinkoob et al., 2015). Unlike the vertebral artery, the ICA branches extensively after entering the cranium, providing the major contribution the Circle of Willis.

On the other hand, the posterior circulation is perfused by a bilateral pair of vertebral arteries (Tortora, 1994). Originating from the subclavian arteries, the vertebral artery ascends through the neck at the level of the upper six cervical vertebrae and enters the cranium at the foramen magnum. The vertebral artery is divided into four segments V1, V2, V3, and V4, with the first three first segments being extracranial (Chandra et al., 2017). V4 enters the cranium through foramen magnum and before uniting with contralateral pair of the vertebral arteries to form the basilar artery, gives rise to two arteries, the posterior inferior cerebellar artery and the anterior spinal artery serving different areas which will be discussed subsequently. The vertebral artery contributes to the posterior cerebral circulation mainly to the upper spinal cord, brainstem, cerebellum, and to parts of the posterior cerebral hemispheres, mainly the occipital lobes. Some of the important areas that the vertebral artery supplies are responsible for regulation of HR, blood pressure (BP) and breathing. As it carries approximately a third of blood going inside the brain, any disruptions can cause severe damages to the autonomic centres that these arteries supply (Chandra et al., 2017). Therefore, the main supply to the central autonomic regions is carried solely by the vertebral artery.

2.2.2 Intracranial Arteries

After entering the cranium, the ICA and the vertebral artery give rise to a vascular anatomical landmark, the Circle of Willis. In the year 1664, Thomas Willis describes an important blood network in the brain which provides connections and allows

redistribution of blood in the brain during disruptions or occlusions (Chandra et al., 2017). The Circle of Willis (Fig 1) is a vascular network formed by the anastomoses of the internal carotid and vertebral arteries allowing redistribution of blood flow if instances of occlusions happens during which major blood conduits to the brain fail (Cipolla, 2010) (Figure 1). The Circle of Willis, provides connections to both circulatory divisions, the anterior circulation provided by the ICA and the posterior circulation provided by the vertebral artery, allowing the option of collateral circulation when there is occlusion of one branch (Cipolla, 2010). This 'collateral' feature is distinctive to the brain vasculature.

The circle of Willis is formed by the anterior communicating artery, ICA, posterior cerebral artery, basilar and the posterior communicating arteries. the anterior communicating artery, and the middle cerebral artery (MCA). The anterior communicating artery supplies blood to some portions of the frontal, and superomedial parietal lobes, namely the hypothalamus, and optic chiasma (Drake et al., 2009, Chandra et al., 2017). The pair of anterior communicating arteries then travel together and form an anastomosis called the anterior communicating artery.

The major cerebral vessel and the largest terminal branch of the ICA, supplying many critical brain regions is the MCA. The MCA main supply is to the caudate nucleus, internal capsule, and the majority of temporal, frontal, and parietal lobes. Cerebrovascular accidents are more common in the MCA and are always associated with high rate of disability and death (Walcott et al., 2014). The MCA supplies 21% of brain with blood, considered as the largest contribution from a cerebral artery (Zarrinkoob et al., 2015). This is one reason why MCA is routinely examined in experimental studies seeking to better understand cerebrovascular function

On the other hand, the vertebral arteries feed the posterior circulation and branches from the subclavian artery which arises at the aortic arch (Tortora, 1994). Both vertebral arteries unite after entering the cranium to form the basilar artery. The cerebral arteries

that branch off the ICA and the basilar artery and their anastomoses are called intracranial arteries. Furthermore, the pair of vertebral arteries unite to form the basilar artery, supplying the pons with penetrating artery branches (Chandra et al., 2017). The basilar artery terminates by branching into three different cerebral arteries, the anterior inferior cerebellar artery, the superior cerebellar artery, and the posterior cerebral artery. The posterior cerebral artery which is considered the terminal branches of the basilar artery supplies some parts of the temporal and occipital lobes, in addition to visual areas of the cerebral cortex. The main link between the anterior and posterior circulation is through the posterior communicating artery which is originated from the ICA and anastomose with the posterior cerebral artery to form the circle of Willis (Figure 1).

Another unique feature of the cerebral circulation is the presence of a barrier that serve as a shield gate to protect the brain from toxic substances found in the blood stream and other important functions which are discussed later (Kulik et al., 2008). Given the limited substrate energy stores in the brain, features such as vascular collaterals, bilateral blood supply, and the blood brain barrier help the brain avoid interrupted blood flow and protect against harmful substances. Unlike other organs, the brain is affected by short disruptions of blood supply. Only a unique vasculature distribution can help ensure continuous blood supply to the brain and avoid minimal disruptions.

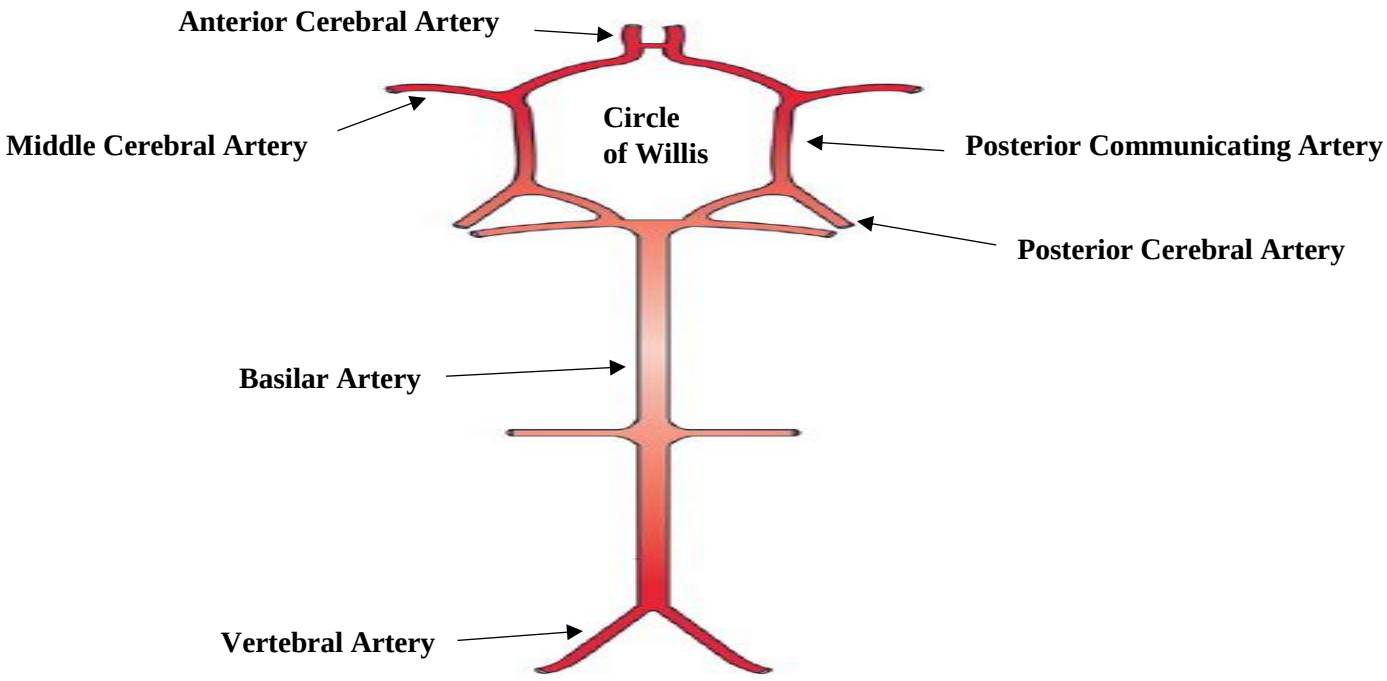


Figure 1 Schematic representation of the major cerebral arteries including anterior, middle, posterior cerebral, posterior communicating, internal carotid, vertebral and basilar arteries. Figure modified from (Roloff et al., 2016).

2.2.3 Blood-Brain Barrier

The exchange of gases and nutrients takes place at an interface between the cerebral microcirculation (vessels >100 µM) and the brain parenchyma within the endothelium (Cipolla, 2010). Unlike the systemic capillaries, cerebral capillaries possess a unique endothelium with tight inter-endothelial junctions (Cipolla, 2010, Veenith and Menon, 2011, Chandra et al., 2017). In addition, endothelial cells are surrounded by a layer consisting of pericytes, astrocytes and microglia (Wolburg et al., 2009). The presence of the blood brain barrier means that brain cells can be sufficiently perfused with nutrients and gases while the transport of pathogens and harmful substances are restricted. O_2, CO_2 and small lipophilic substances can freely enter brain tissue by passive diffusion, similar to other capillary beds in the human body, while hydrophilic molecules are restricted from entering (Cipolla, 2010). In addition, tight junctions restrict other blood

borne substances from entering the brain tissue. While the presence of special endothelial transporters enables selective transport of certain molecules in and out the brain tissue. Insulin, glucose, lactate, and amino acids are allowed into the brain in accordance with the metabolic demand and plasma substrate concentration.

The blood-brain barrier can be disrupted in several conditions, such as in strokes, infections or neurodegenerative diseases (Obermeier et al., 2013). Blood brain barrier dysfunction can lead to an abnormal neuronal activity, due to the imbalance of ions, transmitters and metabolites (Obermeier et al., 2013). Therefore, an intact blood brain barrier is crucial to carry on normal brain function. Interestingly, the blood brain barrier disruption is reversible and can be managed therapeutically, unlike injuries to neurons. A high protein concentration (e.g., albumin) within the brain is indicative of a disruption in the blood brain barrier. Ischemia and acute hypertension can interfere with the hydraulic conductivity of the blood brain barrier losing the transport control of protein to the pressure gradient between the brain and the blood (Cipolla, 2010).

In general, the brain's unique vascularisation and its highly sophisticated features (i.e., blood brain barrier and collateral circulation) are two factors that help maintain normal brain function without interruption. The other characteristic is the highly precise CBF regulation during rest and during different physiological stimuli which help ensure optimal nutrition. In the next sections CBF assessment and CBF regulation during rest and during different physiological stimuli will be discussed.

2.3 Cerebrovascular Assessment

Different approaches have been used to assess CBF and various modalities were developed to understand CBF regulation. Each modality has its own advantages and limitations which are discussed subsequently.

2.3.1 Clearance Techniques

CBF was first quantified by Kety and Schmidt (1945), but it was as early as 1880 that cerebral perfusion observations were first published (Mosso, 1881). Mosso (1881) detected changes in blood volume in patients with skull defects using plethysmography. Subjects performing mathematical tasks displayed increases in blood volume, which was later referred as neurovascular coupling. However, no quantification of CBF could be provided. Later in the 20th century, CBF was first quantified by Kety and Schmidt using the Fick principle (Willie et al., 2011c). An inert tracer, nitric oxide was inhaled and when saturation is reached, the process is terminated. The concentration of the tracers falls back again into zero. This process involves simultaneous collection of carotid and jugular blood samples where differences in rate of appearance and clearance of the tracer are calculated to quantify CBF using the Kety-Schmidt equation (Willie et al. 2010). The Fick Principle is a method developed to quantify CO that was delivered to humans during cardiac catheterisation (Willie et al. 2010); this allowed the first quantification of global CBF in humans. There are several limitations associated with this technique. Firstly, this technique only represents global CBF and not regional CBF, and the invasive nature of this technique makes it a challenging approach. Another limitation is that it does not allow estimation of instantaneous CBF changes since measurements are taken over minutes. Therefore, temporal resolution is poor and assessing dynamic CBF is very difficult.

CBF assessment was improved later by introducing the use of radioactive gases and gamma imaging (Lassen 1959, Harper and Glass 1965). Inert diffusible radioactive substances such as Xe^{133} and Kr^{85} are infused and imaging of the extracranial arteries and veins from the cerebral cortex was performed using extracranial sensors (Ingvar, 1961). The major disadvantage of using this approach is the increase in likelihood of cancer, in addition to its invasive nature of application (Soustiel et al., 2003) and the poor temporal resolution.

2.3.2 Transcranial Doppler

In 1842 Christian Doppler, an Austrian physicist documented what is known now as the Doppler effect. The principle states that the frequency of moving waves depends on the source and the observer's speed. This principle led later to the development of the Doppler ultrasound and then portable Doppler ultrasound during the 1980s (Aaslid et al., 1982). A transducer emitting an ultrasonic beam, using the piezoelectric effect, bounces off different structures producing an image depending on their density. The beam will continue moving through liquid substances and will bounce off hard substances, hence its suitability for investigating soft tissues.

The first observation of blood velocity in the extracranial blood vessels was as early as 1965 (Miyazaki and Kato, 1965). But its use was limited to surgical procedures on children with open fontanels because the skull attenuates ultrasonic wave penetration. Therefore, the use in examining the cerebral vasculature in healthy individuals was very limited. However, Aaslid et al. (1983) later established the first method to quantify cerebral blood flow velocity as a surrogate for CBF using the transcranial Doppler (TCD). They demonstrated that using low frequency range within 1-2 MHz in specific acoustic windows allows insonation of cerebral arteries through specific thin areas of the skull (Tymko et al., 2018).

Nowadays, the utility of TCD is broad due to the significant advantages that this modality can offer over other neuroimaging modalities. TCD offers high temporal resolution and is cost effective, providing a clear-cut advantage over other CBF assessment approaches. The general use of TCD can provide indications of brain health by assessing resting cerebral blood flow velocity and responses to different physiological perturbations (i,e., neurovascular coupling, cerebrovascular reactivity and exercise). In clinical settings, TCD can also be used to assess stenosis, sickle cell death, or to detect an

embolus (Willie et al 2011). Although TCD is a widely common procedure to measure cerebral blood flow velocity, there are several limitations that needs to be addressed with its utility.

The main principle that allows TCD to record cerebral blood flow velocity depends on sound wave reflection. Doppler emits sound waves which are reflected back to the transducers by moving red blood cells, the resulting doppler shift from this reflection is proportional to the speed or velocity of the moving red blood cells (Willie et al., 2011c). The standard TCD only allows cerebral blood flow velocity to be measured; volumetric blood flow cannot be estimated due to the lack of vessel diameter measures. Thus, those who use the TCD generally assume constant blood vessel diameter, a controversial assumption that has apparently been validated in many magnetic resonance imaging studies (Bishop et al., 1986, Valdueza et al., 1997, Aram Ter et al., 1998, Serrador et al., 2000, Verbree et al., 2014). Recently this was challenged by Coverdale et al. (2014), who report an increase in MCA diameter (~8%) during minimal end-tidal carbon dioxide ($P_{ET}CO_2$) manipulations (+7.5 mmHg; $P_{ET}CO_2$) in healthy individuals (Coverdale et al., 2014). However, Verbree et al. (2014) showed significant increases in MCA diameter only during +15 mmHg hypercapnia, by using a 7-T Magnetic resonance imaging . Ainslie and Hoiland (2014a) have therefore suggested that there are boundaries for acceping the 'static diameter' assumption depending on $P_{ET}CO_2$ provocations. Any change in $P_{ET}CO_2$ within ± 5 mmHg results in no notable changes in diameter and therefore this range appears to back the assumption of vessel's diameter constancy. This in turn means that cerebral blood flow velocity examinations that involve a variable $P_{ET}CO_2$ of greater than ± 5 mmHg are more prone to underestimation or overestimation of cerebral blood flow velocity.

Nevertheless, despite these uncertainties with regards the 'diameter' assumption there has been venues of 'opportunity' for TCD in research. TCD provides real-time

assessment of changes in cerebral blood flow velocity, for example, in individuals moving, engaging in any physical activities or during different postural positioning. Ainslie et al. (2008) have found significant mean middle cerebral artery velocity (MCAv) reductions as age increases regardless of physical activity levels. Their findings complement the fact that aging is associated with brain atrophy and less neuronal activity. cerebral blood flow velocity response during different physiological perturbation have been used to assess cerebrovascular function. Also, different exercise intensities have shown to elicit differential perfusion response during an exhaustive incremental cycling exercise (Smith and Ainslie, 2017). This has led to a better understanding of CBF regulation during incremental exercise and factors that may contribute to the exercise hyperaemic response. Simultaneous mean arterial blood pressure (MAP) measurements offer insight to cerebrovascular function by calculating cerebrovascular conductance or resistance. Lower CVC have been reported in sedentary women compared to more active and fit physically women with same age (Brown et al., 2010).

However, there are other factors that are equally important in the application of TCD. User's knowledge of cerebral arteries anatomy is crucial, as well as the knowledge of depth and velocities related to the targeted blood vessel. Collectively, these aforementioned factors enhance the accuracy of TCD measurements and allow more appropriate cerebral blood flow velocity measurements. However, the main limitation associated with TCD use is its poor spatial resolution (Willie et al., 2011c). This limitation has led to the development of other imaging modalities enhancing assessment of brain structure and function.

2.3.3 Multi-modal Imaging

Recently, many imaging techniques assessing brain function have been established with adequate spatial resolution (Willie et al., 2011c). Using accurate

mathematical models and physics, brain hemodynamic such as CBF and cerebral blood volume is quantified. The main imaging techniques used now are duplex Doppler ultrasound, Positron Emission Tomography, magnetic resonance imaging, and Arterial Spinal Labelling. Each technique uses different tracers, apply different techniques, and use different durations of data acquisition (Wintermark et al., 2005).

In conjunction with the use of the TCD, the duplex Doppler ultrasound is a non-invasive and bed side imaging application that can enhance TCD acquisitions with confirming extracranial vessel diameter as shown elsewhere (Prodel et al., 2016d, Flück et al., 2017a). It does not require the administration of any radiation and can be used for prolonged periods. ICA sonography quantifies volumetric blood flow that would correlate with CBF in ipsilateral hemisphere (Wintermark et al., 2005). These tools helped advance the understanding of CBF regulation and how cerebral circulation is influenced by environmental and systemic factors as discussed further in detail later.

2.4 Cerebrovascular Regulation

Uninterrupted delivery of arterial oxygenated blood to the brain is key to normal brain function, hence why simple head-up tilt can cause unexpected light-headedness (Njemanze, 1993). Blurred vision or complete syncope are consequences of acute reductions in CBF, whereas headache, vomiting, and confusion characterise overperfusion (Njemanze, 1993, van Mook et al., 2005). Mechanisms that overlies CBF regulation are multifaceted and depends on hemodynamic, chemical, neurovascular coupling, and autonomic factors as means of coordination to maintain resting CBF (Ainslie and Brassard, 2014b). All these mechanisms coordinate efficiently to ensure continuous CBF (50-60 ml min^{-1} 100 g^{-1}) to the brain (Phillips et al., 2015). These mechanisms that contribute to cerebrovascular regulation at rest will be discussed in detail subsequently.

2.4.1 Hemodynamic factors

CO and BP are crucial in ensuring appropriate blood distribution to organs. But extreme changes in BP can have adverse effects on brain tissue due to skull inflexibility limiting brain tissue and extracellular fluid expansion (Ainslie and Brassard, 2014b). Therefore, maintaining constant CBF even during pressure perturbations is crucial (Ainslie and Brassard, 2014b). Due to the distinctive anatomy of the brain and the skull, the brain possesses its own control over blood supply by changing the diameter of the vessels lumen to control how much flow the brain can withstand at a certain period. This distinctive mechanism that preserves CBF during pressure changes is named cerebral autoregulation. The first observations of cerebral autoregulation were in cats (Fog, 1937, Fog, 1939). A relationship between BP and pial vascular tone was detected irrespective of a neural stimulus. Cerebral autoregulation is often referred to as either dynamic or static, static cerebral autoregulation operates from several minutes to hours, whereas dynamic cerebral autoregulation is referred to the control of sudden pressure changes, taking place over seconds.

Bayliss et al. (1895) describes cerebral autoregulation as ‘a rise in arterial pressure accelerates the flow of blood through the brain, and a fall slackens it’. However, Lassen (1959) later have reported that CBF is constant within a set range of MAP. According to data plotted from seven studies on patients, a plateau region is shown within the ~60-150 mmHg of MAP (Lassen, 1959) . By contrast, Lucas et al. (2010) have reported MCAv changes (0.82 ± 0.35% per millimetre of mercy change in MAP) within the proposed ‘autoregulatory curve’ of Lassen (1959). In fact, Willie et al. (2014) have questioned the existing cerebral autoregulation paradigm proposed. After reanalysis of 41 studies, they describe the relationship between pressure and flow as much more ‘passive’ than

observed traditionally. Indeed, Tan have demonstrated only a small plateau region in the relationship between MAP and CBF (Tan 2012) (Figure 1).

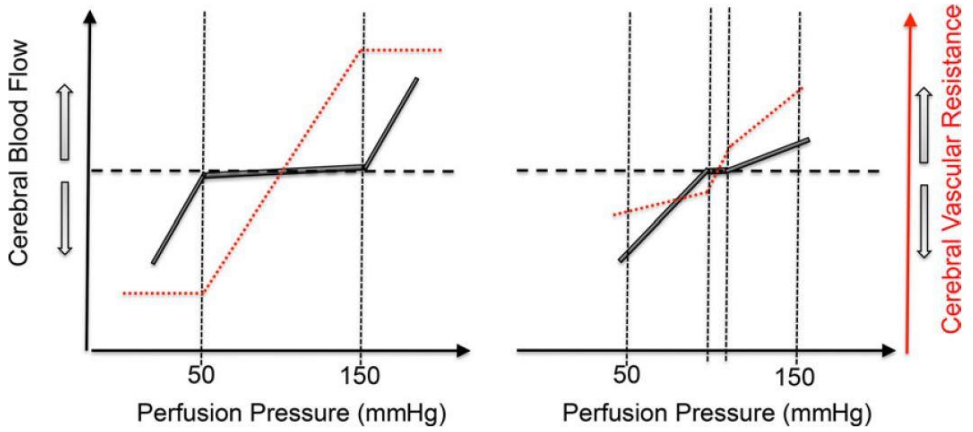


Figure 2 Left represents the classical relationship between mean MAP and CBF and right represents the contemporary relationship between MAP and CBF (Willie et al., 2014).

The proposed mechanistic principle that underlies cerebral autoregulation is the indirect adjustment of cerebrovascular resistance, occurring at the level of pial arterioles, through changes in diameter in accordance with the Hagen-Poiseuille equation (Lassen 1959, Budohoski et al., 2013). Changes in diameter occur through one of four classical mechanisms, myogenic tone, neurogenic, metabolic, or through endothelial mechanisms (Silverman and Petersen 2021). Animal studies have also shown that arteries from the neck and large intracranial arteries are implicated in modulating cerebrovascular resistance during cerebral autoregulation (Mchedlishvili et al., 1973, Faraci et al., 1987). Nevertheless, cerebral autoregulation in humans remain incompletely understood.

Another important regulator of CBF is CO. Studies that investigated CO using different CO manipulations (i.e., lower body negative pressure, albumin, saline) have suggested reductions in CBF when CO was reduced (van Lieshout et al., 2001, Ogoh et al., 2005, Ogawa et al., 2007). But the concomitant influence of BP and the arterial partial pressure of CO_2 (P_{aCO2}) makes it very difficult to not attribute these results to other factors

as well. For example, changes in P_{aCO2} can have an indirect effect on both CO and BP. The assumption that CO does influence CBF regulation is based on 2 haemodynamic principles. Firstly, referring to Ohm's law, perfusion is governed by two integral inputs, BP, and the resistance to flow of any organ's vasculature. Secondly, the metabolic rate of an organ governs the distribution of CO within the organ. However, due to methodological difficulties it is difficult to investigate the effect of CO on intact humans. Therefore, although CO is one of the regulators of CBF, its influence on CBF control remains inconclusive.

2.4.2 Chemical factors

CBF can also be influenced by partial pressures of arterial blood gases (Willie et al., 2011c). Chemoreceptors located in the carotid and aortic bodies monitor arterial levels of O_2 indexed by either arterial partial pressure of oxygen (P_{aO2}) or SpO_2 and also P_{aCO2} (Dampney, 2016). Any reductions of SpO_2 (<90%) or P_{aO2} (<60 mmHg) or increases in P_{aCO2} stimulates chemoreceptor afferent activity (Dampney, 2016). Peripheral chemoreceptor afferents project into nucleus tractus solitarius via cranial nerve IX and X projecting to the rostral ventral lateral medulla via interneurons initiating the chemoreflex (Dampney, 2016).

Arguably the most important regulator of CBF is P_{aCO2}. Cerebral blood vessels are highly sensitive to CO_2, a unique feature found in arteries around the neck, intracranial arteries, pial arterioles and parenchymal vessels (Willie et al., 2014). Many studies have demonstrated enhanced cerebrovascular reactivity relative to hypocapnia using TCD compared to hypercapnia or normocapania in MCA (Ide et al., 2003), posterior cerebral artery (Skow et al., 2013), and duplex Doppler insonation of the ICA and vertebral artery (Sato et al., 2012a, Willie et al., 2012). Congruent responses are reported where cerebral vessel velocity increased (3-6% increases) or decreased (1-3% decrease) corresponding

to CO_2 concentrations above or below baseline (Ide et al., 2003, Sato et al., 2012b, Skow et al., 2013). This suggest that the brain prioritises mechanisms that offers protection from acidosis by finding the balance between cerebral constriction and dilatation, hence maintaining constant pH. However, mechanisms behind the sensitivity of the cerebral vasculature to CO_2 are a subject of debate.

Cerebral vessels are also sensitive to O_2, thus shielding the brain from the deleterious consequences of hypoxia (Willie et al., 2011c). Although this response seems simple, mechanisms are complex and difficult to determine since P_{aCO2} is far more important. When P_{aCO2} is uncontrolled during acute hypoxia, MCAv is reduced (Ainslie et al., 2007), while MCAv increases when P_{aCO2} is controlled (Ogoh et al., 2014), indicating that hypocapnia secondary to increases in ventilation or hyperpnoea overrides the initial increases in MCAv in response to low levels of O_2. Mardimae et al. (2012) suggest that cerebrovascular sensitivity to O_2 during hypoxia depends on levels of P_{aCO2}. A concurrent increase in ventilation during hypoxia alters P_{aCO2} levels, triggering a counter acting cerebral vasoconstriction, thus reducing CBF (Willie et al., 2011c). Hypercapnia increases cerebrovascular sensitivity, whereas hypocapnia decreases cerebrovascular sensitivity during hypoxia.

Interestingly, isocapnic studies that examined at the effect of hypoxia on control of CBF have suggested an increase in CBF (+0.5-2.5% cm s^{-1}) for every percentage point reduction in SpO_2 (Cohen et al., 1967, Shapiro et al., 1970, Willie et al., 2012). However, it should be noted that the effect of P_{aO2} on CBF is negligible when end tidal O_2 ($P_{ET}O_2$) is > ~58 mmHg or SpO_2 > 90% (Gupta et al., 1997a). Mechanisms associated with CBF control and O_2 includes local vasodilatory mechanisms and neurovascular coupling. Local vasodilatory mechanisms consist of factors that are associated with endothelium such as adenosine, NO, and prostaglandins (Rocha et al., 2020). But it is possible that during acute hypoxia chemoreflex-mediated increase in respiratory muscle activity may

increase blood flow to associated areas of the sensory-motor cortex via a neurovascular coupling mechanism. Large motor cortical response following hyperpnoea may evoke a measurable increase in local perfusion, as occurs during other voluntary movements (e.g., counting loudly (Orgogozo and Larsen, 1979) or finger tapping (Vafaee et al., 2012)). Another possibility is the potential effect of ventilation on central hemodynamics (Bilo et al., 2012) or brain activation secondary to breathlessness (von Leupoldt and Dahme, 2005). However, the contribution of ventilation to CBF control during hypoxia remains unclear.

2.4.3 Neurovascular coupling and cerebral metabolism

Mosso (1881) was among the first to hypothesise that brain activity is coupled with precise changes in local blood flow. Indirect assessment of CBF using plethysmography in patients with skull defects, demonstrated increases in cerebral blood volume when the peripheral nerves are stimulated (Mosso, 1881). Almost fifty years later, it was demonstrated that upon opening of the eyes a bruit was auscultated around the occipital area in a patient with *angioma arteriale racemosum*, indicative of increased local blood flow (Fulton, 1928). Modern methods of CBF assessment have advanced the hypothesis that regional CBF is coupled with cerebral activity (Fox and Raichle, 1984), and this phenomenon has been term is known as neurovascular coupling (Willie et al., 2014). Phillips et al. (2015) suggest that neurovascular coupling is an interaction where changes in neuronal activity affects local blood flow (Phillips et al., 2015). A classic example of human neurovascular coupling is the increase in mean posterior cerebral artery velocity by ~20-30% during a simple visual stimulation test (Rosengarten et al., 2001, Boms et al., 2010, Samora et al., 2020).

Neurovascular coupling consists of the interaction between the vascular smooth muscles in arterioles, neurons, and glial cells making up a neurovascular unit (Willie et

al., 2014). Neurons synapse on astrocytes and GABAergic interneurons which synapse on arterioles. Signal transmission occurs via gap junctions between vascular smooth muscle cells initiating vasodilation in the arterioles (Willie et al., 2014). The cellular mechanisms that are associated with neurovascular coupling in humans remains poorly understood (Peterson et al., 2011, Phillips et al., 2015). However, studies on rats investigated astrocytic involvement in neurovascular coupling have found that astrocytic activation causes changes in vascular tone via vasoactive agents such as nitric oxide (Filosa et al., 2004, Filosa et al., 2006). Other molecules such as K^+ , H^+ and also adenosine have also been implicated in CBF regulation via neurovascular coupling (Peterson et al., 2011).

2.4.4 Autonomic factors

Adrenergic and cholinergic fibres provide an extensive innervation of the cerebral vasculature (Willie et al., 2014). This innervation arises primarily from the cervical sphenopalatine and trigeminal ganglia, and provides regulation by the release of neurotransmitters such as noradrenaline and acetylcholine in addition to co-neurotransmitters such as nitric oxide and vasoactive intestinal peptide (Hamel, 2006). Historic assessment of the role of the autonomic nervous system in the regulation of CBF is largely based on animal studies and studies in humans are limited but will be the focus here.

The role of the sympathetic nervous system in the regulation of CBF has been investigated in patients with a gangliectomy or healthy participants administered either local or oral ganglionic blockage. Following gangliectomy, patients have increased CBF compared to prior to the procedure (Shenkin et al., 1951, Shenkin, 1969, Suzuki et al., 1975, Jeng et al., 1999), and similar results were found during some studies using acute local ganglionic blockade (Lindén, 1955, Umeyama et al., 1995, Ide et al., 2000a,

Yokoyama et al., 2004). In contrast, other studies that have used local ganglionic blockade have reported either no change in CBF (Harmel et al., 1949, Scheinberg, 1950, Ohta et al., 1990), or reductions in CBF (Kang et al. (2010). Differences in methodological approach may justify discrepancy in results, however ganglionic excision have increased CBF convincingly in five studies supporting sympathetic role in CBF regulation. Animal studies suggest that sympathetic CBF regulation may seem important in buffering pressure surges in blood flow to the brain through cerebral vasoconstriction (Willie et al., 2014). In addition, there is also a cholinergic regulation of CBF (Roloff et al., 2016). Indeed, cerebral vasodilation was observed during the stimulation of cholinergic fibres in humans (Edvinsson et al., 1976, Tsukahara et al., 1986). Although studies indicate the contribution of the autonomic nervous system, more robust observations are needed to support these findings.

2.5 CBF Responses to Physiological Stressors

2.5.1 Diving

Mammals are air breathers, but a remarkable ‘ability’ to submerge in water and maintain O_2 is key to survival. Diving vertebrates such as seals and ducks can go underwater for long durations, a feature not possible without the mammalian diving response (Michael Panneton, 2013). Powerful autonomic reflexes resulting in a bradycardia, peripheral vasoconstriction, and increases in MAP believed to preserve blood flow to critical organs such as the heart and brain. The world record for underwater diving was held by the Cuviers beaked whale at an astonishing 137 min 30 s (Schorr et al., 2014). However, in a recent observation of underwater diving by Quick et al., (2020) a Cuvier beaked whale demonstrated an extreme 268 mins of underwater diving. While humans, despite their terrestrial origin and limited substrate brain storage, are capable of 11 min 35 s of breath-holding, as recorded by The Association Internationale pour le

Développement de l'Apnée (AIDA). A component of the diving response is trigeminal nerve stimulation, activated by facial cold receptors thought to prioritise blood delivery to heart and brain. However, whether trigeminal nerve stimulation modulate blood delivery to heart and brain, or secondary activation of breath-hold is more important remains inconclusive.

Activating facial cold receptors of the forehead by face immersion or by facial cooling stimulates the trigeminal afferents (Khurana et al., 1980). In humans, facial immersion (9°C) induces a more pronounced bradycardia than diving in warmer temperatures (20°C) (Fagius and Sundlöf, 1986). Furthermore, excision of the anterior ethmoidal nerve a sub-branch of the trigeminal nerve in rats abolishes the diving response (Rybka and McCulloch, 2006). These studies confirm trigeminal nerve stimulation involvement in the diving response. However, breathing is also suppressed following trigeminal nerve stimulation causing a 'reflex apnoea' (Dampney, 2016). Indeed, the full effects of the diving response are not reached without a breath-hold (Asmussen and Kristiansson, 1968, Andersson et al., 2000). Andersson et al. (2002) demonstrated that a 30 s breath-hold alone can reduce HR by 21-42 %. Whether these effects are due to integration of respiratory and cardiovascular centres or stimulation of thoracic stretch receptors relaying back information to the brain stem remains unknown (Gooden, 1994a). Collectively, evidence suggest that both the breath-hold and trigeminal nerve stimulation are important for the full expression of the diving response.

Autonomic control during the diving response

Peripheral activation of facial cold receptors, areas supplied by the trigeminal nerve, convey signals to the nucleus tractus solitarius of the brainstem. Inhibition of the breathing centre, and excitation of vasomotor centres and cardiac vagal motor neurons in the medulla oblongata causes a reflex apnea or a breath-hold, bradycardia, and increases in MAP (Foster and Sheel, 2005a). Secondary activation of other receptors such as the

peripheral chemoreceptors maintain and reinforce the diving response (Foster and Sheel, 2005a). Andersson et al. (2002) have demonstrated augmentation of the bradycardia during a 30 s breath-hold when the face is immersion in 10°C water (-34 vs. -42 %). Hypoxia secondary to breath-holding during the diving response can trigger a chemoreflex mechanism enhancing bradycardia and peripheral vasoconstriction. A reduction in P_{aO2} triggers a chemoreflex mechanism inducing an excitatory effect on vasomotor control causing vasoconstriction in the periphery, hence the increases in the MAP (Foster and Sheel, 2005).

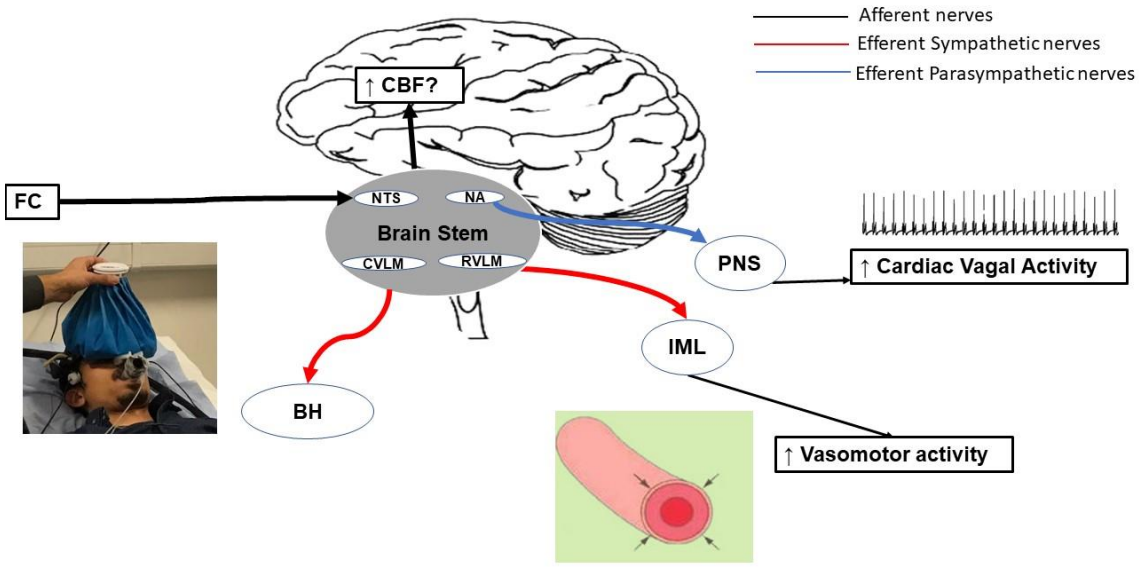


Figure 3 Schematic diagram illustrating the essential afferent and efferent pathways during facial cooling (FC). CBF, cerebral blood flow; NTS, nucleus tractus solitarius, NA, nucleus ambiguous; RC, respiratory centres; RVLM, rostral ventrolateral medulla; IML, imtermediolateral; HR, heart rate; PNS, parasympathetic nerve activity; FC, facial cooling; BH, breath-hold.

CBF control during the diving response

There are several studies that support the modulation of CBF by the diving response. In rabbits, stimulation of the trigeminocardiac reflex causes cerebral

vasodilation (Lapi et al., 2016), indicating a role for trigeminal nerve stimulation in CBF control during diving. Indeed, cold water immersion increased blood flow ((Δ36.6%) in the carotid artery in 14 healthy untrained males (Pan et al., 1997). It is possible that trigeminal nerve stimulation activation stimulated by cold facial receptors influences cerebral perfusion via intrinsic pathways. Indeed, end-feet projections of the trigeminal nerve are across various brain regions, and synapse on extracerebral vessels and in the vasomotor centre in the brain stem (May and Goadsby, 1999). In addition, cutaneous thermoreceptor stimulation may increase local cerebral metabolism and perfusion via neurovascular coupling (Phillips et al., 2015). However, the act of breath-hold during diving may mean that changes in blood gas concentrations (low O_2 concentrations and CO_2 accumulation) are also an important determinant of CBF regulation. Indeed, a breath-hold as short as only 20 s can increase MCA velocity by 47% (Przybylowski et al., 2003). However, it is presently unclear how facial cooling influences CBF in humans.

To date only two studies have examined at the effects of facial cooling on CBF (Brown et al., 2003, Kjeld et al., 2009). The combination of breath-hold and face immersion during cycling augmented MCAv responses (Kjeld et al., 2009). Similarly, Brown et al. (2003) have found increases in MCAv during a one minute of FC. Main concerns with these two studies are issues with the cofounding effects of P_{aCO2} on CBF which are not accounted for, and limitations associated with the investigations using MCAv measurements as a surrogate for MCA flow.

2.5.2 Hypoxia

During either ascent to high altitude, pathology (e.g., heart failure, lung diseases) or the breathing of gas mixture with reduced FiO_2, hypoxemia or low P_{aO2} develops and induces physiological adjustments in autonomic and cerebral control to facilitate O_2 delivery to the brain.

(Hoiland et al., 2016). In this section, changes occurring during acute hypoxia will be highlighted.

Autonomic cardiovascular control during hypoxia

Acute hypoxia increases HR, CO, along with enhancing respiratory frequency (RF) and tidal volume (TV) to defend against the decreases in P_{aO2} (Guyenet, 2000, Fisher et al., 2018b). The peripheral chemoreceptors are regarded as the most O_2 sensitive region of the body and they relay afferent information to the respiratory centre in the brainstem during hypoxia to initiate hyperpnoea (Guyenet, 2014b).

The concurrent activation of lung stretch receptors and changes in P_{aCO2} secondary to hyperpnoea also modulate this response (Guyenet, 2000). Reductions in P_{aCO2} has been shown to enhance cerebral vasoconstriction, suggesting a key role of ventilation during hypoxia. However, how ventilation influences cerebrovascular responses remains incompletely understood. Acute hypoxia is well known to increase sympathetic nerve activity (Saito et al., 1988, Rowell et al., 1989, Fisher et al., 2018b). Hypoxia induced hyperpnoea may modify cardiac autonomic control and thus HR and CO. Siebenmann et al. (2019) have argued that hypoxia elicits vagal withdrawal in humans independently of the hypoxic ventilatory response and hyperpnoea. However, given the classical work in animals, this finding is unexpected. Systemic hypoxia or carotid hypoxic perfusion increases HR in anaesthetised animals (Bernthal et al., 1951, Daly and Scott, 1958, Carmody and Scott, 1974), unless breathing is mechanically controlled or the lungs denervated, a reduction in HR occurs (Bernthal et al., 1951, Daly and Scott, 1958, Carmody and Scott, 1974). Based on such observations it has been proposed that during acute hypoxia the ability of the peripheral chemoreceptors to increase cardiac vagal tone is diminished by an augmented pulmonary stretch receptor mediated vagal withdrawal (Marshall, 1994). To corroborate this view in humans, it would be important to compare

the HR and cardiovagal responses to acute isocapnic hypoxia with and without volitional restrain of chemoreflex mediated increases in ventilation. Indeed, as in previous animal studies (Bernthal et al., 1951, Daly and Scott, 1958, Carmody and Scott, 1974), the restraint of pulmonary stretch receptor activation during peripheral chemoreflex activation may unmask an increase in cardiac vagal tone.

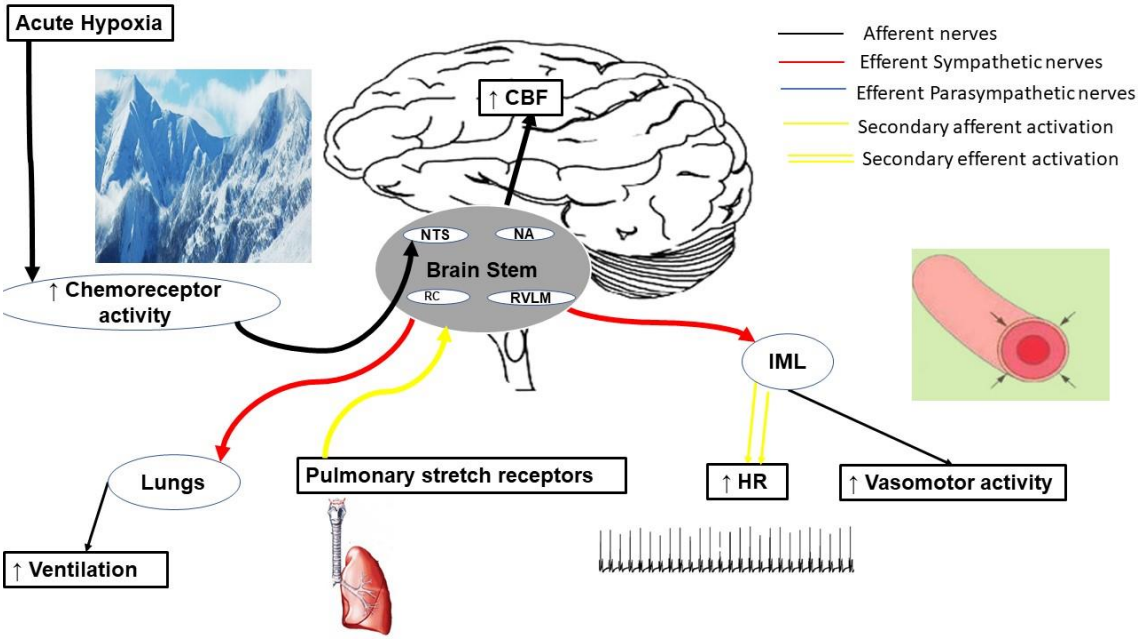


Figure 4: Schematic diagram illustrating the essential afferent and efferent pathways during acute hypoxia. CBF, cerebral blood flow; NTS, nucleus tractus solitarius, NA, nucleus ambiguous; RC, respiratory centres; RVLM, rostral ventrolateral medulla; IML, imtermediolateral cell column; HR, heart rate.

CBF control during hypoxia

Although P_{aO2} and P_{aCO2} are essential inputs of CBF regulation during acute hypoxia, other factors (i.e., neurogenic, hemodynamic, autoregulatory and metabolic) are also involved, making CBF regulation during acute hypoxia highly complex (Ogoh et al., 2014). To protect the brain from acidosis and keeping pH constant, central chemoreceptors modulate the ventilatory response and help adjust constriction and

dilatation (Ogoh et al., 2008). Interestingly, when P_{aCO2} was matched to baseline, hypoxia (FiO_2= 12%) increased MCAv and ICA blood flow (Ogoh et al., 2013). It is well understood that hypoxaemia ($P_{ET}O_2$ < ~58 mmHg or SpO_2 < 90%) enhance cerebral vasodilation CBF through local vasodilatory mechanisms (Gupta et al., 1997a).

Local vasodilatory mechanisms describe the contribution of specific endothelium-derives factors such as adenosine, nitric oxide, and prostaglandins in mediating cerebral vasodilation during acute hypoxia. Indeed in humans, selective blockade of K_{ATP} channels have reduced ICA blood flow in a 5 minute isocapnic hypoxia (FiO_2=10% O_2) (Rocha et al., 2020).

Moreover, activation of higher brain centres during exercise secondary to hyperpnoea can influence CBF through neurovascular coupling (Phillips et al., 2015, Braz and Fisher, 2016, Smith and Ainslie, 2017). During dynamic exercise (Braz and Fisher, 2016), finger tapping (Vafaee et al., 2012) and counting loudly (Orgogozo and Larsen, 1979), part of the hyperaemic response has been attributed to neurovascular coupling secondary to hyperpnoea. Similarly, during hypoxia increases in respiratory muscle activity secondary to chemoreflex activation may influence CBF through activation of the sensory-motor cortex. Whether hypoxia can increase cerebral perfusion independently of changes in arterial oxygen tension in humans remains incompletely understood.

2.5.3 Exercise

Exercise increases the metabolic demands of active muscles to sustain physical activity and performance. During exercise, both BP (+15-25%) and CO (+300-600%) increase progressively from baseline until maximal intensity redistributing blood to active organs (Smith and Ainslie, 2017). The autonomic nervous system plays an important role in adjusting the physiological responses. Alterations in sympathetic and parasympathetic activity help sustain exercise and performance by adjusting HR, BP, CO, and metabolism

(Smith and Ainslie, 2017). Central command, exercise pressor reflex, arterial baroreflex and to a lesser extent the arterial chemoreflex and the respiratory metaboreflex are all neural reflex mechanisms that underlie these autonomic responses during exercise (Hartwich et al., 2011). This section will summarise briefly recent updates with regards mechanisms that are involved in autonomic control and CBF regulation during exercise.

Autonomic adjustments to exercise

Precise alterations in the balance of parasympathetic and sympathetic nervous systems are key to address the metabolic demands of the exercising muscles. During exercise onset in humans, HR increases due to vagal withdrawal (Fisher et al., 2015b). However, the contribution of the sympathetic nervous system to cardiovascular responses during exercise onset remains unclear. In a low intensity steady-state dynamic exercise the cardiac parasympathetic system facilitates vagal withdrawal, hence HR, ventricular contractility and stroke volume (SV) increases. However, as intensity increases cardiac sympathetic nerve activity may increase HR and ventricular contractility (Fisher et al., 2015b). Studies that support this claim have shown increased norepinephrine spill over from different organs vasculature and increases in muscle sympathetic nerve activity during exercise steady state (Galbo et al., 1975, Bloom et al., 1976, Ichinose et al., 2008). Sympathetic nerve activity is also purported to increase HR and peripheral vasoconstriction to allow redistribution of blood flow at steady state exercise (Fisher et al., 2015b). Neural-cardiovascular reflex mechanisms that underlie these autonomic adjustments are discussed subsequently.

Neural cardiovascular control during exercise

During the late 19th century, a control mechanism emanating from the cortex was first proposed that is responsible for the activation of the respiratory centers and voluntary movements (Zuntz and Geppert, 1886). This ‘feed word’ mechanism that was named the

'central command' was later found to influence cardiovascular responses to exercise (Krogh and Lindhard, 1913, Krogh and Lindhard, 1917). Neural signals initiated at the cortex descend to produce a muscle contraction, in addition to activating central neural centers to influence cardiorespiratory responses (Fisher et al., 2015b). An important determinant of the central command response is the perception of an individual's exertion, irrespective of the actual force applied (Fisher et al., 2015b). Recent studies in awake humans involving direct electrical stimulation of areas around the midbrain during neurosurgery have identified potential areas associated with central command activation. The thalamus, subthalamic nucleus, substantia nigra, periaqueductal gray, and periventricular gray (Green et al., 2005, Green et al., 2007, Green and Paterson, 2008). How central command influence cardiovascular responses during exercise will be discussed collectively with skeletal muscle afferents.

The first observation of a reflex to cardiorespiratory responses originating from the skeletal muscle was by the end of the 19th century (Zuntz and Geppert, 1886, Johansson, 1894). However, the first classical experiment showing the contribution of skeletal muscle afferents to the pressor response was by Alam and Smirk (1938). Inflation of an arm cuff to supra-systolic pressure during and after a hand rhythmic exercise elevated systolic blood pressure even when muscles remained quiescent. Studies on cats later identified the components of the afferent and efferent feedback of this 'exercise pressor reflex' (Coote et al., 1971, McCloskey and Mitchell, 1972a, McCloskey and Mitchell, 1972b). This 'exercise pressor reflex' is initiated by thinly myelinated group III afferents which are the mechanoreceptors and thinly unmyelinated group IV metaboreceptors. These afferent signals feeds into the brain into the brain stem via the dorsal horn of the spinal cord (McCloskey and Mitchell, 1972a). Both receptors are stimulated by a different type of stimulus, the metaboreceptors responds to any metabolic stimuli produced by the contracting muscle. While mechanoreceptors respond to any

mechanical stimuli caused by pressure or any stretch to the muscles. Although there are differences in activation theoretically, both skeletal muscle afferent fibres can be stimulated by a mechanical or metabolic stimulus due to their intrinsic polymodal qualities (Fisher, 2015).

During exercise onset, it is well documented that the initial autonomic adjustments are initiated by central command (Raven et al., 2002, Waldrop et al., 2010). But human and animal studies have also demonstrated increases in HR and muscle sympathetic nerve activity in the absence of central command (Krogh and Lindhard, 1917, Matsukawa et al., 1994, Nobrega et al., 1994, Williamson et al., 1995). This is mainly due to mechanoreflex activation in response to mechanical deformation happening during exercise onset. Inhibitory inputs to cardiac parasympathetic neurons from central command and muscle mechanoreflex elicit vagal withdrawal increasing HR (Fisher et al., 2015b). On the other hand, metaboreceptor contribution is considered negligible partly due to the late accumulation of metabolites during exercise onset in addition to the longer latency of mateboreceptors afferent. Although there are several observations by Kaufman and colleagues that showed contribution of metaboreceptors during exercise onset in a low intensity dynamic exercise (Adreani et al. (1997), Adreani and Kaufmann (1998)), isolating the metaboreceptors during exercise onset is complex. However, it is generally accepted that the central command sets the initial autonomic response to exercise, and if the exercise pressor reflex is activated at the onset of exercise, it is likely that this would occur through the mechanoreflex.

However, as exercise progresses an acceleration of accumulation of skeletal muscle metabolites influence sympathetic nerve activity extensively with increasing HR, BP, muscle sympathetic nerve activity and to a lesser extent cardiac sympathetic nerve activity (Fisher et al., 2015b). Experiments that isolated metaboreflex activation by post exercise ischemia (PEI) or by partial blood flow restriction (PFR) during exercise have

demonstrated evidence of increases in BP and muscle sympathetic nerve activity. However, the contribution of metaboreflex in HR response is dependent on either the type of exercise used, or the muscle mass involved. During PEI following handgrip, HR returns to baseline, however when the muscle metaboreflex is augmented during exercise by making the muscle ischemic or partially reducing its perfusion, the HR response is increased or remains elevated during PEI following cycling exercise (Fisher et al., 2010b). Fisher et al. (2015b) provides an insight to neural mechanisms that contribute to these differential responses to HR. The elevation in BP during PEI following handgrip exercise is likely to elicit a baroreflex-mediated excitatory drive to cardiac parasympathetic neurons counteracting inhibitory inputs from central command and muscle mechanoreflex which are absent due to exercise termination. In contrast, when inhibitory inputs from central command and muscle mechanoreflex are present during exercise, metaboreflex activation augments HR. indeed, part of the HR elevation during hypoxic exercise has been attributed to a greater inhibition of cardiac parasympathetic activity. Although the mechanisms for such autonomic alterations are unclear, they can be attributed to increases in metaboreflex activation (Fisher et al., 2015b).

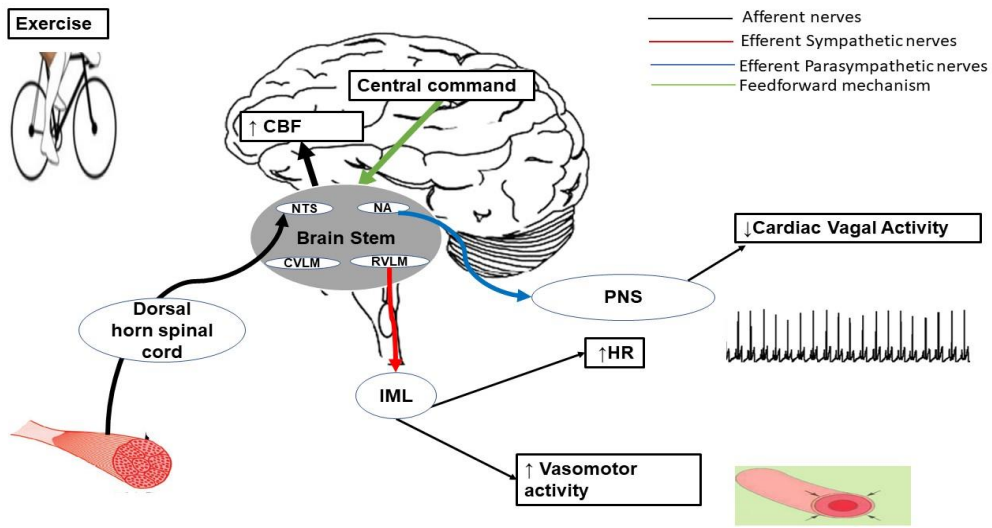


Figure 5 Schematic diagram illustrating the essential afferent and efferent pathways during acute exercise. CBF, cerebral blood flow; NTS, nucleus tractus solitarius, NA,

nucleus ambiguous; RC, respiratory centres; RVLM, rostral ventrolateral medulla; IML, imtermediolateral cell column; HR, heart rate; PNS; parasympathetic nerve activity.

CBF control during exercise

During incremental dynamic exercise, the CBF response is traditionally considered as being biphasic (Smith and Ainslie, 2017). CBF increases up until ~60% of maximal O_2 uptake (VO_2max) and thereafter CBF is either reduced (Subudhi et al., 2008, Smirl et al., 2012, Smith et al., 2012, Fisher et al., 2013b) or plateaus (Larsen et al., 2008, Smith et al., 2014). Several factors have been implicated in the CBF response to exercise and include arterial blood gases (i.e., CO2, O_2), haemodynamic factors (i.e., blood pressure, CO), neurovascular coupling, and neurogenic factors.

An important question is whether activation of other peripheral receptors that occur during exercise and hypoxia (i.e., metaboreflex) modulate CBF increases during hypoxia. A role for group III and IV skeletal muscles afferent has been implicated in the hyperaemic response during exercise (Braz and Fisher, 2016, Prodel et al., 2016d). A local mismatch between oxygen delivery and demand stimulates the release of exercise-induced metabolites, activating the metaboreceptors. The interaction between the chemoreflex and the metaboreflex during exercise has not been established. This is important since its help understand underlying human physiological mechanisms during altitude training or in clinical settings treating heart failure.

Arterial blood gases (P_{aCO2} & P_{aO2})

P_{aCO2} is one of the primary regulators of CBF during both rest and exercise. In an incremental exercise to exhaustion, cerebral blood velocity increases as P_{aCO2} increases up to ~60% of VO_2max (i.e., around the ventilatory threshold), then reductions in cerebral blood velocity accompanies reductions in P_{aCO2} until VO_2max (Subudhi et al., 2011, Smirl

et al., 2012, Fan and Kayser, 2013, Fisher et al., 2013b, Brugniaux et al., 2014, Smith et al., 2014, Smith, 2016). More evidence of P_{aCO2} raising CBF during exercise is by increasing P_{aCO2} during baseline (Siebenmann et al., 2013), or after reaching the ventilatory threshold (Olin et al., 2011, Subudhi et al., 2011). Smith and Ainslie (2017) (Figure 5).

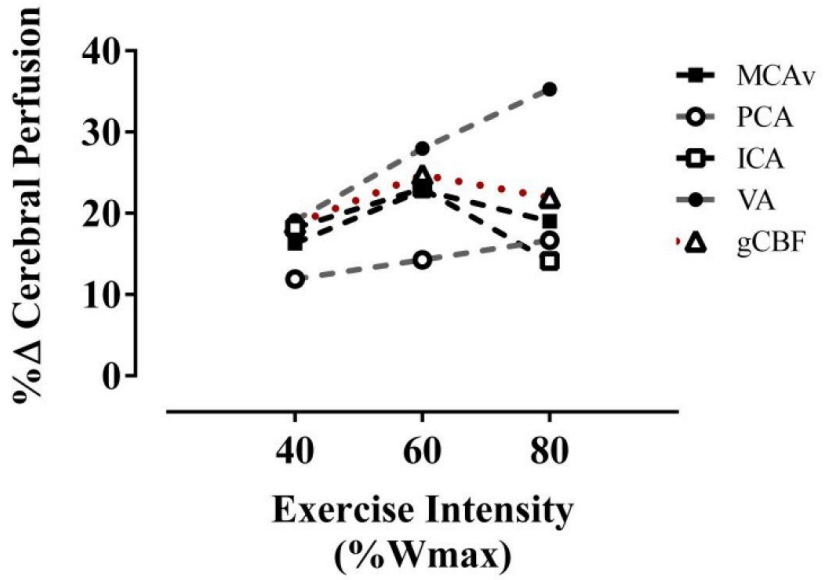


Figure 6 Mean middle (MCAv), posterior (PCA), internal carotid artery (ICA), vertebral artery (VA) velocities and flow during a poikilocapnic incremental exercise. Regional increases are evident by increases in PCA and vertebral artery flow (Smith and Ainslie 2017).

In healthy, recreationally active individuals P_{aO2} is constant and is not affected by exercise. However, in athletes and certain patient groups arterial hypoxemia may develop. Arterial hypoxemia and also hypoxia exposure may affect CBF responses during exercise In an incremental dynamic exercise under acute hypoxic conditions, increases in CBF are greater than during a normoxic exercise (Smith and Ainslie, 2017). This suggest that the initial response to hypoxia is cerebral vasodilation. However, CBF responses to exercise are attenuated during exercise in chronic hypoxia (Smith and Ainslie, 2017). During chronic hypoxia, peripheral chemoreflex activation and reductions in P_{aCO2} secondary to

hyperventilation predominates the hypoxic mediated cerebral vasodilatation. However, during maximal hypoxic exercise, intense progressive reductions in P_{aO2} stimulate a compensatory cerebral vasodilation irrespective of hypocapnia. These discrepant findings explain the combined importance of both arterial blood gases on CBF control during exercise.

BP and CO

CO and CBF responses to incremental exercise are both linearly related to intensity. CBF and CO increases during low-to-moderate intensity workloads, but is either reduced or plateaus after ~60-70% of maximal intensity (Smith and Ainslie, 2017) However, the proportions in increases are incomparable where CO is increased by 300-600% and the change in CBF during exercise is around ~20% only. This might suggest that there is a limited influence of CO on the CBF response to exercise, however there is also evidence to the contrary. For example, when metropolol was administered during a submaximal exercise, no increases in cerebral blood velocity were demonstrated (Ide et al., 2000a). Furthermore, certain exercise modes where BP fluctuates rapidly such as resistance exercise (Edwards et al., 2002) or rowing (Pott et al., 1997), an influence of CO on CBF can be seen. However, it is difficult to discern the effects of CO from that of BP on CBF during exercise.

Neurogenic Regulation

The sympathetic regulation of CBF remains controversial, yet there is some evidence for its importance during exercise. Administration of the sympathetic alpha receptors phenylephrine (0.25 mg) during rest increases MCAv, but when a 0.30 mg dose was administered during both light and moderate steady state exercise, MCAv failed to increase (Brassard et al., 2010). This discrepancy may be attributed to BP differences during rest (~17-23%) and exercise (~9-13%) following the dose of phenylephrine.

However, vasoactive substances released by the contracting muscle can override the initial vasoconstrictor stimuli to allow sufficient local blood flow. A 'functional sympatholysis' phenomenon can explain why MCAv failed to increase during exercise. Interestingly, a sympathetic inhibitor, Prasozin eliminated any increases in MCAv during submaximal exercise (Purkayastha et al., 2013). Although MCAv was reduced, cerebrovascular conductance increased implying that MCA dilation maintained CBF throughout exercise. Nevertheless, it is difficult to interpret these results without understanding volatility of cerebral artery diameter under these interventions given the nature of TCD and MCAv reporting. Also given the cofounding effects of BP, using central agonist or antagonist may help clarify neurogenic involvement in CBF regulation during exercise without perfusion factors.

Metabolic factors

During dynamic exercise in humans, CBF increases were coupled with central neuronal activation and metabolism in the supplementary motor area and other sensorimotor regions (Orgogozo and Larsen, 1979). This is evident in exercising dogs where increases in regional CBF were demonstrated in areas associated with motor control. Indeed, CBF was reduced following Doxapram, a respiratory stimulant that simulated hypocapnia and hypertension observed during exercise (Gross et al., 1980). Increases in CBF following exercise is indicative for a coupling mechanism between neuronal activity in the motor area and local blood flow during muscle contractions. Nevertheless, high intensity exercise increases cerebral O_2 uptake but CBF is reduced, suggesting that cerebral O_2 uptake may compensate for reductions in CBF. Anaerobic metabolism is another option to maintain cerebral metabolism during extreme intensity exercise. In fact, proportional increases in lactate uptake to arterial concentration in the brain has been implicated during exercise (Ide et al., 2000b, Secher et al., 2008). But

during an exercise to exhaustion, cerebral metabolic ratio increased similarly with or without β-blocker (Dalsgaard et al., 2004).

In the elegant review by Secher et al. (2008), how central fatigue affects CBF regulation during maximal exercise is described thoroughly. Hypocapnia secondary to hyperpnoea and arterial desaturation causes reductions in cerebral oxygenation. This limits the brain capacity to carry on normal metabolism due to emptying of glycogen stores in brain astrocytes, and therefore central fatigue occurs. Although cerebral metabolism has shown some degree of CBF regulation during exercise, during maximum intensity exercise hypocapnia secondary to hyperpnoea limits the brain ability to sustain high intensity exercise due to inadequate oxygen, glucose, and lactate delivery to the brain.

2.6 Literature Review Summary

CBF regulation is complex and involves integrated mechanisms to ensure continuous oxygen delivery at rest and during the exposure of different environmental stimuli. An array of factors contributes to CBF regulation comprising of hemodynamic, chemical, metabolic and neurogenic factors (Phillips et al., 2015). Conditions of local ischaemia or systemic hypoxaemia compromise CBF regulation triggering reflex mechanisms to allow uninterrupted O_2 delivery. Therefore, the understanding of how different reflex mechanism effect CBF regulation is crucial since the consequences of such interactions can be relevant clinically to patients with hypertension or heart failure. Also, it can help explain abnormal ventilatory behaviours or exertional dyspnoea exhibited by such patients and ramifications for exercising in high altitude.

2.7 Specific Research Questions

This thesis will investigate the cerebrovascular and cardiorespiratory responses to conditions of local ischaemia and general hypoxaemia. Based on the literature undertaken, the following specific research questions have been generated and will be addressed in this thesis:

1. Does facial cooling mediated stimulation of cutaneous trigeminal afferents associated with the diving response, increase CBF or are factors associated with breath holding (e.g., arterial CO_2 accumulation, pressor response) more important in humans?
2. Does the ventilatory response to moderate acute hypoxia increase cerebral perfusion independently of changes in arterial oxygen tension in humans?
3. Does hypoxia augment cerebrovascular and cardiorespiratory responses to metaboreflex activation.

CHAPTER 3: GENERAL METHODS

3.1 Ethical Approval and Informed Consent

Thesis studies were undertaken in accordance with the principles outlined in the Declaration of Helsinki. Ethical approval was obtained for all studies from the Science, Technology, Engineering and Mathematics Ethical Review Committee at University of Birmingham. All participants were given an information sheet that outlined the purpose of the research and details of the experimental procedures. Participants were also verbally instructed about experimental procedures prior to the study. Written informed consent was obtained from all participants before any experimental procedures took place (Appendix A).

3.2 Participants

The participants were either students or staff at the University of Birmingham. Participants included in these studies were healthy and free from any renal, cardiovascular, respiratory, neurological, or metabolic diseases according to responses given to a general health questionnaire (Appendix B). Participants that were using prescribed or over-the-counter medications were excluded from these studies. For Chapters 4-7, a questionnaire was used to exclude any participants that had either been to high altitude in the past 6 months or had previously been diagnosed with an altitude related condition (e.g., altitude mountain sickness) that would risk their health during the study (Appendix C).

3.3 Experimental protocols

3.3.1 Pre-study procedure

All participants were asked to refrain from alcohol, caffeine, and heavy exercise for 24 hours prior to the studies. Height and weight were assessed to establish body mass

index using the following: (Pietrobelli et al., 1998). Perceived exertion was assessed using the 6-20 point rating of perceived exertion scale (RPE), and perceived pain was assessed using the 10-point pain scale (Noble et al., 1983).

3.3.2 Facial Cooling

The trigeminal nerve divisions innervate the forehead (ophthalmic) and cheeks (maxillary). Therefore, trigeminal nerve stimulation was undertaken using facial cooling applied to these regions using a cold stimulus (i.e., ice pack) of ~0 °C (Appendix D).

3.3.3 Cold Pressor Test

During the cold pressor test, participants were asked to immerse the hand in water at a temperature of ~1 °C as shown in Appendix E. The cold pressor test increases sympathetic nerve activity and BP, therefore is commonly used to assess sympathetic autonomic function (McLean et al., 1992, Frank and Raja, 1994, Freeman and Chapleau, 2013). Participants performed the cold pressor test while lying supine (Chapter 4).

3.3.4 Cycling exercise

Two experimental studies (Chapters 5 and 6) involved cycling exercise carried out on a cycle ergometer **(**Appendix F**)**. The cycle ergometer was custom-made to allow a semi-recumbent positioning using a car seat and electrically braked cycle ergometer (Angio, Lode, Groeningen, The Netherlands; Appendix F). During cycling, the pedalling rate was kept constant at 60 revolutions per minute. Participants were seated comfortably with pillows provided for back support and Velcro straps used to secure the participants' feet to the pedals.

3.3.5 Handgrip

Chapter 6 involved handgrip exercise **(**Appendix G). For this, participants were seated comfortably in an upright position. They were asked to perform 3 maximum voluntary contractions (MVC) separated by a 1 minute of rest. The most forceful contraction was taken as the MVC and used in the calculation of the submaximal target force (i.e., 30%). The target force was displayed on a visual display to provide feedback to the participant.

3.3.6 Dynamic end-tidal forcing

In two experimental studies (Chapter 5 and 7) a dynamic end-tidal forcing system was used to induce hypoxia while keeping $P_{ET}CO_2$ constant. This technique allows user-dependent manipulation of inspired gases.

The dynamic end-tidal forcing system was devised in the 1970's (Swanson and Bellville, 1975) and later modified (Robbins et al., 1982). The physiological principles of this mechanism can be simplified as since the partial pressure of alveolar and arterial (before leaving the lung) O_2 and CO_2 are equal, sampling expired gases can indirectly predict alveolar gases. Therefore, the dynamic end-tidal forcing system modifies the inspired gases to achieve the required end-tidal gas concentration, while monitoring the concentration of the expired gases. These inspired gases values are predicted to achieve the desirable end-tidal gases using a cardio-respiratory algorithm. The actual and desired values are checked and manually corrected as necessary to control and minimise potential errors. As shown in Appendix H, when using the dynamic end tidal forcing system, participants breathed through a mouthpiece while wearing a nose clip worn. To ensure uninterrupted breathing and continuous recording, a built-in saliva trap was incorporated into the mouthpiece.

A standard calibration procedure was followed prior to each experimental session. This involved a 2-point (15% O_2 and 5% CO_2) calibration for the gas analyser and the use of a 1 L syringe for calibration of the flow transducer. A standard dynamic end tidal forcing system procedure was followed with each participant. An initial adjustment period (~15 mins) always took place before the experiment to establish normal end-tidal gases values. These values were used to determine the desired end-tidal values for $P_{ET}O_2$ and $P_{ET}CO_2$ for the different experimental procedures for each study. In terms of participants safety, participants were always wearing a finger pulse oximetry (Magna Medical 1400 Multi Parameter Monitor, Datex-Ohmeda Medical Equipment, USA) to monitor O_2 saturation throughout the whole procedure. Any unusual readings would permit audio-visuals alarms to go on and signal the experimenter that values went beyond pre-set safety levels. HR and BP are monitored continuously, and participants are asked to report any chest pain, shortness of breath, or light-headedness as signs that would immediately terminate the experiment. Nevertheless, no incidence of unexpected symptoms happened during any of the experimental procedures.

3.3.7 Metaboreflex Activation

Two approaches were used for assessing muscle metaboreflex sensitivity (Prodel et al., 2016d). The first approach restricted leg blood flow during exercise by inflating bilateral thigh cuffs to create a mismatch between demand and O_2 delivery, hence activating metaboreceptors (PFR). Rapid cuff inflator (E20, Hokanson Bellevue, WA, USA) were used to inflate cuffs to ~100 mmHg. The other approach involved complete restriction of blood flow to the thighs after exercise while the muscle is inactive (PEI) (Prodel et al., 2016d). This was achieved by using a rapid cuff inflator and inflation of cuffs to a supra-systolic pressure of ~250 mmHg. Custom-made shorts with sewn in cuffs

ensured that cuffs did not move during PFR or PEI. A picture of the shorts that were used during the metaboreflex activation experimental procedure can be found in Appendix (I).

3.4 Measured Variables

3.4.1 Haemodynamic monitoring

HR was recorded using electrocardiography (lead II, ECG). Lead II enhances the visualisation of upward P and R peak waves in healthy individuals (Morris et al., 2009). Since participants included in this thesis experimental studies are healthy and free from any existing cardiac conditions, this methodology is deemed appropriate. BP was monitored using brachial BP recordings using either the oscillometric method (Dinamap ProCare 200, GE Medical Systems, Milwuakee, WI, USA) or an automated BP monitor using the auscultatory R-wave gating algorithm (Tango+; SunTech Medical, Enysham, UK). The non-invasive assessment of BP was made on a beat-to-beat basis with finger photoplethysmography (Finometer Pro; Finapres Medical Systems, Arnhem, the Netherlands). Changes in BP detected using this method are comparable with those derived from intra-arterial BP monitoring during different manipulations (i.e., tilt test) (Petersen et al., 1995). An adjustable table ensured that the hand was positioned at the heart level. Once systolic and diastolic BP have been acquired, MAP was calculated using the following formula:

Finger MAP measurements were corrected and verified using brachial MAP measures by calculating the difference between the assessments and applying the difference to all finger MAP measurements. Finger BP waveform using the Modelflow technique (FMS, Amsterdam, the Netherlands) were used to establish SV. CO and total peripheral resistance (TPR) were calculated using the following formulae:

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3.4.2 Respiratory measurement

During all experimental procedures, participants wore a mouthpiece and nose-clip to allow continuous recording of respiratory variables. $P_{ET}CO_2$ and $P_{ET}O_2$ were used as a surrogate for P_{aCO2} and P_{aO2} respectively. $P_{ET}CO_2$ and $P_{ET}O_2$ were sampled at the mouth and either recorded by a gas analyser (Chapter 4 and 5; model ML206, ADInstruments, Dunedic, New Zealand) or by using rapid response gas analysers (Chapter 5 and 7; Moxus Modular, AEI Technologies, Pittsburg, PA). TV, and RF were either determined using a one-way valve spirometer (fr41s, Flowmetrics Inc., Chatsworth, USA) (Chapter 5 and 7 model ML206, ADInstruments, Dunedic, New Zealand) or by a turbine volume transducer (Chapter 6; VMM400, Interface Associates, Aliso Viejo, CA). VE was calculated offline using the following formula:

.

The gas analyser was calibrated using a two-point calibration using mixtures of 5% CO2, 15% O_2, and 100% Nitrogen. While volume was calibrated using a 3 L syringe before each testing session.

3.4.3 CBF

TCD 2-MHz pulsed Doppler ultrasound probe (Doppler Box X; Compumedics, 144 Singen, Germany) was employed to assess MCAv as a surrogate index of CBF. The temporal window was used for insonation, specifically ~1 cm above the zygomatic arch with depth as 45-65 mm (Willie et al., 2011c). Ultrasound gel was used to help transmit waves from the probe to the skin. Gain and power were adjusted to reduce noise and artefacts and improve the quality of the signal. After obtaining a stable signal, the probe was fixed at the optimum insonation angle using the modifiable head kit.

To quantify volumetric CBF, mean internal carotid artery velocity (ICAv) and internal carotid artery diameter (ICAd) were assessed using duplex Doppler ultrasound

(Terason T3300, Teratech, Burlington, MA, USA). Multi-frequency linear-array transducer (4-15 MHz) with an insonation angle of ~60° relative to the skin was employed. ICA was identified using the common carotid artery bifurcation. The brightness was adjusted, and a longitudinal section of vessel walls was used for ICAd assessment. 1 to 1.5 cm distal to the common carotid artery bifurcation was the area used for recording. For establishing ICAv, the pulse-wave mode was used. Recordings were stored offline and analysed using an automated edge and velocity spectral detection software (FMD Studio, Pisa, Italy). This software has been widely used for ultrasound applications and has shown good agreement with an echo-tracking device in diameter measurements (Rastelli et al., 2012). SAS performed all volumetric measurements and TCD data acquisition.

3.5 Methodological Reproducibility

Doppler ultrasound is user dependent (Aldrich, 2007). Therefore, the main user of ultrasound in all studies (SAS) participated in Liverpool John Moores' summer cardiovascular school to establish skills in Doppler ultrasonography. SAS underwent >50 hours of training before conducting any ultrasonography. Training was initially supervised by Dr James Fisher and Dr Rehan Junejo. To establish reliability of methods used in ultrasonography, it is important to indicate that the results are reproducible. This is accomplished by investigating the degree of agreement of day-to-day measurements.

The test-retest reliability of Doppler ultrasound measurements of ICA used in Chapter 4. Participants were asked to visit the lab twice with at least 24 hours between sessions. During both tests, the experimenter aimed to make the conditions of the two tests similar. This was achieved by asking the participant to avoid any alcohol, caffeine, or vigorous physical activity for 12 hours before the test, and avoid eating 2 hours before the test, and if possible, to eat the same meal. Participants were supine during the

experiment and both left and right ICA were assessed (Appendix J). Agreement between measurements were determined by Bland Altman plots (Bland and Altman, 1986). In addition, coefficient of variation CV, calculated as: was used also to assess agreement between measurements. SD refers to sample standard deviation and is the sample mean. Secondly, reliability was assessed using the intraclass correlation coefficient (ICC) with a one-way analysis of variance (ANOVA).

ICA assessment was carried on 12 arteries on two separate days. ICAd and ICAv were included for offline analysis. Video files were acquired from ICA imaging using a software that has edge tracking features that enable edge detection independent of user control (FMD Studiom Pisa, Italy). ICAd and ICAv both showed high ICC (ICAd: ICC= 0.981, 95% CI 0.935 - 0.994; ICAv: ICC= 0.955, 95% CI 0.850 – 0.987). These results indicate high reliability of test-retest measurements that were conducted by the experimenter. CV results have indicated fair results with 13% ICAd and 26% ICAv. Moreover, Bland-Altman plots showed good agreement (Fig 7), with mean differences and limit of agreements ranging as follows: ICAd mean differences is -0.01 cm, with limits of agreements ranging from -0.04 cm to 0.02 cm. ICAv mean differences is -0.17 cm s^{-1}, with limits of agreement ranging from -7.88 cm s^{-1} to 7.54 cm s^{-1}. On the basis of these investigations it can be concluded that the between-day agreement of ICAd and ICAv is acceptable and that the measurements made are within limits that indicate good reliability (Quan and Shih, 1996).

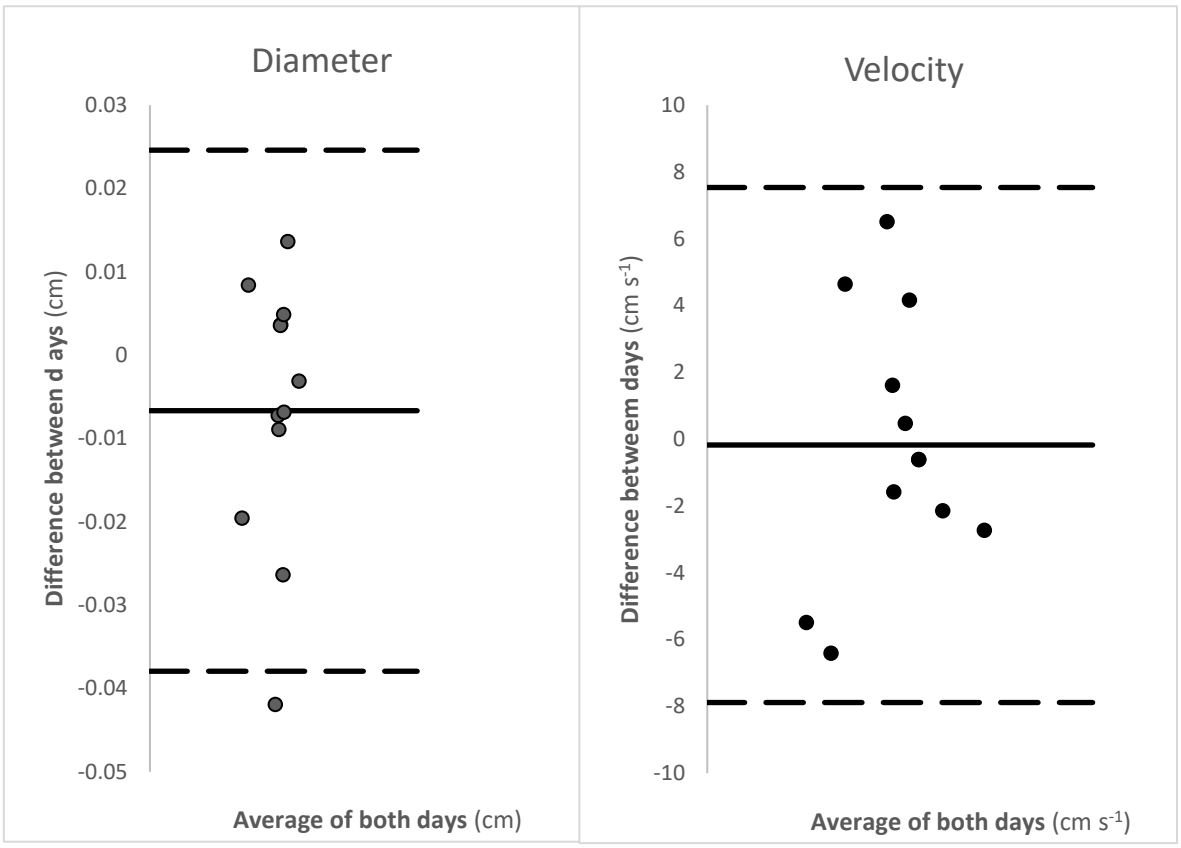


Figure 7 Reproducibility of ICA diameter and velocity between days in 11 participants. Means and limits of agreement are presented by the dashed lines.

3.6 Data and Statistical Analysis

Cardiorespiratory and cerebrovascular measurements were converted from analogue to digital at 1 kHz using a high-performance data acquisition system (Powerlab, ADInstruments, Dunedin, New Zealand). Data were stored offline for analysis (LabChart Pro; ADInstruments). Beat-by-beat and breath-by-breath data were transferred to a spreadsheet (Excel 2018, Microsoft, Redmond, WA, USA) for further analysis.

All statistical analysis were performed by using SigmaPlot (version 13.0, 14.0, and 14.5 SYSTAT Software Inc., Chicago, IL, USA). Specific details of the statistical analyses undertaken are provided in each experimental chapter.

CHAPTER 4: HUMAN CEREBROVASCULAR RESPONSES TO SIMULATED DIVING ARE NOT RELATED TO FACIAL COOLING

A version of this manuscript has been published in Experimental Physiology

4.1 Abstract

Diving evokes a pattern of physiological responses purported to preserve oxygenated blood delivery to vital organs such as the brain. We sought to uncouple the effects of trigeminal nerve stimulation on CBF, from other modifiers associated with the diving response, such as apnoea and changes in arterial CO_2 tension. Thirty-seven young healthy individuals participated in separate trials of; Facial cooling (3 min) and cold pressor test (3 min) under poikilocapnic (Protocol 1) and isocapnic conditions (Protocol 2), facial cooling while either performing a breath-hold (FC +BH) or breathing spontaneously for a matched duration (FC -BH) (Protocol 3), and breath-hold during facial cooling (BH +FC) or without facial cooling (BH -FC) (Protocol 4). Under poikilocapnic conditions neither facial cooling nor cold pressor test evoked a change in middle cerebral artery blood flow velocity (MCAv; transcranial Doppler) ($P>0.05$ vs. baseline). Under isocapnic conditions, facial cooling did not change MCAv ($P>0.05$), whereas cold pressor test increased MCAv by 13% ($P<0.05$). Facial cooling with a concurrent BH markedly increased MCAv (Δ 23%) and internal carotid artery blood flow (ICA_Q; duplex Doppler ultrasound) (Δ26%) ($P<0.001$), but no change in MCAv and ICA_Q were observed when facial cooling was accompanied by spontaneous breathing ($P>0.05$). Finally, MCAv and ICA_Q were similarly increased by breath-hold either with or without facial cooling. These findings suggest that physiological factors associated with breath-hold, and not facial cooling (i.e., trigeminal nerve stimulation) per se, make the predominant contribution to increases in CBF during diving in humans.

4.2 Introduction

Diving evokes a characteristic pattern of physiological responses when the face is immersed in cold water (Gooden, 1994b, Foster and Sheel, 2005b). It is in part activated by a stimulation of the trigeminal nerve that innervates the areas around the forehead and cheeks, but also modulated by mechanisms such as apnoea (Gooden, 1994b, Lemaitre et al., 2015). Activation of the diving response results in a parasympathetically mediated reduction in HR and an increase in sympathetic nerve activity which causes peripheral vasoconstriction and increases MAP (Fagius and Sundlöf, 1986, Shamsuzzaman et al., 2014, Fisher et al., 2015a, Lemaitre et al., 2015, Schlader et al., 2016, Lapi et al., 2016). It is thought that the diving response serves to preserve blood flow and oxygen delivery to the heart and brain in animals (Butler and Jones, 1997). However, whether this is primarily a result of the trigeminal afferent stimulation or mechanisms associated with apnoea remains incompletely understood in humans.

Regulation of CBF is complex and highly integrated involving multiple mechanisms with the aim of ensuring continuous perfusion of oxygenated blood to the brain (Ainslie and Brassard, 2014a). Several mechanisms likely contribute to the regulation of CBF during the diving response, including neurogenic and hemodynamic factors, neurovascular coupling and changes in blood gases (e.g., P_{aCO2}) (May and Goadsby, 1999, Phillips et al., 2015). Notably, underwater submersion in rats causes a redistribution of blood away from peripheral regions such the thoraco-abdominal region towards the head and thorax, a response not exhibited in rats swimming without head submersion (Ollenberger et al., 1998). This is indicative of a key role for the trigeminal nerves in the control of cerebral perfusion during diving and may be explained by the release of vasorelaxant mediators from activated trigeminal nerve cell bodies that project bipolar cells that synapse on extra-cerebral vessels (e.g., MCA) (May and Goadsby, 1999). In addition, with activation of trigeminal afferents and cutaneous thermoreceptors

during facial cooling, regional cortical sites within the central nervous system are activated and increases local metabolism. These are potentially coupled to increases local blood flow via complex series of cellular events, collectively referred to as neurovascular coupling (Phillips et al., 2015). Activation of the sympathetic nervous system along with changes in hemodynamic factors (e.g., BP, CO) can potentially impact CBF during facial cooling (Fisher et al., 2015a). Moreover, arterial blood gases concentrations can play a critical part in cerebral blood regulation. When the diving response is associated with apnoea, hypercapnia and hypoxia evoke cerebral vasodilation, and hypercapnia has been reported as being more important than blood pressure and sympathetic nerve activity in evoking apnoea-induced increases in cerebral perfusion (Pan et al., 1997, Przybylowski et al., 2003, Bain et al., 2016). However, the effect of facial cooling on CBF in humans, remains incompletely understood.

To the authors' knowledge, there are only two studies that have investigated the effects of facial cooling on intra-cranial perfusion in healthy humans (Brown et al., 2003, Kjeld et al., 2009). Brown et al. (2003) reported a small increase in MCAv during cold face stimulation (9%), while Kjeld et al. (2009) reported that MCAv responses to a breath-hold performed during moderate intensity leg cycling exercise, were augmented when undertaken with concurrent facial immersion. However, the contribution of exercise *per se* to the latter MCAv response is unclear. Additionally, these studies did not consider whether thermoreceptor stimulation may have contributed to the responses, and as potential changes in P_{aCO2} were not controlled the powerful effects of P_{aCO2} on CBF regulation secondary to respiratory changes, cannot be excluded. Finally, an important assumption implicit in the use of MCAv as an index of CBF also limit these investigations. That is, in the absence of MCA diameter measurements, it is assumed that MCAv is representative of MCA blood flow.

The purpose of the present study was to determine the contribution of trigeminal nerve stimulation to the diving response associated changes in CBF, and to address the shortcomings mentioned above. To achieve this, cardiovascular and cerebral vascular responses (i.e., MCAv) to stimulation of trigeminal afferents with facial cooling (0°C) were determined. Facial cooling trials were undertaken under control conditions (i.e., spontaneous respiration and poikilocapnia) (Protocol 1) and with isocapnia ensured (Protocol 2). Also, the cardiovascular and cerebral vascular responses to another thermoreceptor stimulus, namely the cold pressor test, were examined (both Protocol 1 and 2). In addition, facial cooling was performed during a breath-hold, in which CO_2 would naturally accumulate and also without a breath-hold (Protocol 3). Finally, breath-hold trials were performed both with and without facial cooling to further elucidate the role of trigeminal nerve stimulation on CBF (Protocol 4). To circumvent issues surrounding the validity of MCAv being representative of CBF, internal carotid artery volumetric blood flow was also measured (Protocols 3 and 4). This series of experimental trials permitted us to test the hypothesis that the stimulation of trigeminal afferents with facial cooling contributes to the CBF increases during the diving response.

4.3 Methods

4.3.1 Ethical approval

All study protocols were approved by the Health, Safety and Ethics Committee at the University of Birmingham, School of Sport, Exercise and Rehabilitation Sciences (22/03/16MW and ERN_19-0700), and were undertaken according with the Declaration of Helsinki, except for registration in a database. Written informed consent was acquired from each participant before the commencement of the study following the provision of a detailed verbal and written overview of the experimental procedures.

4.3.2 Participants

A total of 37 volunteers completed this study. Participants were healthy and free of any renal, neurological, cardiovascular, respiratory, or metabolic diseases, and were not using prescribed or over-the-counter medications. Participants were requested to refrain from any caffeinated, or alcoholic beverages and not to undertake vigorous exercise, for 24 hr before the experimental sessions. Participants were not trained breath-hold divers.

4.3.3 Experimental measures

All participants rested in a semi-supine position throughout the study. Heart rate was monitored using an ECG and beat-to-beat arterial blood pressure assessed using finger photoplethysmography (Finometer Pro; Finapres Medical Systems, Arnhem, the Netherlands). An automated sphygmomanometer (Tango+; SunTech Medical) was used to verify resting blood pressure measures. A mouthpiece and a nose-clip were worn by participants and $P_{ET}CO_2$, used to estimate P_{aCO2}, was sampled at the mouth and was recorded by a calibrated gas analyser (model ML206, ADInstruments, Dunedin, New Zealand). A 2-MHz pulsed Doppler ultrasound probe (Doppler Box X; Compumedics,

Singen, Germany) was used to simultaneously measure the blood flow velocity of the right MCA. The temporal window, approximately 1 cm above the zygomatic arch, was insonated for the MCA (depth 45-65 mm) (Willie et al., 2011b). Once the signal was stable, the probe was fixed using a modifiable head kit that locked the angle of insonation at the optimum position allowing signal stability. All measurements recorded were converted from analogue to digital data at 1 kHz (Powerlab, 16/30; ADInstruments) and were stored in for offline analysis (LabChart Pro; ADInstruments).

Duplex Doppler ultrasound (Terason T3300, Teratech, Burlington, MA, USA) was used to measure left ICAv and ICAd by a single experimenter (SAS). A 4-15 MHz multi-frequency linear-array transducer was used with a constant insonation of 60° angle relative to the skin. ICA recordings were undertaken at a site 1 to 1.5 cm distal to the carotid bifurcation. For ICA localisation, the brightness mode was used on a longitudinal section to clarify the vessel appearance and assess ICAd. The pulse-wave mode was used to determine ICAv. ICA images were captured and stored as video files for offline analysis using automated edge detection software independently of investigator influence (FMD Studio, Pisa, Italy). All video files were analysed by a single operator (SAS).

4.3.4 Experimental protocols

Protocol 1: Facial cooling and cold pressor test under poikilocapnic conditions

Thirteen healthy individuals (11 males, age: 23 [4], height: 174 [7] cm, weight: 74 [8] kg; mean [SD]) undertook trials of facial cooling and cold pressor test in a random order decided with a coin toss. A >15-min rest period was allowed between the trials to allow for the restoration of the measured variables to baseline.

Facial cooling: Following a 3-min baseline period, an ice pack (0°C) was used to simulate the trigeminal nerve stimulation component of the diving response for 3 min. The ice pack was shaped so that it covered the areas innervated by the ophthalmic

(forehead) and maxillary division (cheeks) of the trigeminal nerve. This was followed by a recovery period of 3 min.

Cold pressor test: Following a 3-min baseline period, participants were instructed to immerse their hand up to their wrist into a bucket containing iced-water (4°C) for 3 min. This was followed by removal of the hand and continuation of the data collection for a further 3-min recovery period.

Protocol 2: Facial cooling and cold pressor test under isocapnic conditions

In protocol 2, eight healthy individuals (8 males, age: 23 ± 6, height: 180 ± 7 cm, weight: 74 ± 6 kg) undertook cold pressor test and facial cooling as described for Protocol 1, however $P_{ET}CO_2$ was maintained at baseline values (i.e., $P_{ET}CO_2$ controlled at +1 mmHg baseline) by the manual supplementation of CO_2 to the inspired air.

Protocol 3: Facial cooling with and without breath-hold

Eight healthy individuals completed protocol 3 (7 males, age: 24 ± 3 years, height: 175 ± 4 cm, weight: 72 ± 8 kg). At an initial familiarisation session, participants practiced exhaling and holding their breath for as long as possible on three occasions. At a subsequent experimental session, the cardiovascular and cerebrovascular effects of facial cooling with breath-hold (FC +BH) and without a breath-hold (FC -BH) were determined. The FC +BH trial was always undertaken first because the FC -BH trial was matched in length to the FC +BH trial. For the FC +BH trial, after a 1-min baseline period, participants were instructed to hold their breath at end of a normal expiration and were asked to hold until they reached their maximum comfortable breath-hold duration (i.e., prior to any straining manoeuvre). At the start of the breath-hold an ice pack (0°C) was placed on the face to simulate the trigeminal nerve stimulation component of the diving reflex for the full length of the breath-hold. Following the release of the breath-hold this

protocol was concluded after a 1-min period of recovery. FC +BH and FC -BH trials were separated by a >15-min rest period to allow the restoration of the measured variables to baseline values. The FC -BH trial consisted of a 1-min baseline period followed by facial cooling for the same duration used in the previous trial. Following the completion of facial cooling, this protocol was concluded after a 1-min period of recovery.

Protocol 4: Breath-hold with and without facial cooling

In protocol 4, eight healthy individuals (8 males, age: 24 ± 3 years, height: 175 ± 4 cm, weight: 72 ± 8 kg) undertook breath-hold both with and without facial cooling. At an initial familiarisation session, participants practiced exhaling and holding their breath for as long as possible on three occasions. At a subsequent experimental session, the cardiovascular and cerebrovascular effects of breath-hold with (BH +FC) and without facial cooling (BH -FC) were determined. Trials were randomised with the order decided by a coin toss. Trials were matched in length with the duration of the second trial being matched to the first trial. Each trial was preceded by a 1-min baseline period, then participants were asked to hold their breath at end of a normal expiration, and to hold this until they reached their maximum comfortable breath-hold duration (i.e., prior to any straining manoeuvre) (first trial) or until requested to return to normal breathing (second trial). Following the release of the breath-hold a 1-min recovery period was conducted. For the BH +FC trial only, at the start of the breath-hold an ice pack (0°C) was placed on the face to simulate the facial cooling component of the diving reflex for the full length of the breath-hold. A recovery period (>15-min) was allowed between the trials to allow for the restoration of the measured variables to baseline.

4.3.5 Data analysis

MAP was calculated as:

Volumetric blood flow was calculated as (Flück et al., 2017b):

ICA and MCA CVCi was calculated as (Flück et al., 2017b):

4.3.6 Statistical analysis

Statistical analysis was performed using SigmaPlot (version 13.0, SYSTAT Software Inc., Chicago, IL, USA). Physiological data were statistically analysed using repeated-measures of ANOVA with significant main effects and interactions examined *post hoc* using Student Newman-Kuels tests. More specifically, to determine the physiological responses to facial cooling and cold pressor test under poikilocapnic conditions (Protocol 1) averages were calculated for baseline (3 min), facial cooling and cold pressor test interventions on a minute-by-minute basis, and recovery (3 min). A two-way repeated-measures ANOVA was used in which the factors were condition (Facial cooling, cold pressor test) and time (baseline, intervention min 1-3, recovery), as well as the interaction between them. Given the known association between changes in $P_{ET}CO_2$ and MCAv, Pearson correlations were used to examine the change from baseline in MCAv and $P_{ET}CO_2$ during the 3rd minute of both facial cooling and cold pressor test. To better understand the $P_{ET}CO_2$-independent influence of facial cooling and cold pressor test on the cerebrovascular response, Protocol 2 was used to determine the physiological responses to facial cooling and cold pressor test under isocapnic conditions. Averages were calculated over the same time points, and the same ANOVA approach used, as described for Protocol 1. In Protocol 3, facial cooling was examined both with and

without a breath-hold, because during diving a breath-hold accompanies facial cooling. ICA_Q was also measured along with MCAv, thus variables were averaged at baseline (1 min), the last 10 cardiac cycles of either facial cooling with (FC +BH) or without (FC -BH) a breath hold, and during recovery (1 min). A two-way repeated-measures ANOVA was used in which the factors were condition (FC +BH, FC -BH) and time (baseline, facial cooling, recovery), as well as the interaction between them. In Protocol 4, physiological responses to a breath-hold were examined when undertaken with and without facial cooling. A two-way repeated-measures ANOVA was used in which the factors were condition (BH +FC, BH -FC) and time (baseline, BH, recovery), as well as the interaction between them. To compare responses across protocols, a one-way ANOVA was used to compare the change in MCAv from baseline for the pokilocapnic facial cooling (Protocol 1), isocapnic facial cooling (Protocol 2), FC -BH (Protocol 3), FC +BH (Protocol 3 and Protocol 4), and a breath-hold alone trial. Data are displayed as mean ± SD, unless otherwise indicated. Differences were considered significant when $P<0.05$.

4.4 Results

4.4.1 Protocol 1: Facial cooling and cold pressor test under poikilocapnic conditions

Cardiovascular and cerebrovascular responses to facial cooling and cold pressor test under poikilocapnic conditions are shown in Table 1. During facial cooling, MCAv, MAP, MCA CVCi and $P_{ET}CO_2$ remained unchanged, while HR was numerically reduced (P=0.13 baseline vs. min 3). During cold pressor test, MCAv remained unchanged, while MAP (P<0.05 vs. baseline at min 2-3, P<0.01) and HR were increased (P<0.05 vs. facial cooling), and MCA CVCi (P<0.05 baseline vs. min 2-3) and $P_{ET}CO_2$ were reduced (P<0.05 baseline vs. min 2-3).

Given that reductions in $P_{ET}CO_2$ are well known to result in cerebral vasoconstriction, the association between changes in $P_{ET}CO_2$ and MCAv and during the facial cooling and cold pressor test conditions was examined. A moderate positive correlation was observed between the change from baseline in $P_{ET}CO_2$ and MCAv measured during the last minute of both facial cooling (r=0.59: P=0.04) and cold pressor test (r=0.64: P=0.03).

4.4.2 Protocol 2: Facial cooling and cold pressor test under isocapnic conditions

Given the observation made in Protocol 1 that facial cooling and cold pressor test mediated changes in $P_{ET}CO_2$ are significantly associated with response in MCAv, trials of facial cooling and cold pressor test were repeated in Protocol 2 under isocapnic conditions, the aim being to unmask the $P_{ET}CO_2$-independent influence of facial cooling and cold pressor test on the cerebrovascular response. Accordingly, the cardiovascular and cerebrovascular responses to facial cooling and cold pressor test performed under isocapnic conditions are shown in Table 2.

During isocapnic facial cooling, MCAv was unchanged (P>0.05), MAP was increased (P<0.05 baseline vs. min 2 and 3), while HR (P<0.05 baseline vs. min 2) and

MCA CVCi ($P<0.05$ baseline vs. min 2 and 3, respectively) decreased. During isocapnic cold pressor test, MCAv ($P<0.05$ baseline vs. min 1 and 2), MAP ($P<0.05$ baseline vs. min 1 and 2), and HR ($P<0.05$ cold pressor test vs. facial cooling) were increased, while MCA CVCi was reduced ($P<0.05$ baseline vs. min 2 and 3).

4.4.3 Protocol 3: Facial cooling with and without breath-hold

To better discern the cerebrovascular consequences of facial cooling, ICA_Q was measured along with MCAv in Protocol 3. In addition, because during diving a breath-hold accompanies facial cooling, in Protocol 3 facial cooling was examined both with (FC +BH) and without (FC -BH) a breath-hold. The apnoea was held for 26 ± 4 s. MCAv and ICA_Q were only increased when facial cooling was accompanied by a breath-hold ($P<0.05$ vs. baseline), while ICA_Q and ICA_v were different between trials ($P<0.05$ FC +BH vs FC -BH) (Table 3). MAP was elevated numerically during FC -BH trial ($P=0.23$ vs baseline), while MAP increased during the FC +BH trial ($P<0.05$ vs. FC -BH). HR was unchanged during the FC –BH trial ($P>0.05$ vs. baseline) but declined in the FC +BH trial ($P=0.01$ vs. baseline). MCA CVCi and ICA CVCi remained unchanged in both trials ($P>0.05$ vs. baseline).

4.4.4 Protocol 4: Breath-hold with and without facial cooling

To further understand the cerebrovascular effects of facial cooling, in Protocol 4 the responses to a breath-hold were determined both with and without facial cooling (Table 4). The apnoea was held for 28 ± 4 s. A breath-hold undertaken either with facial cooling (+FC) or without facial cooling (-FC) increased MCAv, ICA_Q, MAP, MCA CVCi, and ICA_v from baseline ($P<0.05$) with no difference between conditions.

4.4.5 Comparison of MCAv responses in Protocols 1-4

As illustrated in Figure 8, MCAv responses to poikilocapnic facial cooling (Protocol 1), isocapnic facial cooling (Protocol 2), and facial cooling without a breath-hold (FC -BH; Protocol 3) were minimal, and lower than that evoked by facial cooling when accompanied by a breath-hold (FC +BH, Protocol 3; BH +FC, Protocol 4), and a breath-hold undertaken in the absence of facial cooling (BH -FC, Protocol 4) ($P<0.05$).

4.5 Discussion

We sought to determine the contribution of facial cooling (i.e., trigeminal nerve stimulation) to changes in CBF during simulateddiving response. In order to examine the influence of potentially modulatory factors associated with diving (e.g. apnoea, changes in $P_{ET}CO_2$, thermoreceptor stimulation), we implemented different protocols to isolate these variables. The major novel findings are that in young healthy individuals, 1) MCAv did not increase during facial cooling or cold pressor test under poikilocapnic conditions (Protocol 1), 2) under isocapnic conditions MCAv did increase during thermoreceptor stimulation with cold pressor test, but not during facial cooling (Protocol 2), 3) both MCAv and ICA_Q were increased when facial cooling was combined with a breath-hold, but not when facial cooling was performed with spontaneous breathing (Protocol 3), and 4) similar increases in MCAv and ICA_Q were observed during a breath-hold when performed either alone or in combination with facial cooling (Protocol 4). Collectively, our findings suggest that physiological factors associated with breath holding (e.g., pressor response, CO_2 accumulation) make the predominant contribution to diving response mediated-increases in CBF in humans.

4.5.1 Cerebral perfusion during facial cooling

During diving, a multitude of mechanisms can contribute to the regulation of CBF, including neurogenic and hemodynamic factors, neurovascular coupling, and changes in blood gases (Bain et al., 2018). The findings of Ollenberger *et al.* (1998) in rats indicate that trigeminal nerve stimulation can play a role in regulation of CBF. They observed a redistribution of blood flow away from the periphery to the brain during swimming, but only if the head was submerged (i.e., with trigeminal nerve stimulation / facial cooling) and not when the head remained above water. As reviewed by Lapi et al. (2016), stimulation of the trigeminal cardiac reflex, involving sensory ending of the trigeminal

nerve, evokes a (partly) nitric oxide-mediated cerebrovascular vasodilatation in rabbits. Interestingly, the direct stimulation of the trigeminal root has been reported not to cause dilatation of the pial arteries in cats and monkeys, whereas stimulation of either the facial nerve root or the vagus nerve does evoke cerebral vasodilatation (Cobb and Finesinger, 1932). How trigeminal nerve stimulation can regulate cerebral flow in humans remains less well studied. Brown et al. (2003) reported that trigeminal afferent activation increases MCAv (by 9%) when evoked with the cold face test under poikilocapnic conditions in healthy individuals. In contrast, in our study we found no increases in MCAv during facial cooling under poikilocapnic conditions. A potential explanation for these contradictory findings may be differences in $P_{ET}CO_2$, well recognised as a powerful dilator of the cerebral vasculature. In the present study we observed a moderate positive relationship between $P_{ET}CO_2$ and MCAv ($r=0.59$; $p=0.04$) during facial cooling (Protocol 1), and although overall under poikilocapnic conditions no differences from baseline in $P_{ET}CO_2$ were noted, a significant degree of between-subject variability was observed (i.e., responses ranged from +3.5 to -5.5 mmHg) likely a result of a heterogeneous ventilatory response. To further examine the influence of $P_{ET}CO_2$ on cerebral perfusion during facial cooling, we repeated the facial cooling under isocapnic conditions (Protocol 2). However, even under consistent isocapnic conditions no changes in MCAv were observed during trigeminal nerve stimulation by facial cooling.

Another possible explanation for previous reports of an increase in CBF during the cold face test is activation of thermoreceptors. Signals from cutaneous thermoreceptor afferents are integrated within the central nervous system (e.g., within hypothalamic and medullary regions) and lead to activation of cortical sites (Di Piero et al., 1994), which may increase local perfusion by neurovascular coupling. Under poikilocapnic conditions MCAv remained unchanged (Protocol 1), likely as a result of a hyperventilation induced fall in $P_{ET}CO_2$ decreased secondary to hyperventilation, whereas, under isocapnic

conditions (i.e., $P_{ET}CO_2$ controlled at +1 mmHg baseline) MCAv increased during cold pressor test (Protocol 2). Such findings agree with those of Tymko et al. (2017) and highlight the importance of nociceptor mediated alterations in ventilation and thus $P_{ET}CO_2$, on blunting the cerebral perfusion response to the cold pressor test. A striking example of the balance between the effects of ventilation (and thus $P_{ET}CO_2$) on cerebral perfusion in the cold has been provided by Datta and Tipton (2006). They reported that reductions in MCAv observed in hyperventilating participants immersed up to the neck in cold water (12°C) were less marked than when reductions in $P_{ET}CO_2$ were matched in control experiments undertaken in either thermoneutral water (35°C) or room air (24°C). Such findings suggest that under conditions of more extreme cold stress, the vasoconstrictor effects of hyperventilation on the cerebral vessels may at least be partially offset by other factors, such as neurovascular coupling and MAP.

4.5.2 CBF and apnoea

Several studies show that an apnoea robustly increases cerebral perfusion (Pan et al., 1997, Przybylowski et al., 2003, Kjeld et al., 2009, Bain et al., 2016). For example, Przybylowski et al. (2003) reported dramatic increases in MCAv (by 42 %) during a short 20 s apnoea, while Kjeld et al. (2009) have shown that MCAv increased from a baseline of 37 ± 23 cm s^{-1} to 103 ± 15 cm s^{-1} during a maximal apnoea. In the present study we observed that when facial cooling was undertaken in combination with an apnoea, MCAv increased by 23 % (Protocol 3). In addition, we observed that ICA_Q also increased during facial cooling with a concurrent apnoea (by 26 %) (Protocol 3). In fact, MCAv and ICA_Q only increased when facial cooling was accompanied by an apnoea and did not increase during a cold face stimulation with uncontrolled breathing (poikilocapnic conditions). Moreover, when an apnoea was performed either alone or in combination with facial cooling similar increases in MCAv and ICA_Q were observed (Protocol 4). Such findings

suggest that physiological factors associated with breath holding make the predominant contribution to diving response mediated-increases in CBF in humans.

The CBF responses to an apnoea may be attributed to a number of factors, which include metabolic, neurogenic and hemodynamic factors, neurovascular coupling, and changes in blood gases (Bain, *et al.* 2018). Increases in MAP were noted during breath-holding and these may be partially responsible for the increase in cerebral perfusion during apnoea. Indeed, Przybylowski et al. (2003) demonstrated that ganglionic blockade with trimethaphan eliminated the increase in MAP during a 20 s apnoea, and the MCAv response was significantly blunted (62% of hyperaemic response without ganglionic blockade). Thus, in addition to increases in P_{aCO2} alteration in MAP likely makes a contribution to the increase in cerebral perfusion noted during apnoea.

4.5.3 Methodological considerations

The findings of the present study should be considered in light of the following:

1) Study population: Care should be taken when generalising the findings of the current study to a population beyond the young healthy group studied. The sympathetic and blood pressure responses to facial cooling are reportedly modified in some disease states (Prodel et al., 2017) and therefore it is quite likely that the cerebrovascular responses are altered too. In rat models of traumatic brain injury, trigeminal nerve stimulation was reported to increase CBF and reduce the development of secondary injury symptoms, such as oedema, blood-brain barrier disruption, and lesion volumes (Chiluwal et al., 2017). In humans, therapeutic use of trigeminal nerve stimulation using external electrical stimulation has been examined in neurologic, cardiovascular and psychiatric conditions such as, epilepsy, depression, attention deficit hyperactivity disorder and post-traumatic stress disorder (Grahame and Hann, 1978, Cook et al., 2015, Borsody and Sacristan, 2016, Cook et al., 2016). This approach resulted in reduced CBF in regions

attributed with initiation and propagation of seizures, whereas CBF was enhanced in other cortex regions where metabolism is low because of depression (Cook et al., 2016). In addition, elegant work by Schaller (2005) has documented that stimulation of the trigeminal nerve during craniofacial surgery in anaesthetised patients can evoke a trigemino-cardiac reflex, with potential implications for CBF (Schaller, 2004). Comparisons of these clinical studies to the present work are difficult due to differences in the mode of trigeminal afferent activation and the presence of pathology. We acknowledge that it would have been ideal for all participants to take part in each experimental session, however due to logistical reasons this was not possible. Finally, we acknowledge that the majority of participants in the present study were men and we have not been able to include a comparison of sex-differences in the present analysis. Whether there are sex-differences in the CBF responses to facial cooling requires further study.

2) $P_{ET}CO_2$: P_{aCO2} was not directly measured and instead was indexed using $P_{ET}CO_2$. Young et al. (1991) identified similar hypercapnic cerebrovascular reactivity when either P_{aCO2} or the surrogate $P_{ET}CO_2$ was used. However, this relationship was only consistent while participants maintained a fixed supine position. Therefore, in our study subjects remained in a comfortable supine position throughout the data collection period.

3) Assessment of CBF: Cerebral perfusion was principally assessed using transcranial Doppler ultrasound measures of MCAv, which, in the absence of a direct measurement of MCA diameter, can only be assumed to reflect MCA blood flow. However, studies were also included where ICA measures of blood flow were derived from simultaneous duplex Doppler ultrasound measurements of ICAd and ICAv (Protocols 3 and 4, but not Protocols 1 and 2). Of note, the facial cooling and facial cooling with concomitant apnoea evoked very similar responses in ICA_Q to that exhibited in MCAv. However, it remains to be determined whether the MCAv and ICA_Q responses described are representative of perfusion changes in other major cerebral arteries (e.g.,

vertebral and posterior cerebral arteries), which given the known regional differences in cerebral vascular regulation may not be the case.

4) PaO_2 levels: Lack of PaO_2 assessments and the shortness of the BH (~30 seconds) performed during this study might not be sufficient to elicit cerebral vasodilation. Indeed, according to animal research (mostly in rabbits) (Lapi et al., 2016) cerebral vasodilation is reached when O2-sensitive neurons in the rostral ventrolateral medulla are activated. However, perfusion was similar whether the BH was performed with or without FC arguing against the role for TGS in CBF control.

5) Unilateral assessment of blood flow: Cerebral perfusion was principally assessed using the right side of the neck for MCA and ICA. Recent studies have shown individual measurement error of 37% when comparing bilateral neck arteries flow (ICA and VA) (Friend et al., 2021). Therefore, to minimise measruemtn error, future studies should consider assessing the vessel with the larger resting blood flow (Friend et al., 2021).

4.5.4 Summary

The findings of the present study indicate that factors associated with breath-holding (e.g., arterial CO_2 accumulation, pressor response), rather than stimulation of cutaneous trigeminal afferents, makes the predominate contribution to diving response mediated increases in CBF in humans.

Table 1. **Cardiorespiratory and cerebrovascular responses to facial cooling (FC) and cold pressor test (CPT) under poikilocapnic conditions (Protocol 1).**

		Baseline	Intervention (min)			Recovery	P-Value		
			1	2	3		Condition	Time	Int.
MCAv (cm s^{-1})	FC	52 ± 7	53 ± 8	52 ± 9	52 ± 8	52 ± 7	0.61	0.59	0.7
	CPT	53 ± 16	55 ± 15	52 ± 13	52 ± 13	53 ± 15			
MAP (mmHg)	FC	86 ± 7	86 ± 9	88 ± 8	89 ± 10	86 ± 8	0.16	**<0.01**	**<0.01**
	CPT	85 ± 5	88 ± 11	101 ± 17*†‡	98 ± 10*†‡	87 ± 7			
HR (b min^{-1})	FC	68 ± 12	66 ± 11	66 ± 12	64 ± 12	69 ± 12	**0.03**	0.21	**<0.01**
	CPT	69 ± 10	73 ± 9*†	71 ± 9†	69 ± 11†‡	68 ± 11			
MCA CVCi (cm s^{-1}/mmHg)	FC	0.60 ± 0.14	0.60 ± 0.13	0.59 ± 0.12	0.58 ± 0.11	0.61 ± 0.13	0.63	**<0.01**	**<0.01**
	CPT	0.63 ± 0.18	0.62 ± 0.10	0.52 ± 0.10*†‡	0.54 ± 0.12*‡	0.61 ± 0.17			
$P_{ET}CO_2$ (mmHg)	FC	39 ± 4	40 ± 4	40 ± 5	40 ± 5	39 ± 4	**<0.01**	0.08	**<0.01**
	CPT	40 ± 4	39 ± 4†	37 ± 5*†‡	38 ± 4*†‡	39 ± 3			

MCAv, middle cerebral artery mean flow velocity; MAP, mean arterial pressure; HR, heart rate; CVCi, cerebrovascular conductance; $P_{ET}CO_2$, partial pressure of end-tidal CO_2; Int, interaction. Values are mean ± SD. P values represent two-way repeated ANOVA results. * P<0.05 vs. Baseline, † P<0.05 vs. FC, ‡ P<0.05 vs. Min 1.

Table 2. Cardiorespiratory and cerebrovascular responses to facial cooling (FC) and cold pressor test (CPT) under isocapnic conditions (Protocol 2).

		Baseline	Intervention (min)			Recovery	P-Value		
			1	2	3		Condition	Time	Int.
MCAv (cm s^{-1})	FC	57 ± 12	55 ± 13	54 ± 14	56 ± 15	56 ± 12	0.06	0.10	**<0.01**
	CPT	58 ± 8	63 ± 13*†	65 ± 11*†	65 ± 13*†	60 ± 10			
MAP (mmHg)	FC	87 ± 6	91 ± 12	102 ± 8*†‡	100 ± 8*†‡	89 ± 5	**0.00**	**<0.01**	**0.02**
	CPT	90 ± 6	97 ± 13*†	112 ± 13*†‡	111 ± 10*†‡	96 ± 7			
HR (b min^{-1})	FC	65 ± 10	65 ± 11	58 ± 9*‡	60 ± 11	64 ± 12	**0.04**	**<0.01**	**<0.01**
	CPT	67 ± 11	77 ± 14*†	70 ± 12†	66 ± 9	61 ± 6†			
MCA CVCi (cm s^{-1}/mmHg)	FC	0.80 ± 0.23	0.79 ± 0.30	0.64 ± 0.19	0.67 ± 0.21	0.77 ± 0.21	0.58	**<0.01**	0.54
	CPT	0.75 ± 0.14	0.76 ± 0.21	0.66 ± 0.12*	0.66 ± 0.12*	0.71 ± 0.12			
$P_{ET}CO_2$ (mmHg)	FC	41 ± 5	41 ± 5	40 ± 5	41 ± 5	41 ± 5	0.89	0.37	0.37
	CPT	41 ± 4	42 ± 5	41 ± 5	40 ± 6	41 ± 4			

MCAv, middle cerebral artery mean flow velocity; MAP, mean arterial pressure; HR, heart rate; CVCi, cerebrovascular conductance; $P_{ET}CO_2$, partial pressure of end-tidal CO_2; Int, interaction. Values are mean ± SD. P values represent two-way repeated ANOVA results. * P<0.05 versus Baseline, † P<0.05 vs. FC, ‡ P<0.05 vs. Min 1.

Table 3. Cardiorespiratory and cerebrovascular responses to facial cooling (FC) undertaken without (-BH) and with (+BH) a breath-hold (Protocol 3).

		Baseline	FC	Recovery	P-Value		
					Condition	Time	Int.
MCAv (cm s^{-1})	-BH	66 ± 21	66 ± 21	66 ± 23	0.51	**<0.01**	**<0.01**
	+BH	67 ± 26	82 ± 24*	66 ± 25			
ICAQ (ml min^{-1})	-BH	182 ± 68	177 ± 70	181 ± 74	0.12	**<0.01**	**<0.01**
	+BH	185 ± 72	232 ± 95*†	180 ± 70			
MAP (mmHg)	-BH	87 ± 5	91 ± 6	88 ± 6	0.21	**<0.01**	**<0.01**
	+BH	88 ± 5	101 ± 11*†	86 ± 5			
HR (b min^{-1})	-BH	67 ± 10	66 ± 10	66 ± 9	0.76	0.07	0.13
	+BH	72 ± 5	65 ± 11	66 ± 5			
MCA CVCi (cm s^{-1}/mmHg)	-BH	0.70 ± 0.23	0.69 ± 0.22	0.69 ± 0.26	0.71	0.78	0.08
	+BH	0.77 ± 0.30	0.81 ± 0.22	0.77 ± 0.31			
ICAv (cm s^{-1})	-BH	34 ± 10	32 ± 11	33 ± 12	0.37	0.06	**<0.01**

	+BH	35 ± 10	41 ± 14*†	34 ± 10			
ICAd (cm)	-BH	0.50 ± 0.08	0.50 ± 0.07	0.50 ± 0.08	0.46	0.16	0.2
	+BH	0.47 ± 0.07	0.48 ± 0.08	0.47 ± 0.08			
ICA CVCi (ml min^{-1}/mmHg)	-BH	2.1 ± 0.8	2.3 ± 0.8	2.1 ± 0.9	0.35	0.87	0.12
	+BH	2.1 ± 0.8	2.0 ± 1.0	2.1 ± 0.8			

MCAv, middle cerebral artery mean flow velocity; ICA_Q, internal carotid artery flow; MAP, mean arterial pressure; HR, heart rate; CVCi, cerebrovascular conductance; ICA_v, internal carotid artery velocity; ICA_d, internal carotid artery diameter; -BH, facial cooling without breath-hold; +BH, facial cooling with breath-hold; Int, interaction. Values are mean ± SD. P values represent two-way repeated ANOVA results. * $P<0.05$ vs. Baseline, † $P<0.05$ vs. -BH.

Table 4 Cardiovascular and cerebrovascular responses to breath-hold undertaken without (-FC) and with (+FC) facial cooling (Protocol 4).

		Baseline	Breath-hold	Recovery	P-Value		
					Condition	Time	Int.
MCAv (cm s^{-1})	-FC	47 ± 7	59 ± 11	47 ± 8	0.62	**<0.01**	0.94
	+FC	46 ± 7	57 ± 14	47 ± 8			
ICA_Q (ml min^{-1})	-FC	190 ± 107	236 ± 150	203 ± 139	0.48	**<0.01**	0.29
	+FC	206 ± 60	227 ± 89	187 ± 70			
MAP (mmHg)	-FC	90 ± 8	96 ± 9	90 ± 9	0.36	**0.03**	0.37
	+FC	90 ± 9	103 ± 20	98 ± 20			
HR (b min^{-1})	-FC	73 ± 14	69 ± 16	72 ± 14	0.2	**<0.01**	0.36
	+FC	76 ± 13	71 ± 16	73 ± 14			
MCA CVCi (cm s^{-1}/mmHg)	-FC	0.53 ± 0.10	0.62 ± 0.14	0.53 ± 0.10	0.48	**<0.01**	0.53
	+FC	0.52 ± 0.10	0.59 ± 0.19	0.50 ± 0.15			
ICAv (cm s^{-1})	-FC	24 ± 9	28 ± 11	24 ± 7	0.48	**0.01**	0.53
	+FC	28 ± 6	31 ± 11	26 ± 8			

ICAd (cm)	-FC	0.53 ± 0.08	0.52 ± 0.08	0.52 ± 0.09	0.63	0.71	0.61
	+FC	0.53 ± 0.08	0.53 ± 0.09	0.52 ± 0.08			
ICA CVCi (ml min^{-1}/mmHg)	-FC	2.2 ± 1.2	2.6 ± 1.8	2.3 ± 1.6	0.83	0.11	0.42
	+FC	2.3 ± 0.8	2.3 ± 1.1	2.1 ± 0.9			

MCAv, middle cerebral artery mean flow velocity; ICA_Q, internal carotid artery flow; MAP, mean arterial pressure; HR, heart rate; CVCi, cerebrovascular conductance; ICA_v, internal carotid artery velocity; ICA_d, internal carotid artery diameter; -FC, breath-hold without facial cooling; +FC, breath-hold with facial cooling; Int, interaction. Values are mean ± SD. P values represent two-way repeated ANOVA results. * $P<0.05$ versus Baseline.

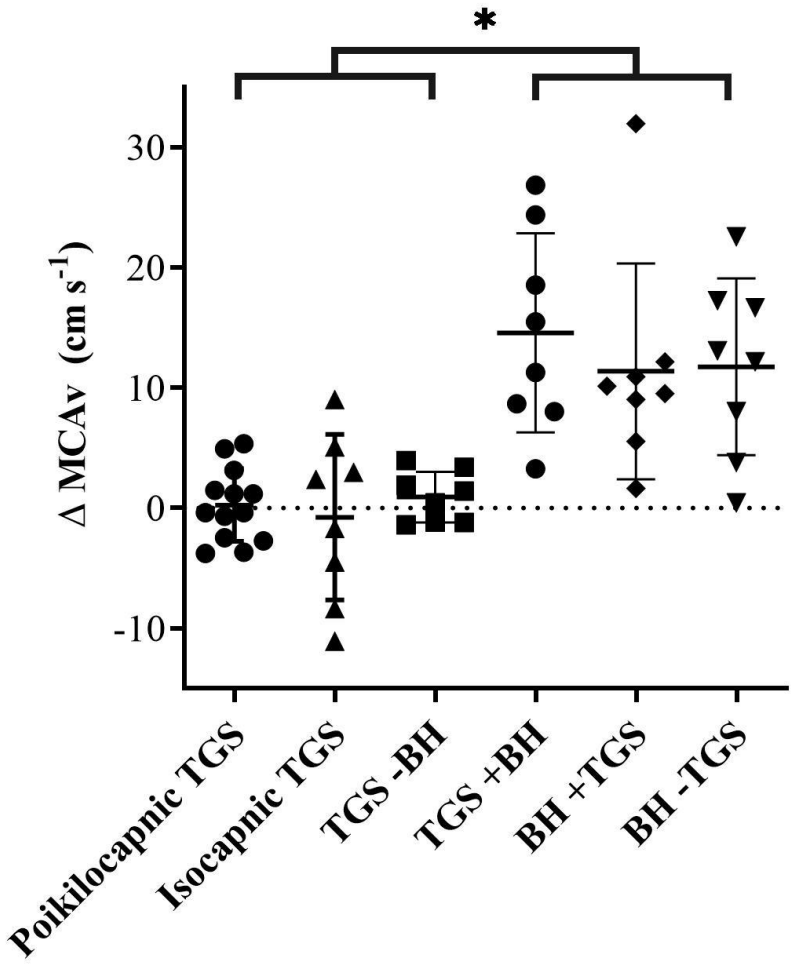


Figure 8 Comparison of the MCAv responses evoked during combinations of facial cooling and breath-hold. MCAv responses to poikilocapnic facial cooling (Protocol 1), isocapnic facial cooling (Protocol 2), and facial cooling without a breath-hold (FC -BH; Protocol 3) were minimal, and significantly attenuated in comparison to facial cooling with a breath-hold (FC +BH, Protocol 3; BH +FC, Protocol 4), and a breath-hold undertaken in the absence of facial cooling (BH –FC, Protocol 4). * $P<0.05$, FC +BH, BH +FC, BH –FC conditions were all significantly different from Poikilocapnic FC, Isocapnic FC and FC –BH conditions. Horizontal bars show mean and SD

CHAPTER 5: MIDDLE CEREBRAL ARTERY VELOCITY RESPONSE TO ACUTE NORMOBARIC HYPOXIA OCCURS INDEPENDENTLY OF CHANGES IN VENTILATION IN HUMANS

A version of this manuscript has been published in Experimental Physiology

5.1 Abstract

Hypoxia induces ventilatory, cardiovascular and cerebrovascular adjustments to defend against reductions in systemic oxygen delivery. We aimed to determine whether the ventilatory response to moderate acute hypoxia increases cerebral perfusion independently of changes in arterial oxygenation. Eleven young healthy individuals were exposed to four 15-minute experimental conditions; 1) normoxia [partial pressure of end-tidal oxygen; $P_{ET}O_2$ = 100 mmHg], 2) hypoxia [$P_{ET}O_2$ = 50 mmHg], 3) normoxia with breathing volitionally matched to levels observed during hypoxia [hyperpnoea; $P_{ET}O_2$ = 100 mmHg], and 4) hypoxia [$P_{ET}O_2$ = 50 mmHg] RF and tidal volume were volitionally matched to levels observed during normoxia (i.e., restricted breathing [RB]). Isocapnia was maintained in all conditions. MCAv assessed by transcranial Doppler ultrasound), was increased during hypoxia (58 ± 12 cm s^{-1}, P=0.04) and hypoxia + RB (61 ± 14 cm s^{-1}, P<0.001) compared to normoxia (55 ± 11 cm s^{-1}), while it was unchanged during hyperpnoea (52 ± 13 cm s^{-1}, P=0.08). MCAv was not different between hypoxia and hypoxia + RB (P>0.05). These findings suggest that the hypoxic ventilatory response does not increase cerebral perfusion, indexed using MCAv, during moderate isocapnic acute hypoxia beyond that elicited by reduced oxygen saturation.

5.2 Introduction

Hypoxia induces ventilatory, cardiovascular and cerebrovascular adjustments to defend against reductions in convective oxygen delivery to organs such as the heart, brain, and kidneys (Marshall, 1994, Guyenet, 2000, Guyenet, 2014b). Although the cardiorespiratory responses to acute hypoxia have been well described, partitioning the contribution of primary effects and secondary consequences is challenging, particularly in humans.

Isocapnic hypoxia increases internal carotid and vertebral artery blood flows (Ogoh et al., 2013), along with MCAv (Willie et al., 2012). It is possible that, in addition to the local vasodilatory effect of hypoxaemia (Kety and Schmidt, 1948, Rocha et al., 2020), the increase in ventilation contributes to the cerebral hyperaemic responses to isocapnic hypoxia. Indeed, the breathing changes that occur in hypoxia lead to changes in ventilation efficiency, blood oxygenation, systemic and pulmonary pressure (Bilo et al., 2012); all with the potential to increase cerebral blood flow (Willie et al., 2014). Furthermore, during isocapnic hypoxia the activation of brain regions accompanying heightened respiratory muscle activity and sensations of "air hunger" may enhance brain blood flow. Indeed, descending inputs from sensorimotor higher brain centres (e.g., primary motor cortex, premotor area, supplementary area, insular cortex, anterior cingulate, cerebellum and amygdala) can influence the neural network located within the brainstem that controls respiratory rhythm and pattern (Colebatch et al., 1991, Guz, 1997, Pattinson et al., 2009a, Pattinson et al., 2009b, Smith et al., 2013). Studies employing a variety of experimental manipulations (e.g., hypercapnia, mechanical ventilation, resistive loads) have identified the involvement of a complex network of brain regions in the sensation of dyspnoea (Evans et al., 2002, von Leupoldt and Dahme, 2005). However, when Ogoh et al. (2014) asked participants to wilfully restrain chemoreflex mediated increases in ventilation during pokilocapnic hypoxia, rather than being attenuated,

internal carotid artery blood flow and MCAv were augmented relative to pokilocapnic hypoxia with unrestricted breathing. However, the $P_{ET}CO_2$ was also higher during the restricted breathing condition potentially confounding the results. It thus remains unclear whether ventilation independently modulates the cerebrovascular response to acute hypoxia.

Herein, we sought to isolate the contribution of hypoxia, from the possible secondary effects of increased ventilation, on MCAv during acute normobaric isocapnic hypoxia. Four experimental conditions were conducted in which participants, breathed spontaneously in normoxia [Normoxia]; breathed spontaneously in hypoxia [Hypoxia]; were exposed to normoxia with ventilation matched to that observed during Hypoxia [Hyperpnoea]; and were exposed to hypoxia with ventilation matched to that observed during normoxia [Hypoxia with restricted breathing (+RB)]. Isocapnia was ensured throughout to avoid potential confounding effects of changes in $P_{ET}CO_2$. This experimental approach enabled us to test the hypothesis that enhanced ventilation independently increases MCAv during acute isocapnic hypoxia.

5.3 Methods

5.3.1 Ethical approval

The Science, Technology, Engineering and Mathematics Ethical Review Committee at the University of Birmingham (ERN_18-2066) approved the study protocol, which was performed according to the Declaration of Helsinki, except for registration in a database. Before starting the study, written informed consent was obtained from each participant after they had been provided with a detailed verbal and written overview of the experimental procedures.

5.3.2 Participants

A total of 11 male volunteers (23 ± 3 years, 173 ± 7 cm, 73 ± 9 kg: mean ± SD) completed this study. Prior to inclusion they completed a screening (Medical Health Questionnaire) to ensure that they were healthy and not taking any prescription or over-the-counter medications. Instructions were provided to participants to refrain from alcohol, caffeine and heavy exercise for >24 hr before the study.

5.3.3 Experimental protocol

At an initial familiarisation session, participants, breathed through a mouthpiece for a 10-minute period to establish normal breathing values. They were then familiarised to the Normoxia, Hypoxia, Hyperpnoea, and Hypoxia + RB conditions (described below) for 15 minutes each.

At the start of the experimental session, participants again breathed through the mouthpiece for 10 min in order to establish normal values for $P_{ET}CO_2$ and partial-pressure of end-tidal oxygen ($P_{ET}O_2$). The four experimental conditions were then undertaken with $P_{ET}CO_2$ and $P_{ET}O_2$ controlled using a dynamic end-tidal forcing system to manipulate inspired (humidified) gases on a breath-by-breath basis (Robbins et al., 1982, Prodel et

al., 2016a). In all conditions, $P_{ET}CO_2$ was held at baseline +1 mmHg. Conditions were; 1) Normoxia: participants breathed spontaneously at a normal rate and depth while inspiring a gas that controlled $P_{ET}O_2$ at baseline levels to ensure there were no effects of isocapnia on $P_{ET}O_2$. 2) Hypoxia: participants breathed spontaneously while $P_{ET}O_2$ was clamped at 50 mmHg. 3) Hyperpnoea: participants were instructed to increase their respiratory rate and depth to target values observed during Hypoxia, while $P_{ET}O_2$ was maintained at baseline levels. 4) Hypoxia + RB: participants were instructed to maintain their RF and TV at values observed during Normoxia, while $P_{ET}O_2$ was clamped at 50 mmHg.

A 10-minute recovery period was undertaken between conditions. Conditions 1 and 2 were always performed before conditions 3 and 4, since the target RF and TV in conditions 3 and 4 were based on values obtained during conditions 1 and 2. However, a coin toss was used to randomize the order of conditions 1 and 2 (Normoxia and Hypoxia), and conditions 3 and 4 (Hyperpnoea and Hypoxia + RB), respectively. RF was guided using a metronome, while TV was guided using an oscilloscope. During conditions 1 and 2, participants were able to view a screen showing an innocuous documentary to shift their attention away from breathing.

5.3.4 Experimental measures

All participants rested on a chair in an upright position throughout the study. HR was monitored by electrocardiography (lead II, ECG) and beat-to-beat arterial BP assessed using finger photoplethysmography (Finometer Pro; Finapres Medical Systems, Arnhem, the Netherlands). SV was derived from the finger BP waveform using the Modelflow technique (FMS, Amsterdam, the Netherlands) (Bogert and van Lieshout, 2005). An automated sphygmomanometer (Tango+; SunTech Medical) was used to verify resting blood pressure measures. A mouthpiece and a nose-clip were worn by

participants and the partial pressures of $P_{ET}CO_2$ and $P_{ET}O_2$ determined using a rapid response gas analysers (Moxus Modular; AEI Technologies, Pittsburg, PA). VE, TV and RF were determined using a turbine volume transducer (VMM400; Interface Associates, Aliso Viejo, CA). Finger pulse oximetry was used to establish O_2 saturation (S_pO_2) (Magna Medical 1400 Multi Parameter Monitor, Datex-Ohmeda Medical Equipment, USA). A 2-MHz pulsed Doppler ultrasound probe (Doppler Box X; Compumedics, Singen, Germany) was used to measure the right MCAv through the temporal window (Willie et al. 2011). The probe was fixed using a modifiable head kit that locked the angle of insonation at the optimum position to ensure signal stability, and once the signal was acquired no further adjustments were made during the protocol.

5.3.5 Data analysis

All measurements recorded were converted from analogue to digital data at 1 kHz (Powerlab, 16/30; ADInstruments) and stored for offline analysis (LabChart Pro; ADInstruments). Respiratory data were extracted on a breath-by-breath basis, while cardiovascular and cerebrovascular data were extracted on a beat by beat basis. The last 5 minutes of each condition were used for analysis and averaged for data representation.

MAP was calculated as:

CO was calculated as and TPR calculated as . CVCi was calculated as (Flück et al., 2017b).

5.3.6 Statistical analysis

A two-way repeated measures ANOVA was used to examine the main effects of oxygenation (hypoxia, normoxia), breathing (spontaneous, controlled) and their interaction (oxygenation x breathing). Significant interactions were explored *post hoc* using Student Newman-Keuls tests. Statistical analysis was performed using SigmaPlot (version 14.0, SYSTAT Software Inc., Chicago, IL, USA). Data are displayed as mean ± SD, unless otherwise indicated. Differences were considered significant if $P<0.05$.

5.4 Results

$P_{ET}O_2$, $P_{ET}CO_2$, RF, VE and TV were successfully controlled to the target values (Figure 9). By design, $P_{ET}O_2$ was matched between Normoxia (101 ± 2 mmHg) and Hyperpnoea (100 ± 0.1 mmHg; P=0.92) as well as between Hypoxia (53 ± 0.1 mmHg) and Hypoxia + RB (52 ± 0.3 mmHg; P=0.84). SpO_2 was lower during hypoxic conditions (Hypoxia 87 ± 2%; Hypoxia + RB 85 ± 1%) than normoxic conditions (Normoxia 98 ± 1%; Hyperpnoea 98 ± 1%; P<0.001), but no main effect of breathing (spontaneous vs. controlled; P=0.407) or interaction (breathing x oxygenation; P=0.598) was observed. $P_{ET}CO_2$ was not different between all four conditions (38.7, 39.0, 38.8 and 38.9 mmHg for Normoxia, Hypoxia, hyperpnoea and Hypoxia + RB, respectively; P=0.34). As expected, TV and VE were elevated in Hypoxia compared to Normoxia (P=0.01 and P<0.001, respectively), whereas, by design, there were no differences in TV, RF and VE between the Normoxia and Hypoxia + RB, and between the Hypoxia and Hyperpnoea (all P>0.05).

Compared to Normoxia, MCAv was increased during Hypoxia (P=0.042), unchanged during Hyperpnoea (P=0.08) and increased during Hypoxia + RB (P<0.001) (Figure 10). MCAv was not different between Hypoxia and Hypoxia + RB (P=0.11). CVCi was lower (P=0.026; Figure 11), and MAP was higher (P=0.02; Figure 12), while CO (P=0.15) and HR (P=0.90) were unchanged, during the controlled breathing conditions (Hyperpnoea, Hypoxia + RB) compared to the spontaneous breathing conditions (Normoxia, Hypoxia). CVCi (P=0.002), CO (P=0.002) and HR (P<0.001) were higher during the hypoxic conditions (Hypoxia, Hypoxia + RB) than the normoxic conditions (Normoxia, Hyperpnoea).

5.5 Discussion

We sought to determine the contribution of the increased ventilation to cerebrovascular responses to acute normobaric isocapnic hypoxia in humans. Our major novel finding is that MCAv was increased to the same extent by Hypoxia and Hypoxia + RB, and not increased by Hyperpnoea. These findings indicate that increases in ventilation do not independently influence the cerebrovascular response to acute normobaric hypoxia in humans.

CBF increases when $P_{ET}O_2$ falls below ~58 mmHg and/or SpO_2 falls below ~90% (Gupta et al., 1997a). This response may be governed by local vasodilatory mechanisms (e.g., factors associated with the endothelium such as adenosine, nitric oxide, and prostaglandins (Ainslie and Ogoh, 2010, Rocha et al., 2020)). However, as a potential contribution from the secondary effects of an increase in ventilation might be possible, we tested the hypothesis that enhanced ventilation independently increases MCAv during acute isocapnic hypoxia. Contrary to this hypothesis, MCAv did not increase during Hyperpnoea, and the increases in MCAv during Hypoxia and Hypoxia + RB were similar. Interestingly, when participants were asked to wilfully restrain increases in ventilation during poikilocapnic hypoxia, internal carotid artery and MCAv increased to a greater extent than during poikilocapnic hypoxia with unrestricted breathing (Ogoh et al., 2014). However, $P_{ET}CO_2$ was higher when ventilation was wilfully restricted, which could have explained the increased cerebral blood flow; therefore, to discern the effects of arterial CO_2 and ventilation, we matched ventilation during hypoxia to normoxia while controlling $P_{ET}CO_2$.

We observed that Hypoxia and Hypoxia + RB evoked similar increases in MCAv. Previously, Bilo et al. (2012) demonstrated that 15 min of breathing at a reduced rate of 6 breaths per minute while at high altitude (4559-5400m) enhanced SpO_2 (+6-9%) and reduced pulmonary artery pressure (-4 mmHg). Such changes in systemic oxygenation

and central hemodynamics might be expected to modify cerebral blood flow, although this was not measured by Bilo et al. (2012). In contrast to Bilo et al. (2012), in our Hypoxia and Hypoxia + RB conditions $P_{ET}O_2$ and SpO_2 were experimentally matched, allowing us more precisely evaluate the distinct influence of breathing pattern on MCAv. Similar to MCAv, CVCi was increased in Hypoxia and Hypoxia + RB relative to the normoxic conditions (Normoxia, Hyperpnoea), suggesting that these hemodynamic changes were not secondary to changes in MAP. However, CVCi was lower and MAP was elevated during the breathing manipulation trials (Hyperpnoea and Hypoxia + RB vs. Normoxia and Hypoxia). A main effect of hypoxia on CO was observed (i.e., CO elevated in the Hypoxia and Hypoxia + RB trials relative to the Normoxia and Hyperpnoea trials) and a contribution to the elevated MCAv in these conditions is possible.

Dyspnoea arises from the activation of multiple sensory afferent mechanisms (e.g., pulmonary vagal afferents, respiratory muscle mechanoreceptors) and its perception involves several cortical sites (von Leupoldt and Dahme, 2005). These mechanisms could be another pathway by which the ventilatory response could increase cerebral blood flow during hypoxia. Indeed, respiratory discomfort induced with loaded breathing increases blood flow in the right anterior insula, cerebellar vermis and medial pons (Peiffer et al., 2001), while active inspiration and expiration increases cortical blood flow in discrete regions when compared with a passive condition (i.e., mechanical ventilation) (Ramsay et al., 1993). Functional magnetic resonance imaging has been used to identify the central pattern of activation induced by hypoxia (Critchley et al., 2015), but the extent to which this is explicitly related to changes in ventilation has not been explored. Herein, we observed increases in MCAv during Hypoxia, but not during either Hypoxia + RB or Hyperpnoea, suggesting that changes in breathing rate and depth do not contribute. However, it is possible that neither the hypoxic stimulus nor the ventilatory response in

our study evoked a sufficiently large activation of higher brain centres perfused by the middle cerebral artery.

5.5.1 Methodological considerations

There are several methodological issues that should be considered. MCAv was used to assess cerebral perfusion and the relative strengths and weaknesses of this technique have been discussed (Willie et al., 2011a, Ainslie and Hoiland, 2014b). In brief, in the absence of MCA diameter measurements we can only assume that the MCAv data are representative of MCA flow. Given that acute hypoxia can cause cerebral vasodilatation (Wilson et al., 2011) it is possible that the magnitude of the increases MCAv underestimate the true increases in cerebral blood flow under these conditions. However, as this would presumably have affected the Hypoxia and Hypoxia + RB conditions equally, it is not likely that it could affect the findings of this study. Further studies are warranted using advanced brain imaging technologies (e.g., magnetic resonance imaging, arterial spin labelling). Finally, care should be taken when considering our findings gathered under conditions of acute normobaric hypoxia in the context of high altitude conditions of chronic hypobaric hypoxia (Coppel et al., 2015).

In conclusion, the results of this study indicate that during moderate isocapnic acute hypoxia the hypoxic ventilatory response does not increase cerebral perfusion beyond that elicited by reduced oxygen saturation.

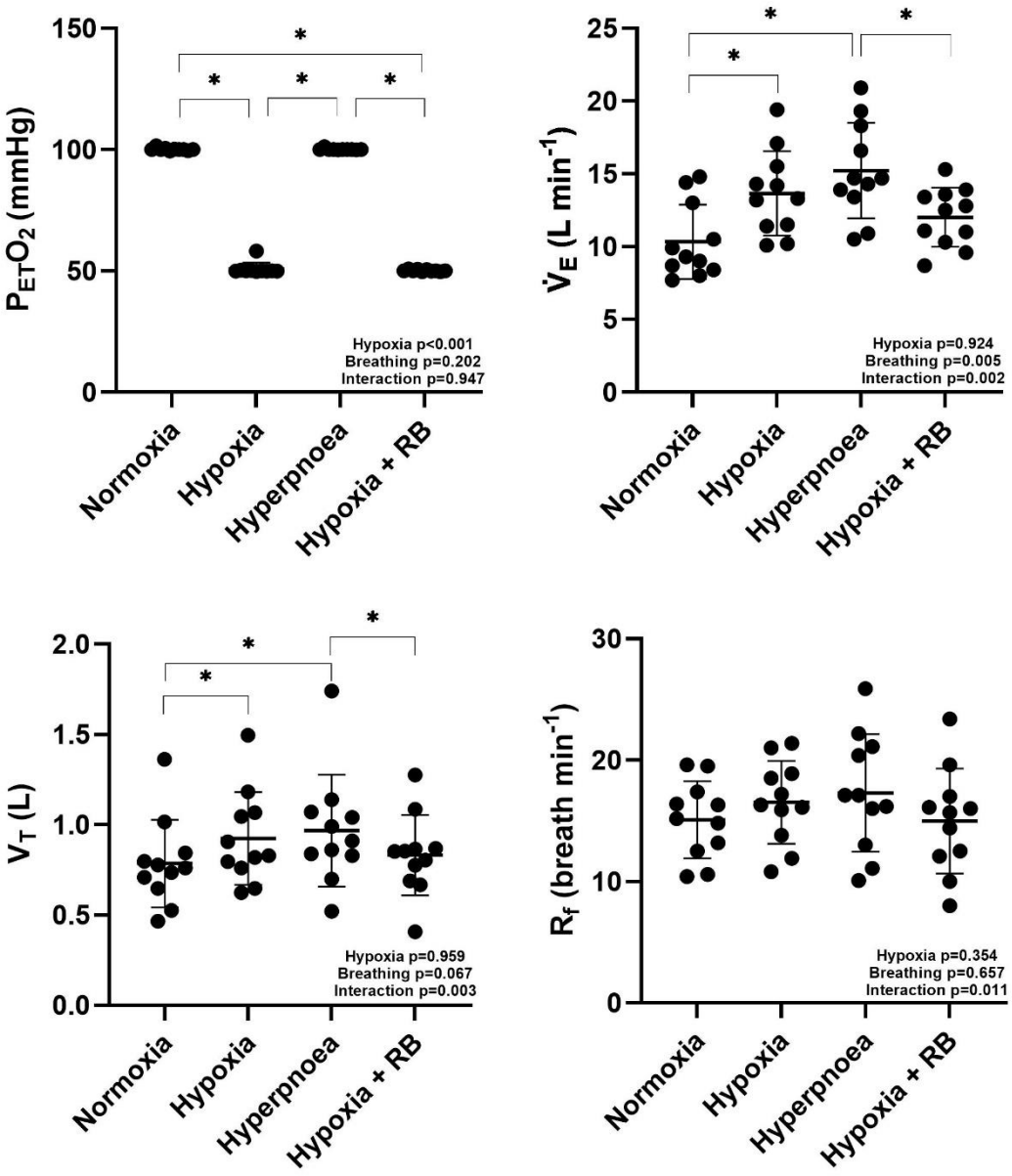


Figure 9 TV, RF, VE , and $P_{ET}O_2$ during the Normoxia, Hypoxia, Hyperpnoea and Hypoxia + RB conditions. * represents P<0.05. Horizontal bars show mean and SD.

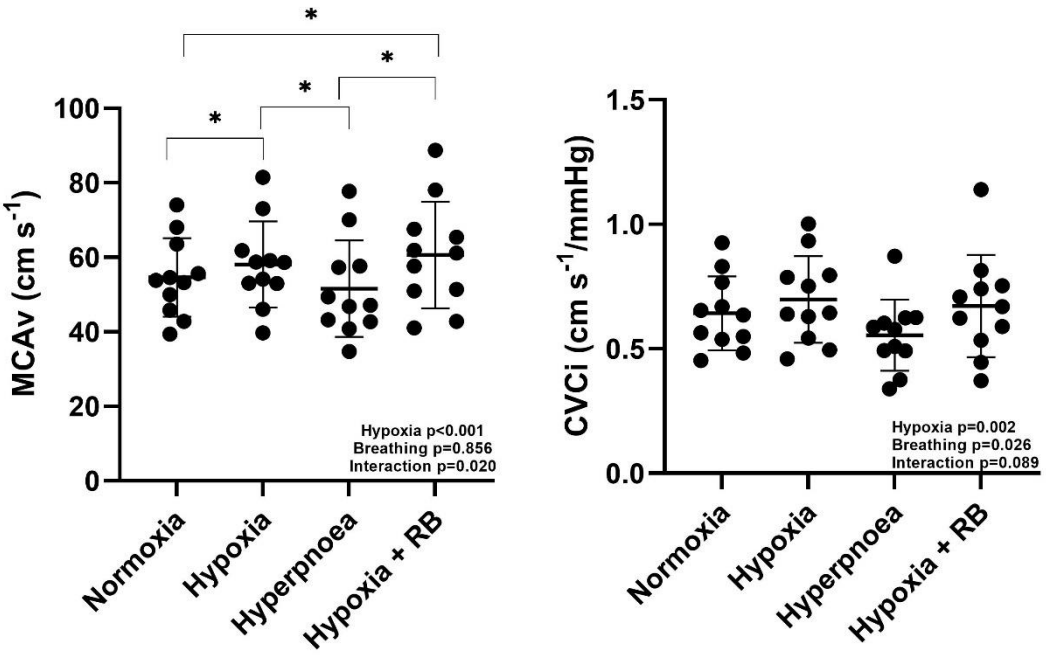


Figure 10 MCAv and CVCi during the Normoxia, Hypoxia, Hyperpnoea and Hypoxia + RB conditions. * represents P<0.05 Horizontal bars show mean and SD.

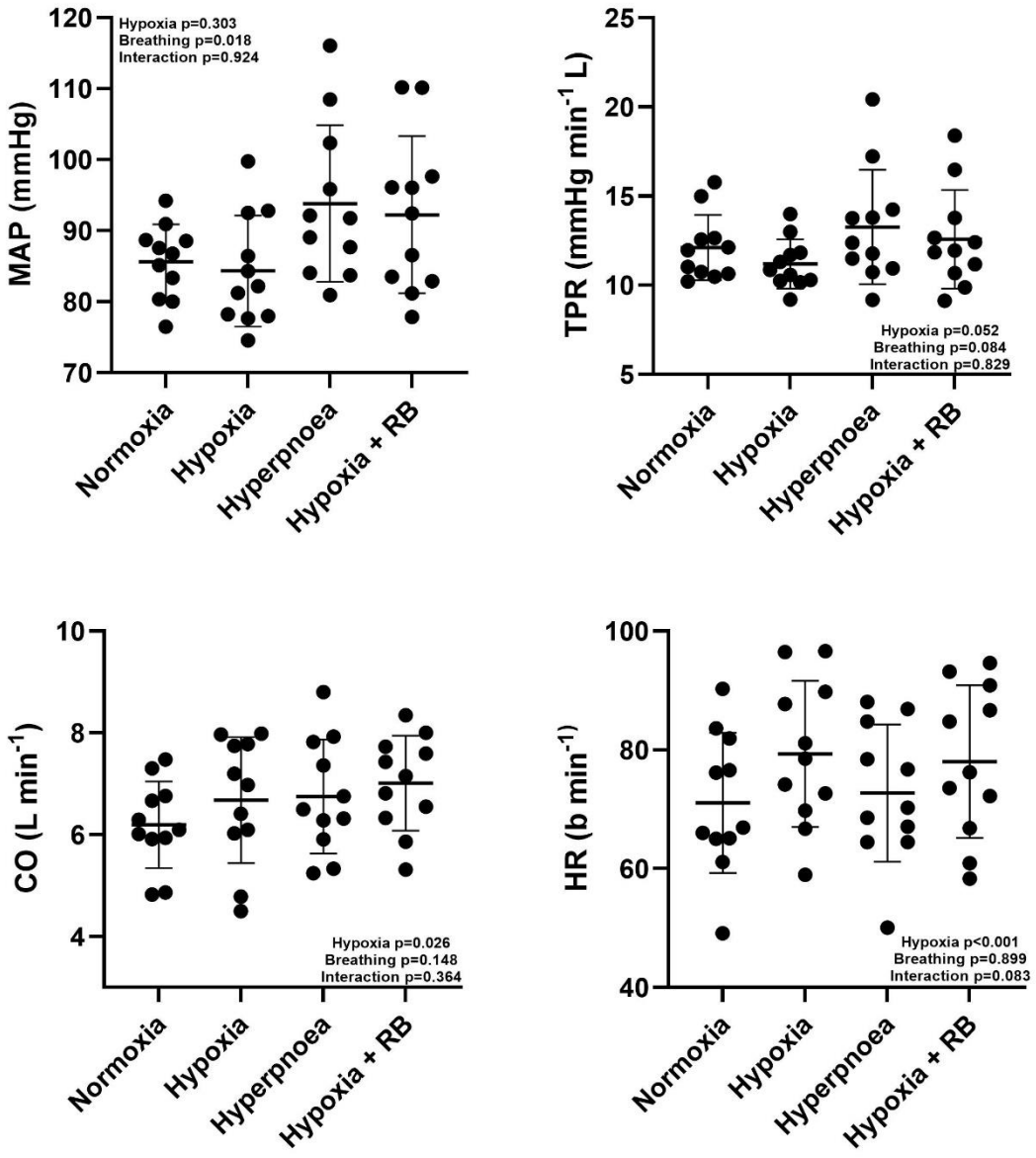


Figure 11 MAP, TPR, CO and HR during the Normoxia, Hypoxia, Hyperpnoea and Hypoxia + RB conditions. * represents P<0.05 Horizontal bars show mean and SD.

CHAPTER 6: MUSCLE METABOREFLEX CONTROL OF CARDIORESPIRATORY AND CEREBROVASCULAR RESPONSES IN MODERATE NORMOBARIC HYPOXIA

6.1 Abstract

Local ischaemia and systemic conditions of hypoxemia trigger compensatory cardiorespiratory and cerebrovascular responses, however the interaction between these systemic and local stimuli is not completely understood. During exercise, a local mismatch between oxygen delivery and demand activates the metabolically sensitive skeletal muscle afferents (muscle metaboreflex). During hypoxia, a systemic reduction in O_2 activates chemoreceptor afferents (chemoreflex). The aim of this study was to determine the influences of hypoxia on cardiorespiratory and cerebrovascular responses to muscle metaboreflex activation. Nine young healthy participants completed two experimental conditions in normobaric poikilocapnic normoxia and hypoxia (FiO_2=13.5%) with trials of; 1) leg cycling with PFR, 2) leg cycling with PEI, and 3) static hand grip (IHG) with PEI. SpO_2 during normoxia (97 ±1%) was significantly higher than hypoxia (87 ±3%). Compared to leg cycling, PFR increased HR, MAP, MCAv, and VE ($P<0.05$ vs exercise) similarly in normoxia and hypoxia. Furthermore, PEI following leg cycling partially maintained exercise-induced increases from baseline in HR, MAP, MCAv, and VE ($P<0.05$ vs baseline) to similar extents in normoxia and hypoxia. During PEI following IHG, MAP was maintained above baseline ($P<0.05$ vs baseline), while HR, MCA, and VE ($P>0.05$ vs baseline) returned to baseline, with differences between normoxia and hypoxia. These findings suggest that moderate poikilocapnic normobaric hypoxia does not augment cardiorespiratory or cerebrovascular responses to muscle metaboreflex activation and that this is the case irrespective of the mode muscle metaboreflex activation.

6.2 Introduction

Systemic and local conditions of hypoxemia trigger compensatory cardiorespiratory and cerebrovascular responses. During exercise, a condition of local ischaemia develops where a mismatch occurs between oxygen delivery and demand by the exercising skeletal muscles. This mismatch causes an accelerated interstitial accumulation of the chemical by-products of contraction, resulting in an enhanced stimulation of metabolically sensitive group III/IV skeletal muscle afferents that are known to evoke powerful reflex autonomic cardiovascular effects (Kaufman et al., 1996, Fisher and White, 2004, Fisher et al., 2015c). Furthermore, during general hypoxemia, the sensing of hypoxia by the peripheral chemoreceptors leads to reflex ventilatory and cardiovascular adjustments safeguarding against the deleterious consequences of reductions in tissue oxygenation (Guyenet, 2014a). Along with increasing RF and TV, acute hypoxia heightens cardiac and vasoconstrictor sympathetic nerve activity causing elevations in resting heart rate HR, CO, and less so blood pressure (Guyenet, 2014a, Fisher et al., 2018a). However, the interaction between these systemic and local stimuli is not completely understood and are highly relevant to several chronic pathological conditions (such as heart failure) and to altitude or extreme exercise, in which the sensitivity of both the carotid chemoreflex and skeletal muscle metaboreflex are enhanced (Delaney et al., 2010, Schultz and Marcus, 2012, Paton et al., 2013, Amann et al., 2014).

The mode of metaboreflex activation is an important determinant of cerebrovascular and cardiovascular responses. When the muscle metaboreflex is isolated during PEI following handgrip in normoxia, HR returns towards baseline values, however when the muscle metaboreflex is augmented during exercise by making the muscle ischemic or partially reducing its perfusion, the HR response is increased (Fisher et al., 2010a, Fisher et al., 2013a). Moreover, when PEI is imposed following leg exercise, HR remains elevated (Fisher et al., 2013a). These differential responses are likely attributable

to differential activities of the cardiac sympathetic and parasympathetic nerves under these distinct experimental conditions (Fisher, 2014). During PEI following handgrip the elevated blood pressure is believed to provide a baroreflex-mediated excitatory drive to cardiac parasympathetic neurons at a time when inhibitory inputs from central command and muscle mechanoreflex are lost (Fisher et al., 2010a, Fisher et al., 2013a). In contrast, inhibitory inputs to cardiac parasympathetic neurons from central command and muscle mechanoreflex are present when the muscle metaboreflex is activated during leg exercise, meaning that HR is increased.

It was recently demonstrated that the HR response to hypoxic exercise was augmented, partly due to a greater inhibition of cardiac parasympathetic activity (Siebenmann et al., 2015b). The mechanism for such autonomic alterations remains to be determined, but one possibility is an increase in muscle metaboreflex activation (Fisher, 2015). Indeed, epidural anaesthetic administration attenuated the HR response during concurrent interaction of hypoxia (SpO_2 79%) and 60% workload single leg extension exercise (Wan et al., 2020b). In contrast, Keller-Ross et al. (2019) have shown no effect of hypoxia (FiO_2=12.5%) on HR during a 30% peak workload cycling and 'vascular occlusion' (metaboreflex activation). Such findings suggest that the differences in the mode of metaboreflex activation are important determinants of HR during exercise in hypoxia.

The metaboreflex has also been implicated in CBF responses during exercise (Braz et al., 2014, Prodel et al., 2016b). Braz et al. (2014) reported increases in MCAv during fatiguing handgrip exercise and PEI, but only when $P_{ET}CO_2$ is controlled to baseline levels. Similarly, Prodel et al. (2016b) demonstrated that both ICA_Q and MCAv increased during leg cycling with PFR, only when $P_{ET}CO_2$ is maintained to isocapnic levels. But whether hypoxia augments MCAv to metaboreflex activation remains unknown.

In the present study we sought to determine the influences of hypoxia on cardiorespiratory responses to muscle metaboreflex activation. Secondly, whether cardiorespiratory responses depend upon the metaboreflex mode of activation (during vs. post-exercise), the preceding exercise (static vs. dynamic) and the mode of exercise (handgrip vs. leg cycling). Thirdly, we sought to determine the influences of hypoxia on cerebrovascular responses to muscle metaboreflex activation and whether these responses depend upon the metaboreflex mode of activation. To achieve this, young healthy participants undertook separate bouts of dynamic leg cycling exercise in which the muscle metaboreflex was activated with partial flow restriction or followed by PEI to selectively isolated muscle metaboreflex activation (Fisher et al., 2013a, Prodel et al., 2016b). For comparison with previous studies a trial of isometric static handgrip exercise followed by PEI was also included. Studies were performed under normoxic or hypoxic conditions (FiO_2 = 13.5%). Isocapnia was not maintained and as such our experiments more closely reflect conditions experienced at altitude and by hypoxaemic patients. This would enable us to test the hypothesis that hypoxia would augment cerebrovascular and cardiorespiratory responses to muscle metaboreflex activation.

6.3 Methods

6.3.1 Ethical approval

This study was performed in accordance with the most recent revision of the *Declaration of Helsinki* and the experimental protocol was approved by the Science, Technology, Engineering and Mathematics Ethical Review Committee at the University of Birmingham (ERN_17-0810). Each participant provided written consent after a receiving a detailed written and verbal explanation of the study purpose and potential risks.

6.3.2 Participants

Nine males (age 23±7 yr; height 178±6 cm; weight 78±8 kg; mean ± SD) that were free from cardiovascular, pulmonary, renal, metabolic and neurological conditions took part in this study. Participants were non-smokers and were not taking any prescription or over-the-counter medications. Prior to experimental sessions they were requested to avoid strenuous exercise and alcohol consumption for 24 hours, caffeine intake for 12 hours, and food intake for 2 hours prior to testing.

6.3.3 Experimental Measurements

HR was assessed using a lead II electrocardiogram (ECG; BioAmp, AD Instruments, Bella Vista, NSW, Australia). Beat-by-beat was obtained from the middle finger of the left hand (Portapres, Finapres Medical Systems, Amsterdam, The Netherlands). Portapres measures were verified using brachial artery BP measured using an automated sphygmomanometer (Tango+; SunTech, Morrisville, NC, USA). Participants wore a mouthpiece and nose clip to permit breath-by-breath assessment of VE, TV and RF via an ultrasonic phase-shift flowmeter (model FR-41s, Flowmetrics, Chatsworth, CA, USA). 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). Peripheral capillary SpO_2 was determined using finger pulse oximetry. Doppler ultrasound probe (2-MHz probe; Doppler Box X; Compumedics, Singen, Germany) was fixed ~1 cm above the zygomatic arch for simultaneous MCAv measurement (depth 45-65 mm) (Willie et al., 2011b). This area was insonated and once the signal is stable a modifiable head kit enabled locking of the signal for stability. All measurements recorded were converted from analogue to digital data at 1 kHz (Powerlab, 16/30; ADInstruments) and were stored in for offline analysis (LabChart Pro; ADInstruments).Participants provided rating of perceived exertion during baseline, exercise, and metabolic activation using a 6–20 scale (Borg, 1982).

6.3.4 Experimental Protocol

A comprehensive familiarisation session was undertaking with all participants prior to the experimental protocol, during which all the study procedures were conducted. The experimental protocol was conducted over two laboratory visits separated by ~2-7 days but performed at the same time of day for each participant. Experimental sessions were performed under both normoxic (FiO_2 = 21%) and hypoxic conditions (FiO_2 = 13.5%; equivalent of altitude of 3,500m) conditions. The normoxic condition was conducted first and on the second experimental visit the hypoxic condition was undertaken, according to a single blind design. This permitted the normoxic leg cycling exercise workload, which was based on target HR, to be established and then matched during the hypoxic condition. All experiments were undertaken in the same environmental chamber (Sporting Edge, Basingstoke, UK) with a constant mean ambient temperature of ~21 °C and relative humidity of 40%. At each experimental session the experimental protocols described below were undertaken according to a randomised design using a random number generator app. Participants were acclimatized to the

chamber for a minimum of 20 min before the first protocol commenced and remained in the chamber throughout the experimental session.

Protocol 1 – Leg cycling with partial flow restriction: Whilst seated in a semi-recumbent position, participants rested for a 3 min baseline period, following which they performed leg cycling exercise at 60 revolutions per minute at a low workload (target HR of 100 beats·min^{-1} in normoxia; 64±21 Watts) (Angio, Lode, the Netherlands) (Hartwich et al., 2011, Fisher et al., 2013a, Prodel et al., 2016b). It is generally accepted that lower workloads (<100 beats·min^{-1}) reflect the relative contribution of cardiac parasympathetic activity during a dynamic exercise (Fisher et al. 2015). The first few minutes (~3 ½ min) of cycling was used to adjust the workload in order to achieve the target HR in normoxia, and in hypoxia a similar adjustment period was employed to reach the target workload (i.e., in Watts) for a given participant. Then a steady-state period of cycling was performed for 3 ½ min (Ex1), after which bilateral thigh cuffs were inflated to 100 mmHg (Hokanson Inc., Bellevue, WA, USA) to partially restrict blood flow to the exercising muscles and engage the metaboreflex (Hartwich et al., 2011, Fisher et al., 2013a, Prodel et al., 2016b). This approach created an increase in accumulation of exercise-induced metabolites due to the mismatch between oxygen delivery and demand caused by the inflatable cuffs. Therefore, an increase in activity of metabolically sensitive skeletal muscle afferent. The thigh cuffs were then deflated after 3 ½ minutes and another 3 ½ min of steady-state leg cycling conducted under free-flow conditions (Ex2). This timing allows minute to minute analysis and a 30 second period to discriminate the effect of cuff inflation for analysis and data cleaning. Rating of perceived exertion was obtained at the end of both periods of free-flow leg cycling and leg cycling with partial flow restriction.

Protocol 2 - Leg cycling and PEI: As described for Protocol 1, after a baseline period (3 min) participants performed seated semi-recumbent leg cycling at 60 rpm for ~14 min. The first few minutes (~3 ½ min) of cycling were used to adjust the workload in order to achieve the target HR in normoxia (100 beats·min^{-1}; 64±21 Watts), and in hypoxia a similar adjustment period was employed to reach the target workload (i.e., in Watts). Once this was established ~10 min of steady-state cycling was performed. Just prior to the end of the exercise bout (~15 s), bilateral thigh cuffs were inflated to 250 mmHg and they remained inflated for ~3 ½ min, for muscle metaboreflex activation. In contrast to PFR where muscles are engaged during exercise, during PEI the muscles remain inactive, and metabolites are trapped by cuffs activating the metaboreflex in isolation.

Protocol 3 – Isometric handgrip and PEI: Participants were seated in semi-recumbent position with a dynamometer held in their right hand. The MVC was determined as the greatest of 3-5 efforts, each separated by 1 min. A visual display was positioned in front of the participants and after a 3 min resting baseline period, participants performed 3 min of isometric handgrip at 30% MVC. Just prior to the end of handgrip (~15 s) a cuff placed around the upper arm (Hokanson Inc., Bellevue, WA, USA) was inflated to 250 mmHg, to occlude the circulation to the exercising muscles (PEI) for ~3 min to maintain the activation of the muscle metaboreflex.

Protocol 4 – Control experiments. Control experiments were conducted to evaluate cardiorespiratory responses to pneumatic cuff inflation around the limbs under both hypoxic and normoxic conditions. These involved 3 min of 1) bilateral thigh cuff inflation to 100 mmHg (as used in protocol 1); 2) bilateral thigh cuff inflation to 250 mmHg (as used in protocol 2) and 3) arm cuff inflation to 250 mmHg (as used in protocol

3). Each trial was preceded by a 3 min rest period and followed by a 3 min recovery period.

6.3.5 Data and Statistical Analysis

Analog data were digitally converted at 1 kHz and stored on a PC for offline analysis (Powerlab 16/30 and LabChart Pro, ADInstruments, Dunedin, New Zealand). Data expressed as mean (standard deviation) unless otherwise stated. Statistical analyses was conducted using SigmaPlot (version 14.0, SYSTAT Software Inc,. Chicago, IL, USA). $P<0.05$ was considered statistically significant. Significant main effects and interactions were explored using post hoc Student Newman Keuls test. Statistical significance was set at $P < 0.05$.

6.4 Results

6.4.1 Protocol 1 – Leg cycling with partial flow restriction

Cardiorespiratory and cerebrovascular responses to leg cycling with PFR are demonstrated in Table 6 and figure 15. Baseline SpO_2 and $P_{ET}CO_2$ were lower during hypoxia (87±3 %; 40±1 mmHg) than normoxia (97±1 %; 42±1 mmHg $P<0.05$) and HR was greater during hypoxia than normoxia (73 ± 4 vs 67 ± 3 $b \cdot min^{-1}$; $P<0.05$). However, baseline MAP, MCAv, CVCi and $P_{ET}CO_2$ were not different between normoxia and hypoxia ($P>0.05$).

Leg cycling under free-flow conditions significantly increased MAP, HR, VE and MCAv ($P<0.05$ vs. baseline), with no differences observed between hypoxic and normoxic trials ($P>0.05$). CVCi remained unchanged in both conditions ($P>0.05$) and $P_{ET}CO_2$ was increased during normoxia ($P<0.05$ vs Baseline) but remained unchanged during hypoxia ($P>0.05$). HR (Δ9±3 vs. Δ8±3 $b \cdot min^{-1}$; $P=0.251$), MAP (Δ14±3 vs. Δ16±3 mmHg; $P=0.215$), $P_{ET}CO_2$ (Δ-1.1±1 vs. Δ-3.4±2 mmHg; $P=0.596$), VE (Δ3±4 vs. Δ3±4 $L \cdot min^{-1}$; $P=0.446$) and MCAv (Δ4±5 vs. Δ4±8 $cm \cdot s^{-1}$; $P=0.408$) responses to PFR were

not different in hypoxia and normoxia. $P_{ET}CO_2$ remained unchanged during PFR in hypoxia and was reduced during normoxia. VE increased in both conditions but was significantly higher in hypoxia (33±4 $L \cdot min^{-1}$) than normoxia (29±4 $L \cdot min^{-1}$). Paired t-test shows the change between both conditions was not significant (P=0.40).

6.4.2 Protocol 2 - Leg cycling and PEI

Cardiorespiratory and cerebrovascular responses to leg cycling and PEI are provided in Table 7 and figure 16. Baseline SpO_2 and MAP were lower during hypoxia (86±3 %) than normoxia (97±1 % P<0.05). While HR was numerically greater in hypoxia than normoxia (P=0.129), but MCAv, CVCi, $P_{ET}CO_2$ and VE were not different between trials during baseline (P>0.05). Leg cycling under free-flow conditions significantly increased HR, MAP, VE and MCAv (P<0.05 vs. baseline), with no differences observed between hypoxic and normoxic trials (P>0.05). However, CVCi remained unchanged in both conditions (P>0.05) and $P_{ET}CO_2$ increased in normoxia during leg cycling under free flow conditions but remained unchanged during hypoxia (P>0.05).

During PEI in both normoxia and hypoxia, HR and VE decreased but remained significantly higher than baseline levels. MCAv returned to baseline levels during PEI with hypoxia and normoxia (P>0.05 vs baseline). CVCi was reduced during PEI in both normoxia and hypoxia (P<0.05). MAP remained similarly elevated during PEI in both conditions (P<0.05 vs baseline and P>0.05 vs exercise). VE was slightly higher during PEI in normoxia and hypoxia (Δ6±2 and Δ5±2; P>0.05) while PEI reduced $P_{ET}CO_2$ significantly from baseline (-Δ3±1 mmHg and Δ-2±2 mmHg) and exercise (Δ-7±1 mmHg and Δ-3±2 mmHg; P>0.05) in both conditions.

6.4.3 Protocol 3 – IHG and PEI.

Cardiorespiratory and cerebrovascular response to IHG and PEI are show in Table 8 and figure 17. At baseline, SpO_2 was lower during hypoxia (88±2 %) than normoxia (97±1 %; P<0.05) and HR was higher during hypoxia (78±4 $b \cdot min^{-1}$) than normoxia (69±4 $b \cdot min^{-1}$). VE was numerically greater in hypoxia than normoxia (P=0.155) and $P_{ET}CO_2$ tended to be greater in normoxia than hypoxia (P=0.241), but MAP, MCAv, and CVCi were not different between conditions (P>0.05).

IHG increased MAP, HR, VE and MCAv (P<0.05 vs. baseline), and reduced $P_{ET}CO_2$ (P<0.05 vs. baseline) with no differences observed between hypoxic and normoxic conditions (P>0.05). CVCi was numerically but non-significantly reduced during both conditions (P=0.068). MAP remained similarly elevated during PEI in both conditions (P<0.05 vs baseline), while HR, MCAv and VE returned to baseline (P>0.05 vs baseline and P<0.05 vs exercise). $P_{ET}CO_2$ remained lower than baseline in both hypoxia and normoxia (P<0.05 vs baseline and P>0.05 vs exercise) and CVCi was numerically lower than baseline similarly in both conditions (P=0.068).

6.4.4 Protocol 4 – Control experiments.

During bilateral thigh cuff inflation to 100 mmHg, SpO_2 was lower during hypoxia (87±1 %) than normoxia (97±1 %; P<0.05) and HR higher during hypoxia than normoxia (P=0.035). In contrast, MAP, MCAv, CVCi, $P_{ET}CO_2$, and VE were not different between normoxia and hypoxia (P>0.05). HR, MAP, MCAv, CVCi, $P_{ET}CO_2$, and VE responses to bilateral cuff inhalation to 100 mmHg were not different between hypoxia and normoxia.

During bilateral thigh cuff inflation to 250 mmHg SpO_2 was lower during hypoxia (87±1 %) than normoxia (97±1 %; P<0.05) and HR was numerically higher during hypoxia than normoxia (P=0.122), while $P_{ET}CO_2$ was numerically reduced in hypoxia

from normoxia (P=0.114). But MAP, CVCi, VE and MCAv were not different between trials (P>0.05). HR, MAP, MCAv, CVCi, $P_{ET}CO_2$, and VE responses to bilateral cuff inhalation to 250 mmHg were not different between hypoxia and normoxia.

During unilateral arm cuff inflation to 250 mmHg SpO_2 was lower during hypoxia (87±1 %) than normoxia (97±1 %; P<0.05) and HR higher during hypoxia than normoxia, while $P_{ET}CO_2$ was numerically reduced in hypoxia from normoxia. But MAP, MCAv, CVCi, and VE were not different between trials (P>0.05). HR, MAP, MCAv, CVCi, $P_{ET}CO_2$, and VE responses to unilateral arm cuff inhalation to 250 mmHg were not different between hypoxia and normoxia.

6.4.5 Comparison of physiological variables in protocols 1-4

Figure 12 provides a summary of the HR, MAP, MCAv, and VE responses to PFR in hypoxia and normoxia. There was no difference in the effect of PFR on HR, MAP, MCAv, and VE between normoxia and hypoxia (P>0.05). Figure 13 illustrates the HR, MAP, MCAv, and VE responses to PEI following cycling exercise in normoxia and hypoxia. There was no difference in the effect of PEI on HR, MCAv, and VE between the normoxia and hypoxia conditions (P>0.05). However, MAP during normoxia was greater than hypoxia (P=0.040). Figure 14 shows the HR, MAP, MCAv, and VE responses to PEI following static hand grip exercise in the normoxia and hypoxia conditions. There was no difference in the effect of PEI following handgrip on HR, MAP, MCAv, and VE between normoxia and hypoxia (P>0.05).

6.5 Discussion

In the present study we sought to determine the influences of hypoxia on cardiorespiratory responses to muscle metaboreflex activation. Our main finding is that hypoxia failed to augment cardiorespiratory responses to muscle metaboreflex activation independent of mode of activation (during vs. post-exercise) or type of the preceding exercise (static vs. dynamic).

6.5.1 Cardiorespiratory responses to exercise in hypoxia

Exposure to acute hypoxia evokes increases in ventilation, HR, CO, and skeletal muscle blood flow during exercise elevating convective oxygen delivery to counteract reductions in arterial P_{aO2} (Seals et al., 1991, Joyner and Casey, 2014). In the present study, baseline HR was greater during hypoxia than normoxia (+6 bpm), FiO_2 (13.5%) and SpO_2 (87%). Given the evidence elsewhere (SpO_2<90% (Biscoe and Sampson, 1967, Gupta et al., 1997b)) these parameters should be sufficient to elicit a chemoreflex response. Nevertheless, the hypoxia exposure used in this study is considered mild in comparison with other studies. Comparable studies have used a varying FiO_2 (~10%), with Gujic et al. (2007) reporting a SpO_2 of no greater than 84% and Wan et al. (2020b) reporting a lower SpO_2 (<80%).

An augmented activation of the sympathetic nervous system was seen as being key to the cardiac adjustments to hypoxic exercise on the basis that circulating catecholamine concentrations (Clancy et al., 1975, Escourrou et al., 1984) and vasoconstrictor sympathetic outflow (Katayama et al., 2011) are elevated. However, given recent observations that β-adrenergic blockade with propranolol failed to reduce the additional increase in exercising HR with hypoxia, whereas it was partially attenuated by the cholinergic muscarinic blocker glycopyrrolate (Siebenmann et al., 2015b). This suggests that part of the reason why HR is elevated during hypoxic exercise compared to

normoxic exercise is because of a marked withdrawal of cardiac parasympathetic nerve activity.

6.5.2 Hypoxia, metaboreflex activation and HR

One mechanism for an augmented reduction in cardiac parasympathetic activity during hypoxic exercise may be enhanced pulmonary stretch receptor activation secondary to the increased VE (Guyenet, 2014a) that would serve to overcome any primary effect of chemoreflex activation on increasing cardiac parasympathetic activity (Kollai and Koizumi, 1979). An another possibility for the elevated HR in hypoxic exercise is an increase in the activation of the muscle metaboreflex (Fisher, 2015). Indeed, during exercise in acute hypoxia there is an accelerated anaerobic muscle metabolism and lactic acid production as the normal mismatch between the skeletal muscles' demand for oxygen during exercise and its delivery by the circulatory system is exaggerated (Siebenmann et al., 2015a). For example, Siebenmann et al. (2015a) have demonstrated a difference of 4.5 mmol/l of lactate during hypoxia compared to normoxia (workload ~160 W). In fact, Lagier-Tessonnier et al. (1993) demonstrated that the spontaneous discharge of metabolically sensitive group III/IV skeletal muscle afferents was markedly enhanced by hypoxaemia induced by a reduction in the inspired F_iO_2 of 10% in N_2 for 3 min in a cat model. In contrast, Hill et al. (1992) demonstrated that discharge frequency of contraction sensitive group III/IV skeletal muscle afferents was only trivially increased by hypoxia (F_IO_2 of 3-5% in N_2 for 3-3 ½ min) at rest and during static hind limb contraction. Such findings may be related to the failure of this hypoxic intervention to effect femoral venous hydrogen ion or lactic acid concentration. Indeed, it is well established that lactic acid and ischemia markedly potentiate group III/IV skeletal muscle afferents response to contraction (Longhurst and Zelis, 1979, Thimm et al., 1986, Sheriff et al., 1987, Kaufman and Rybicki, 1987, Rotto et al., 1989, Kaufman et al., 1996).

Moreover, skeletal muscle acidification is potent stimulus for exercise pressor reflex (Victor et al., 1988, Sinoway et al., 1989, Victor and Seals, 1989, Fadel et al., 2003). On that basis we hypothesised that hypoxia would augment the rate of anaerobic metabolism and accumulation of skeletal muscle metabolites and therefore cardiorespiratory and cerebrovascular responses would be augmented.

Following administration of a cholinergic muscarinic antagonist (e.g., glycopyrrolate) HR remains elevated above baseline values during PEI after handgrip, presumably because the muscle metaboreflex-mediated increase in cardiac sympathetic nerve activity can be expressed (Fisher et al., 2010a, Fisher et al., 2013a). The contribution of the muscle metaboreflex to the regulation of HR has historically been the subject of controversy. Intriguingly, HR consistently falls to baseline values during post-exercise ischemia while exercise-induced increases in BP remain elevated, which led to the suggestion that the muscle metaboreflex does not influence HR (Rowell and O'Leary, 1990). In contrast, when the muscle metaboreflex is activated during exercise, by making the muscle ischemic or partially reducing its perfusion, HR does increase (Prodel et al., 2016b). Recent work has provided the explanation for these contrasting effects and elucidated how the autonomic nervous system is differentially modulated when the muscle metaboreflex is activated 'during' vs. 'following' exercise (Fisher et al., 2010a, Fisher et al., 2013a). Thus, when considering how the muscle metaboreflex controls HR both experimental paradigms must be considered.

In our study following IHG exercise, HR returned to baseline, no effect was observed during PEI following IHG during either normoxia or hypoxia. Our findings extend the work of other studies that have found no influence on HR during PEI after IHG (Houssiere et al., 2005, Houssiere et al., 2006, Gujic et al., 2007). Our study supports the claim that metaboreflex influence on HR depends on the group of muscle activated (Fisher et al., 2013a). Indeed, HR was either augmented (PFR) or remained elevated

(PEI). This is consistent with other studies showing similar HR responses during PEI and PFR (Fisher et al., 2013a, Prodel et al., 2016b). However, the interaction of hypoxia and metaboreflex failed to effect HR during both modes of metaboreflex activation. Interestingly, the interaction of metaboreflex and hypoxia induced a 'hyper-additive' effect on HR as reported by (Wan et al., 2020b). A possible explanation to these contrasting results is the differences in chemoreflex (SpO_2 91% vs 79%) and metaboreflex activation (medical blockers vs 100 vascular occlusion). Indeed, the HR response during the concurrent interaction of hypoxia (SpO_2 79%) and exercise (60% workload single leg extension exercise) is greater than the summation of the HR response during exercise and hypoxia supporting that concomitant hypoxia enhances the metaboreflex response. But it is fair to suggest that hypoxia level in this study was not sufficient to initiate an interaction between the two reflexes. Nonetheless, there are differences in the experimental procedures used in this work and Wan et al. (2020b) that could lead to discrepant results. Dynamic exercise (cycling) causes intermittent variations in intramuscular force, which increases skeletal muscle blood flow. Isometric exercises (leg extensions) cause substantial intramuscular force, which reduces skeletal muscle blood flow. These unique contractile characteristics, as well as changes in hemdodynamics, could explain why results are different.

6.5.3 Hypoxia, metaboreflex activation and BP

Despite elevated sympathetic nerve activity directed to the skeletal muscle vasculature (Hanada et al., 2003) muscle blood flow and resting BP is typically unchanged or elevated to a minor degree during acute hypoxia. Indeed, in resting humans, hypoxaemia also prevents sympathetic vasoconstriction (Rowell & Blackman 1986; Weisbrod et al. 2001; Dinenno et al. 2003). During rhythmic handgrip exercise increases in muscle sympathetic nerve activity were potentiated by hypoxia, but muscle

sympathetic nerve activity was not increased by hypoxia at rest suggesting a more profound role for muscle metaboreflex in hemodynamic response during exercise (Seals et al., 1991). Interestingly, MAP increased similarly in the same study during both exercising conditions, with the magnitude of increase is identical implicating the role of skeletal muscle afferent in the pressor response. But how hypoxia and metaboreflex interaction during exercise affects MAP remains unclear. The recent elegant work of Wan et al. (2020b) suggested a hyper-additive effect of the interaction of metaboreflex and hypoxia during lumbar epidural anaesthetic administration. During a 60% workload single leg extension exercise, the summation response of hypoxia only and exercise only is less than the MAP response during the concurrent interaction of hypoxia and exercise. Also, systolic BP in subjects who performed dynamic exercise in hypoxic conditions was found to elevated (Boushel et al., 2001, Vogel et al., 1974). Collectively, these results imply that hypoxia enhances the metaboreflex response during exercise. It is possible that our results at this level of hypoxia (13,500 m), the interaction of the metaboreflex and hypoxia was not sufficient to influence MAP. In fact, MAP increases were identical in hypoxia compared to normoxia during PFR but were less in hypoxia during PEI. However, MAP returned to baseline during handgrip PEI suggesting that the mode of metaboreflex activation and the muscle group are important determinants for cardiovascular responses.

6.5.4 Hypoxia, muscle metaboreflex and ventilation.

The role of group III/IV skeletal muscle afferent feedback in the ventilatory response to exercise is debated. In earlier studies (Braz et al., 2014, Prodel et al., 2016b), VE is augmented with PFR under normoxic conditions (leg cycling at target HR of 120 $b.min^{-1}$) and VE remained elevated above baseline values during PEI, following either leg cycling (after leg cycling at target HR of 120 $b.min^{-1}$) (Prodel et al., 2016b) or

fatiguing isometric handgrip at 40% MVC (Braz et al., 2014). A muscle metaboreflex control of ventilation has also been demonstrated by several previous studies (Duncan et al., 1981, Adams et al., 1987, Piepoli et al., 1999). Therefore, we hypothesised that hypoxia would increase the sensitisation of the metaboreceptors and VE would be augmented. In fact, epidural anaesthetic administration attenuated ventilation during a normocapnic single-leg extension exercise in hypoxia (Wan et al., 2020a). Similarly, our result show increases in VE during both hypoxia and normoxia trials, with a greater increase demonstrated during the hypoxia trial (+4 l/min^{-1}). However, as increases in VE during PFR were accompanied by increases in RPE, it is likely that central command might contribute these responses (Williamson, 2010). On the contrary, VE was not different between normoxia and hypoxia during both IHG and PEI following dynamic cycling. Our results show that at this level of hypoxia (SpO_2 ~87% vs ~79% (Wan et al., 2020a)) was not sufficient to influence augment the metaboreflex induced changes in VE during IHG and PEI .

6.5.5 Hypoxia, muscle metaboreflex and CBF

The role of III/IV skeletal muscle afferent feedback in CBF control during exercise in hypoxia remains obscure. Previous studies (Braz et al., 2014, Prodel et al., 2016c) have reported increases in CBF during exercise during both modes of metaboreflex activation (PFR and PEI) when $P_{ET}CO_2$ is controlled suggesting metaboreflex contribution in these responses. It is possible that in addition to the increases in brain activation and high metabolism in motor regions of the brain (Gross et al., 1980), increases in metaboreceptors afferent activity increases local metabolism, and thus increasing perfusion through a complex neurovascular coupling mechanisms . Indeed, activation of distinctive regions (i.e., cortex and brain stem) have been reported during PEI after a sub-maximal 2-min hand grip exercise. However, whether this applies to

exercising in hypoxia remains unknown. Our results show no interactive effect of systemic hypoxia and the metaboreflex on CBF. However, given the known effect of $P_{ET}CO_2$ on CBF, it is possible that $P_{ET}CO_2$ experienced in this study may restrict metaboreflex changes in CBF. Indeed, as shown in earlier studies metaboreflex mediated increases in CBF are only present when exercise-induced hyperpnoea is avoided (Braz et al., 2014, Prodel et al., 2016b). In our study metaboreflex activation reduced $P_{ET}CO_2$ secondary to hyperpnoea, which may prevent increases in CBF during hypoxia.

6.5.6 Experimental considerations

There are several experimental considerations that needs to be addressed. First, no direct measurement of metabolites was made. Nevertheless, MAP either increased or remained elevated during occlusion indicating the success of muscle metaboreflex activation. Second, the level of hypoxia used in the present study would reasonably be expected to increase the afferent discharge of carotid body chemoreceptors (Biscoe and Sampson, 1967). However, the level of hypoxia exposure is considered modest compared to other similar hypoxia studies. Thirdly, the duration of hypoxic exposure is an important consideration. Seals et al. (1991) have found no increases in muscle sympathetic nerve activity in a short duration of exposure. However, the duration of the present study (i.e., ~210 min) may suggest that the participants adapted to hypoxia. Indeed, Tamisier et al. (2007) have demonstrated alterations in cardiovascular responses during an 8hr application of hypoxia. Nevertheless, the order of experiments was randomised, and baselines were not different. Finally, hypoxia was applied throughout the hypoxic condition rather than being discreetly applied during muscle metaboreflex activation. Gujic et al. (2007), Houssiere et al. (2005), and Houssiere et al. (2006) used a normoxic PEI, whereas both Handa et al. (2003) and Seals (1991) did apply hypoxia during PEI. In

this present study, we applied hypoxia for the whole trial which makes it difficult to examine how both reflexes interact.

In conclusion, the results of this study suggest that moderate normobaric hypoxia does not augment the cardiorespiratory and cerebrovascular responses to muscle metaboreflex activation.

Table 5 Cardiorespiratory and cerebrovascular during leg cycling before (Ex1) and following (Ex2) partial flow restriction (PFR), during hypoxic and normoxic conditions

	Experimental Phase				P Value		
	Rest	**EX1**	**PFR**	**EX2**	**Condition**	**Time**	**Interaction**
HR (b·min^{-1})							
Normoxia	67 ± 3	102 ± 2	110 ± 3	105 ± 2	**0.034**	**<0.001**	0.650
Hypoxia	73 ± 4	110 ± 3	119 ± 3	111 ± 3			
MAP (mmHg)							
Normoxia	85 ± 3	96 ± 2	112 ± 3	96 ± 2	0.639	**<0.001**	0.931
Hypoxia	81 ± 4	91 ± 3	105 ± 3	89 ± 3			
VE (l min^{-1})							
Normoxia	10 ± 2	26 ± 4*	29 ± 4$^{*‡\$}$	30 ± 4*	0.231	**<0.001**	**0.030**
Hypoxia	10 ± 2	30 ± 4*†	33 ± 4$^{*†‡\$}$	29 ± 4*			
MCAv (cm·s^{-1})							
Normoxia	58 ± 4	65 ± 5	69 ± 8	63 ± 7	0.621	**<0.001**	0.992

Hypoxia	61 ± 6	68 ± 7	72 ± 5	67 ± 4			
CVCi (cm·s^{-1}/mmHg)							
Normoxia	0.68 ± 0.15	0.69 ± 0.20	0.65 ± 0.19	0.67 ± 0.17	0.420	0.256	0.857
Hypoxia	0.76 ± 0.25	0.75 ± 0.19	0.69 ± 0.23	0.76 ± 0.25			
$P_{ET}CO_2$ (mmHg)							
Normoxia	42 ± 1	45 ± 1	42 ± 2	44 ± 1	**0.025**	**0.019**	0.386
Hypoxia	40 ± 1	41 ± 1	40 ± 1	41 ± 1			

Values represent means ±SEM at rest (3 min) and last minute of each experimental phase. HR, heart rate; MAP, mean arterial pressure; VE, minute ventilation; $P_{ET}CO_2$, partial pressure of end-tidal carbon dioxide; MCAv, mean middle cerebral artery velocity. P-values are derived from ANOVA examining main effects of condition and time and interaction (condition × time). Values are mean ± SE. P values represent two-way repeated ANOVA results. * P<0.05 versus Baseline, † P<0.05 vs. normoxia, ‡ P<0.05 vs. EX1, $ P<0.05 vs. EX2.

Table 6 Cardiorespiratory and cerebrovascular during leg cycling before (Ex1) and following (Ex2) partial flow restriction (PEI), during hypoxic and normoxic conditions.

	Experimental Phase			P Value		
	Rest	**EX1**	**PEI**	**Condition**	**Time**	**Interaction**
HR (b·min^{-1})						
Normoxia	65 ± 3	107 ± 2	86 ± 4	0.129	**<0.001**	0.221
Hypoxia	71 ± 3	115 ± 3	85 ± 4			
MAP (mmHg)						
Normoxia	85 ± 2	96 ± 2	101 ± 4	**0.038**	**<0.001**	0.282
Hypoxia	80 ± 1	92 ± 3	92 ± 4			
VE (l min^{-1})						
Normoxia	10 ± 1	32 ± 4	16 ± 2	0.192	**<0.001**	0.843
Hypoxia	8 ± 1	34 ± 4	13 ± 1			
MCAv (cm·s^{-1})						
Normoxia	63 ± 9	68 ± 11	58 ± 9	0.882	**0.004**	0.835

Hypoxia	59 ± 6	69 ± 9	59 ± 7			
CVCi (cm·s^{-1}/mmHg)						
Normoxia	0.75 ± 0.35	0.72 ± 0.36	0.58 ± 0.33	0.464	**0.005**	0.135
Hypoxia	0.74 ± 0.24	0.75 ± 0.28	0.66 ± 0.27			
$P_{ET}CO_2$ (mmHg)						
Normoxia	41 ± 1	44 ± 1*	38 ± 1*‡	0.292	**<0.001**	**0.033**
Hypoxia	39 ± 1	40 ± 1	37 ± 2*‡			

Values represent means ±SEM at rest (3 min) and last minute of each experimental phase. HR, heart rate; MAP, mean arterial pressure; VE, minute ventilation; $P_{ET}CO_2$, partial pressure of end-tidal carbon dioxide; MCAv, mean middle cerebral artery velocity. P-values are derived from ANOVA examining main effects of condition and time and interaction (condition × time). Values are mean ± SE. P values represent two-way repeated ANOVA results. * P<0.05 versus Baseline, † P<0.05 vs. normoxia, ‡ P<0.05 vs. EX1.

Table 7 Cardiorespiratory and cerebrovascular variables during static hand grip exercise (IHG) and post-exercise (PEI) during hypoxic and normoxic conditions.

	Experimental phase			P value		
	Rest	**IHG**	**PEI**	**Condition**	**Time**	**Interaction**
HR (b·min^{-1})						
Normoxia	69 ± 4	86 ± 5*	66 ± 5‡	0.095	**<0.001**	**0.044**
Hypoxia	78 ± 4†	90 ± 5*	73 ± 5‡			
MAP (mmHg)						
Normoxia	83 ± 3	105 ± 4	97 ± 3	0.267	**<0.001**	0.356
Hypoxia	81 ± 2	101 ± 4	93 ± 4			
VE (l min^{-1})						
Normoxia	9 ± 1	14 ± 1	9 ± 2	0.155	**<0.001**	0.954
Hypoxia	11 ± 1	15 ± 1	12 ± 1			
MCAv (cm·s^{-1})						
Normoxia	63 ± 9	68 ± 11	58 ± 9	0.541	0.058	0.204

Hypoxia	59 ± 6	69 ± 9	59 ± 7			
CVCi (cm·s^{-1}/mmHg)						
Normoxia	0.74 ± 0.19	0.64 ± 0.25	0.63 ± 0.23	0.732	0.068	0.913
Hypoxia	0.77 ± 0.24	0.70 ± 0.29	0.72 ± 0.35			
$P_{ET}CO_2$ (mmHg)						
Normoxia	41 ± 1	39 ± 1	37 ± 1	0.241	**<0.001**	0.892
Hypoxia	39 ± 1	37 ± 2	36 ± 2			

Values represent means ±SEM at rest (3 min) and last minute of each experimental phase. HR, heart rate; MAP, mean arterial pressure; VE, minute ventilation; $P_{ET}CO_2$, partial pressure of end-tidal carbon dioxide; MCAv, mean middle cerebral artery velocity. P-values are derived from ANOVA examining main effects of condition and time and interaction (condition × time). Values are mean ± SE. P values represent two-way repeated ANOVA results. * P<0.05 versus Baseline, † P<0.05 vs. normoxia, ‡ P<0.05 vs. IHG.

Figure 12 Comparisons of ΔHR, ΔMAP, ΔMCAv, and VE responses to exercise with partial flow restriction (PFR) in normoxia and hypoxia. * represents P<0.05. Horizontal bars show mean and SD.

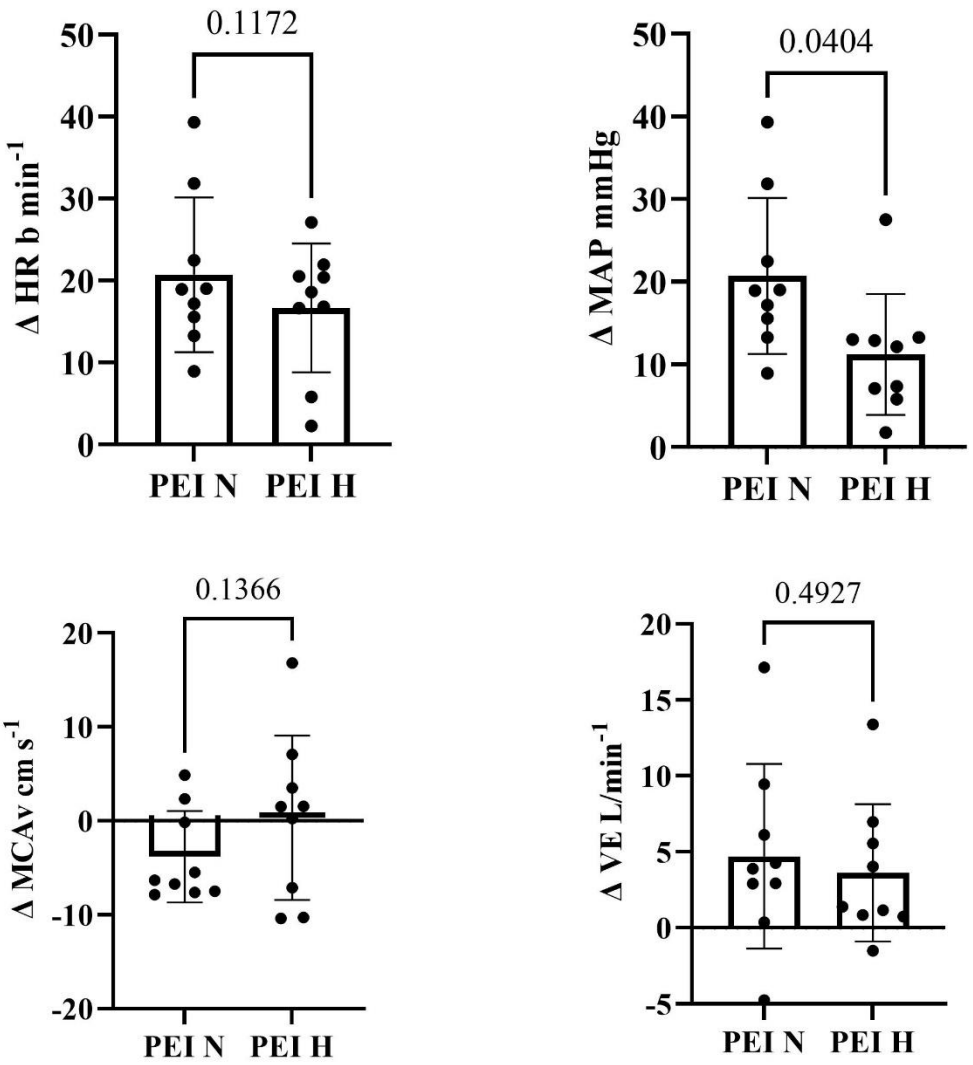


Figure 13 Comparisons of ΔHR, ΔMAP, ΔMCAv, and VE responses to post exercise ischemia (PEI) following leg cycling exercise in normoxia and hypoxia. * represents P<0.05. Horizontal bars show mean and SD.

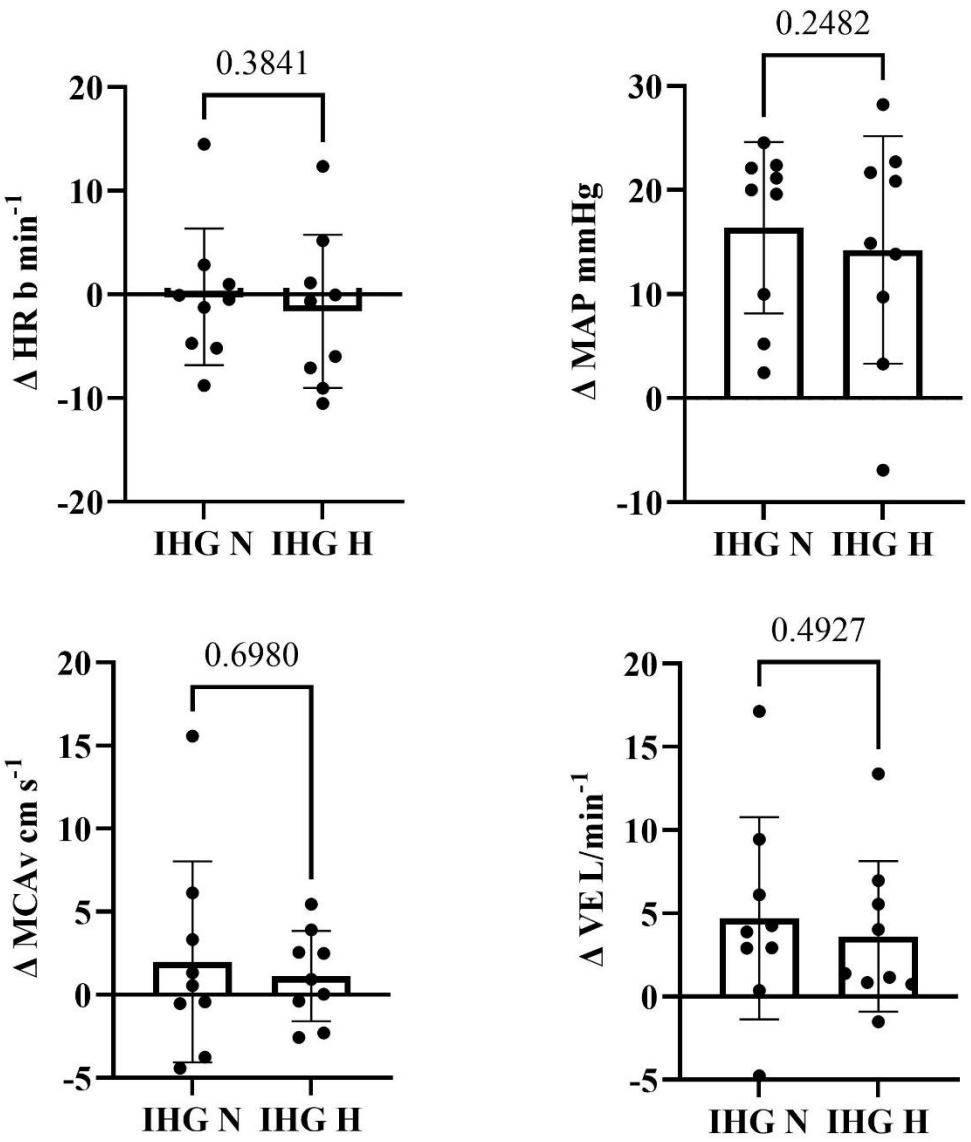


Figure 14 Comparison of ΔHR, ΔMAP, ΔMCAv, and VE responses to post exercise ischemia (PEI) following static hand grip (IHG) in normoxia and hypoxia. * represents P<0.05. Horizontal bars show mean and SD.

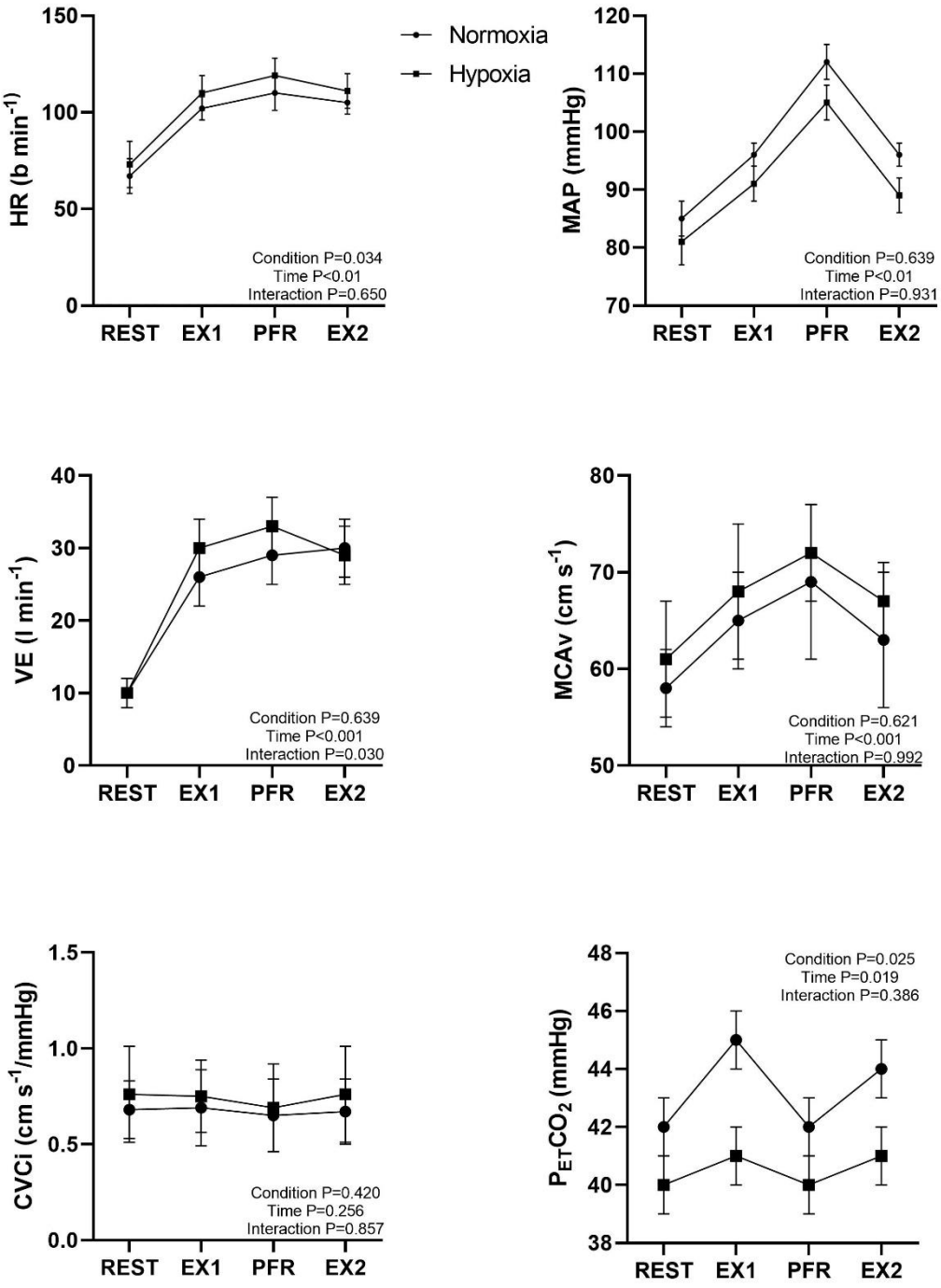


Figure 15 Cardiorespiratory and cerebrovascular during leg cycling before (Ex1) and following (Ex2) partial flow restriction (PFR), during hypoxic and normoxic conditions

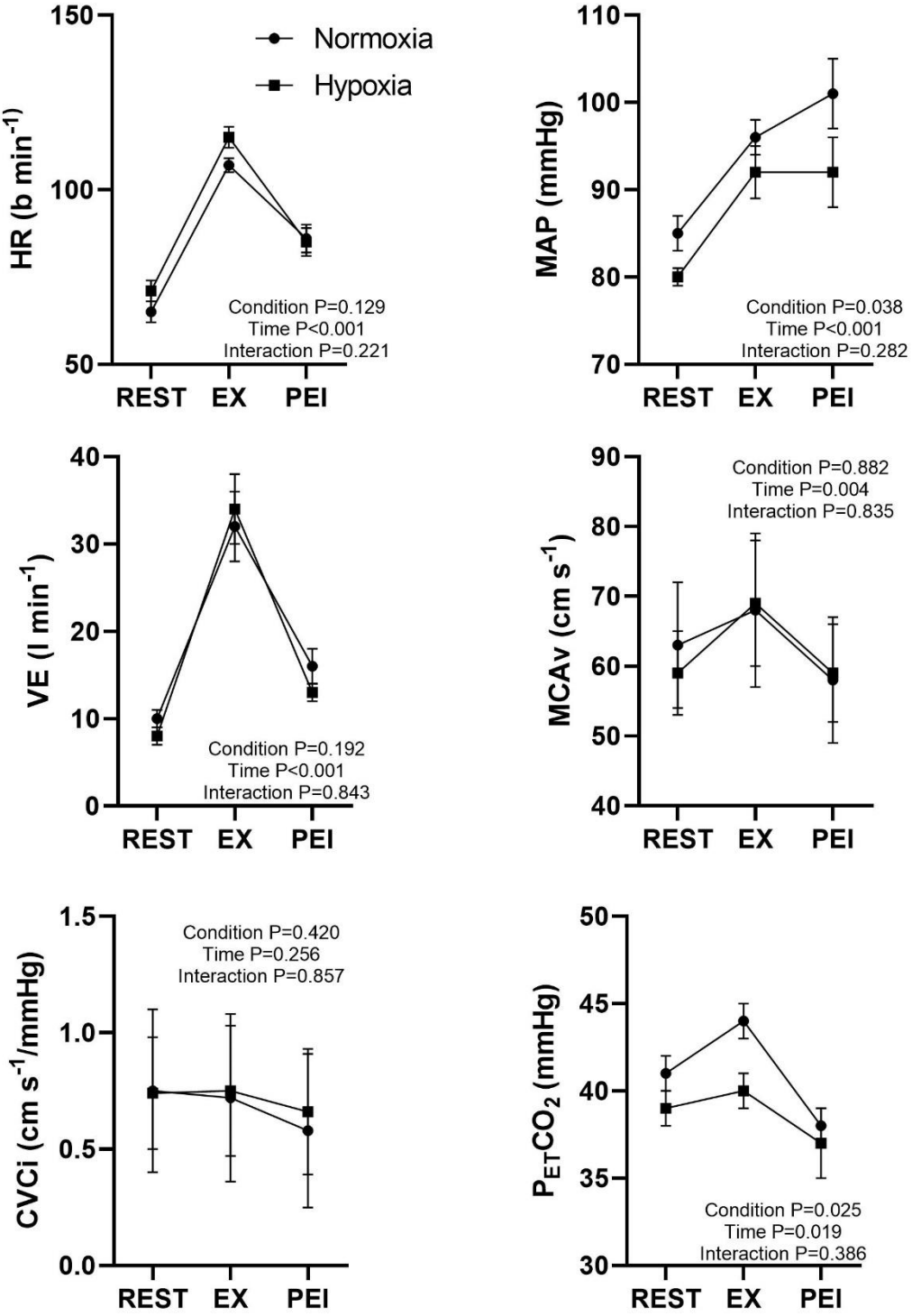


Figure 16 Cardiorespiratory and cerebrovascular during leg cycling before (Ex1) and following (Ex2) partial flow restriction (PEI), during hypoxic and normoxic conditions

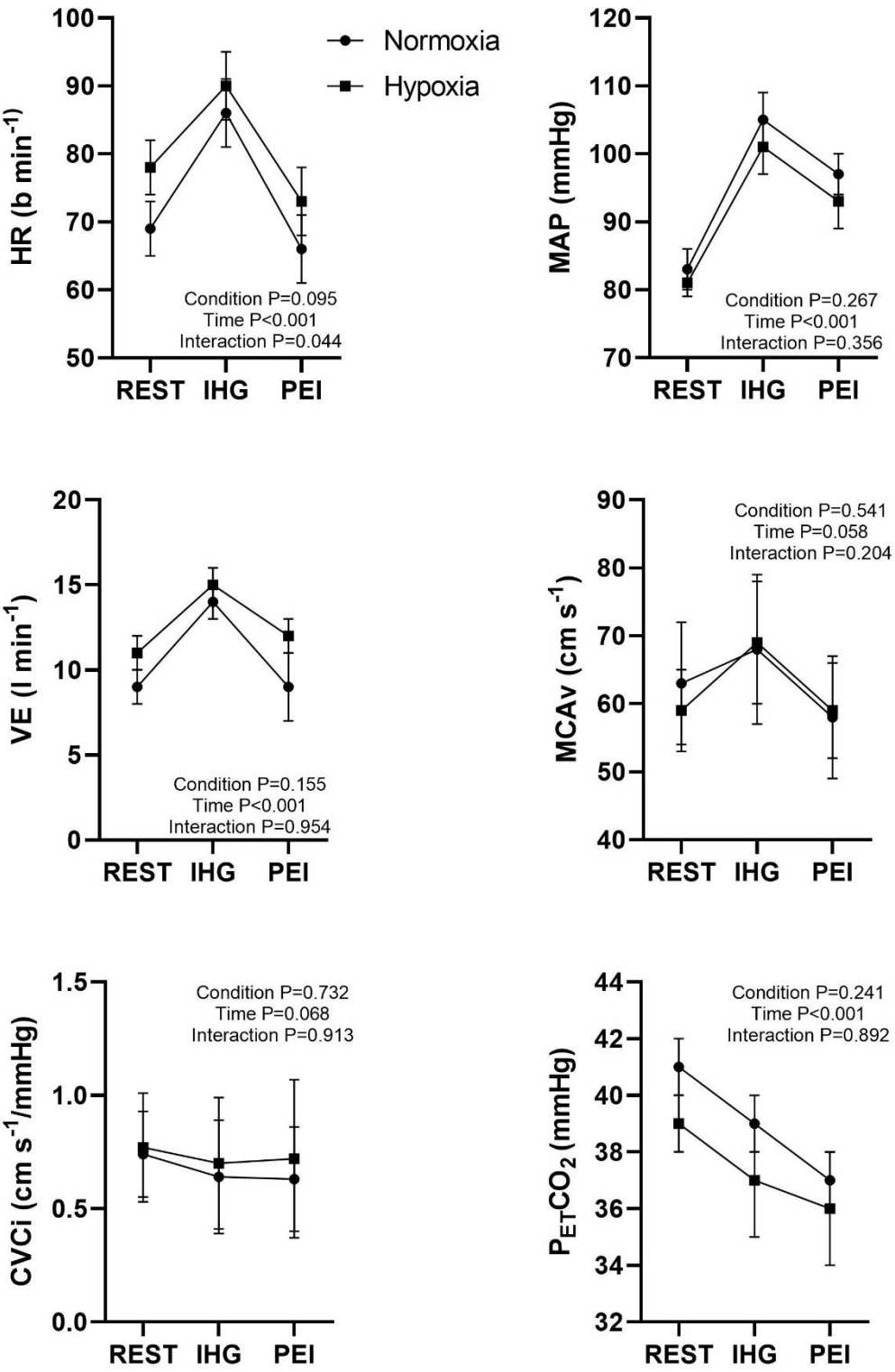


Figure 17 Cardiorespiratory and cerebrovascular variables during static hand grip exercise (IHG) and post-exercise (PEI) during hypoxic and normoxic conditions.

CHAPTER 7: DOES HYPOXIA AUGMENT CARDIORESPIRATORY AND CEREBROVASCULAR RESPONSES TO MUSCLE METABOREFLEX ACTIVATION?

7.1 Abstract

As discussed in the previous chapter, the peripheral chemoreflex is activated by decreases in arterial oxygenation during systemic hypoxia and metabolically sensitive skeletal muscle afferents (metaboreflex) are activated by a local mismatch between O_2 delivery and demand during exercise. The aim of this study was to resolve the key limitations of the previous Chapter and to determine the influences of isocapnic normobaric hypoxia (FiO_2=10.5%) on cardiorespiratory and cerebrovascular responses to muscle metaboreflex activation. Nine young healthy participants (1 women) completed four trials of leg cycling exercise in which the metaboreflex was activated either during exercise in normoxic conditions (PFR+Normoxia) and hypoxia conditions (PFR+Hypoxia), or post-exercise in normoxic (PEI+Normoxia) and hypoxic conditions (PEI+Hypoxia). Isocapnia was maintained in all trials. HR responses to PFR+Hypoxia (Δ52±9 b min^{-1}) and PEI+Hypoxia (Δ24±7 b min^{-1}) were greater than during PFR+Normoxia (Δ40±9 b min^{-1}) and PEI+ Normoxia (Δ9±9 b min^{-1}). However, the HR response to PFR+Hypoxia (Δ52±9 b min^{-1}) and PEI+Hypoxia (Δ24±7 b min^{-1}) was not different than the sum of hypoxia and PEI or PFR +Normoxia (Δ50±14 and Δ19±12 b min^{-1} respectively). VE was greater in PFR+Hypoxia (Δ32±17 L min^{-1}) than PFR+Normoxia (Δ18±12 L min^{-1}), however the response was not different than the sum of hypoxia and metaboreflex alone (Δ32±17 vs Δ25±20 L min^{-1}). These findings suggest that isocapnic normobaric hypoxia augments HR and VE responses to metaboreflex activation.

7.2 Introduction

The findings of the previous Chapter suggest that hypoxia does not augment cardiorespiratory and cerebrovascular responses to metaboreflex activation. However, there are several experimental limitations that limit the veracity of this conclusion. First, the degree (FiO_2= 13.5%) of the hypoxia used may not be sufficient to augment the cardiorespiratory and cerebrovascular responses to metaboreflex activation. In addition, as the study was not isocapnic, changes in $P_{ET}CO_2$ secondary to the ventilatory response may affect CBF responses observed. Finally, the duration of hypoxia was relatively long (~3.5 hr) and not applied discreetly during the muscle metaboreflex activation, but throughout the preceding rest and exercise period meaning that potential carry over effects may have occurred.

To address these limitations, in the present study four trials of leg cycling exercise were performed in which the muscle metaboreflex was either activated during exercise in normoxic (PFR+Normoxia) and hypoxic (PFR+Hypoxia) conditions; or post exercise in normoxic (PEI+Normoxia) and hypoxic conditions (PEI+Hypoxia). To avoid the potential confounding effects of $P_{ET}CO_2$ isocapnia was maintained. This would enable to test the hypothesis that isocapnic normobaric hypoxia would augment the cardiorespiratory and cerebrovascular responses to metaboreflex activation.

7.3 Methods

7.3.1 Ethical approval

Science, Technology, Engineering and Mathematics Ethical Review Committee at the University of Birmingham approved the study (ERN_19-0326). The study was completed in accordance with the most recent revision of the *Declaration of Helsinki*, except for registration in database. All participants were provided a written and verbal explanation of the study objectives and possible risks, then written consent was received from each participant before starting the study.

7.3.2 Participants

Nine participants (1 Female) (age 25±4 yr; height 176±10 cm; weight 79±9 kg; mean ± SD) participated in this study. All participants were healthy, non-smoking and free from any pulmonary, renal, metabolic, cardiovascular, and neurological conditions, in addition not under any kind of prescription or medications. Participants were instructed to refrain from alcohol and vigorous exercise (<24 hrs), caffeine (<12 hrs), and food (<2 hrs) before starting the study.

7.3.3 Experimental Measurements

HR was recorded using electrocardiography (II ECG; BioAmp, AD Instruments, Bella Vista, NSW, Australia). Finger was used to assess Beat-to-beat MAP (Portapres, Finapres Medical Systems, Amsterdam, The Netherlands) and were corrected by an automated sphygmomanometer using brachial artery BP (Tango+; SunTech, Morrisville, NC, USA). Participants were instructed to wear a nose-clip and mouthpiece. $P_{ET}CO_2$, and $P_{ET}O_2$ were used as estimates of P_{aCO2} and P_{aO2} and were established by rapid response gas analysers (Moxus Modular; AEI Technologies, Pittsburgh, PA). VE was established on a breath-to-breath basis using a turbine volume transducer (VMM400; Interface

Associates, Aliso Viejo, CA). SpO_2 was determined using finger pulse oximetry (Magna Medical 1400 Multi Parameter Monitor, Datex-Ohmeda Medical Equipment, USA). MCAv was measured using a 2-MHz pulsed doppler ultrasound probe (Doppler Box X; Compumedics, Singen, Germany). The probe was placed over the temporal window and once the signal was stable the probe was fixed using an adjustable head kit. During each protocol phase (baseline, exercise, and metabolic activation) participants were asked for a rate of Rating of perceived extension using a 6-20 scale (Borg, 1982).

7.3.4 Experimental Protocol

A thorough familiarisation session consisted of a run through of all four experimental protocols in a non-randomised order. Within ~2-7 days the experimental protocol was scheduled, and the order of the protocols were determined using random number generator app. An initial 10-minute adjustment period was first prescribed during which participants were asked to breath using a mouthpiece to establish normal $P_{ET}CO_2$ and $P_{ET}O_2$. $P_{ET}CO_2$ and $P_{ET}O_2$ were controlled during all four experimental protocols using a dynamic end-tidal forcing system, which enabled inspired (humidified) gases to be manipulated on a breath-to-breath basis (Prodel *et al.*, 2016) :

Protocol 1 – PFR+Normoxia: In a semi-recumbent position, following a 3-min baseline, participants cycled for 3 min at a 60 revolution per minute and at a given moderate workload (target HR of 100 beats·min^{-1}) (Angio, Lode, the Netherlands) (Hartwich et al., 2011, Fisher et al., 2013a, Prodel et al., 2016b). Once the target HR has been achieved, participants continued to cycle at steady state period for 3 ½ min (Ex1), then cuffs were inflated to 100 mmHg (Hokanson Inc., Bellevue, WA, USA) to stimulate the metaboreflex by partially restricting blood flow to the exercising muscles as demonstrated elsewhere (Hartwich et al., 2011, Fisher et al., 2013a, Prodel et al., 2016b).

After 3 ½ minutes, cuffs were then deflated, and participants continued cycling at a steady state for another 3 ½ min (Ex2).

Protocol 2 -PEI+Normoxia: Again, after the initial baseline and adjustment period to achieve the target HR (100 beats·min^{-1}), participants cycled for a ~10 min steady-state period. Before the end of cycling (~15 s) bilateral thigh cuffs were inflated to 250 mmHg and they remained inflated for ~3 ½ min, to isolate muscle metaboreflex response.

Protocol 3 – PFR+Hypoxia: Protocol 1 was repeated, however concomitant hypoxia ($P_{ET}O_2$ was clamped at 50 mmHg) was administered during cycling with PFR for ~3 ½ min.

Protocol 4 – PEI+Hypoxia: Protocol 2 was repeated, however concomitant hypoxia ($P_{ET}O_2$ was clamped at 50 mmHg) was administered with bilateral thigh cuffs inflation (250 mmHg) for ~3 ½ min after steady state cycling.

Protocol 5 – Control protocols. Control experiments were performed to assess cerebrovascular and cardiorespiratory responses to hypoxia and normoxia. A 3 min hypoxia administration (FiO_2 = 10.5%) and a 3 min of normoxia (similar FiO_2 levels observed in the initial adjustment period).

In all protocols $P_{ET}CO_2$ was adjusted to baseline level +1 mmHg and $P_{ET}O_2$ was adjusted to baseline levels except during hypoxia. To help achieve this, participants were requested to wear a mouthpiece and nose-clip during all protocols.

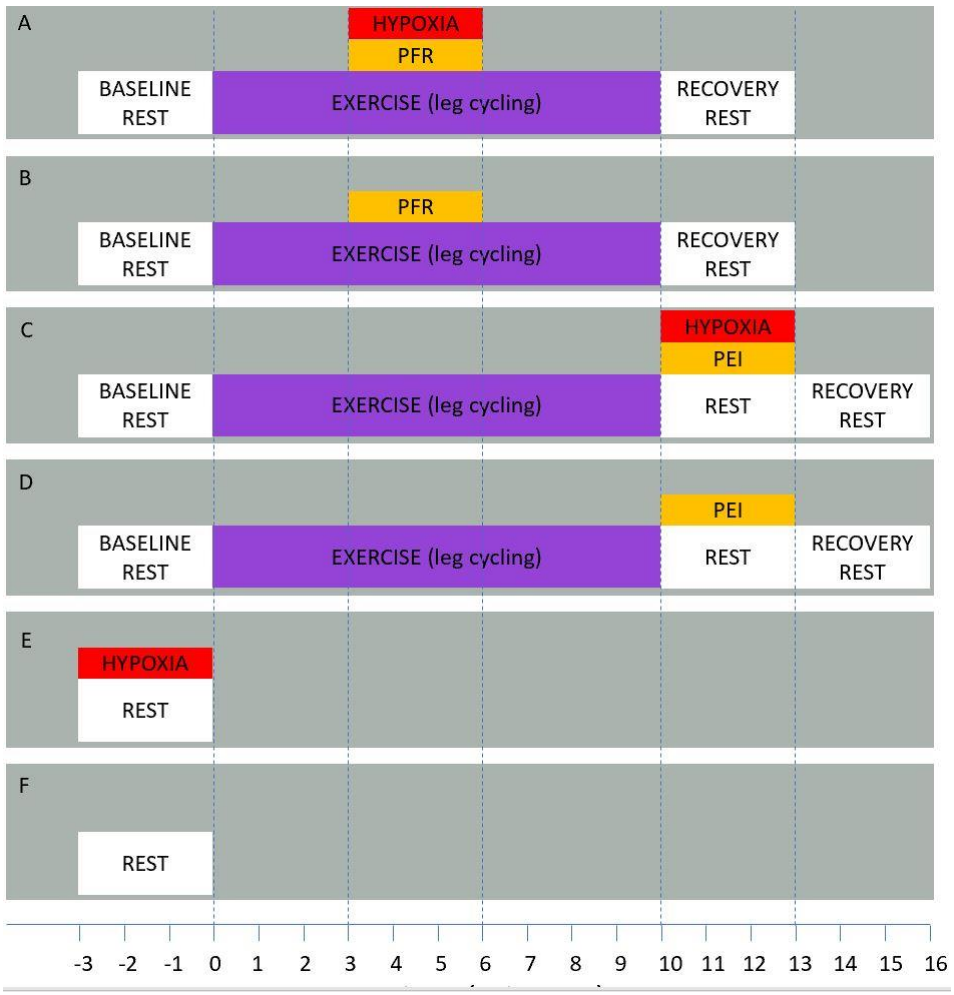


Figure 18. Schematic presentation of the experimental protocol. Panel (A) and (B) represent protocol 1 and protocol 3. Panel (C) and (D) represent protocol 2 and 4. Control experiments are represented by Panel (E) and (F). Red represents any hypoxic application applied in the experiment. Orange identifies metaboreflex applications applied during the experimental protocols. Purple represents the exercise protocol followed during the experimental protocol.

7.3.5 Data and Statistical Analysis

All data collected were digitally converted from analogue at 1 kHz and then were stored for offline analysis (Powerlab 16/30 and LabChart Pro, ADInstruments, Dunedin, New Zealand). Statistical analysis was conducted using SigmaPlot (Version 14.0, SYSTAT software Inc,. Chicago, IL, USA). A two-way repeated measure ANOVA was applied, and significant main effects and interactions were assessed *post hoc* using

Student Newman-Kuels test. Differences between muscle metaboreflex responses were examined by a one-way repeated measure ANOVA and significant main effects and interactions were assessed *post hoc* using Student Newman-Kuels test. Control experiments were examined using paired t-test. All data are demonstrated as mean ± SD, unless stated otherwise. Differences in results were considered significant when $P<0.05$.

To investigate the interactive responses of hypoxia and metaboreflex we assumed that baseline normoxia represents minimal peripheral chemoreceptors and III/IV skeletal muscle afferent activity. This enabled to calculate the differences in physiological responses during the interaction of hypoxia and metaboreflex. Therefore, we calculated change from baseline normoxia as follows: 1) Difference from Hypoxia (ΔHypoxia) is hypoxia control minus normoxia control), 2) Difference from PEI (ΔPEI+Hypoxia) or PFR (ΔPFR+Hypoxia) in hypoxia (response to concomitant hypoxia and PFR or PEI minus hypoxia control), 3) ΔPFR or ΔPEI (response to normoxia and PFR or PEI [PFR+Normoxia or PEI+Normoxia] minus normoxia control), and 4) sum of ΔHypoxia and ΔPFR, or sum of ΔHypoxia and ΔPEI. The last minute of each phase of all physiological responses were averaged for analysis.

7.4 Results

7.4.1 Leg cycling with PFR

Cardiorespiratory and cerebrovascular responses to PFR during cycling exercise in hypoxia and normoxia are illustrated in Table 9 and figure 21. As expected, cycling increased HR during both conditions (P<0.05 Ex1 and Ex2 vs baseline), and was further increased during PFR in both conditions (P<0.05 PFR+ Normoxia and PFR+ Hypoxia vs Ex1 and Ex2), however HR was greater during hypoxia than normoxia (P<0.001 PFR+ Hypoxia versus PFR+ Normoxia). MCAv, MAP, and CVCi were not different between normoxia and hypoxia at any phase (Table 9). Cycling increased MAP and reduced CVCi in both conditions (P<0.05, Ex1 vs Baseline), and PFR further increased MAP in both conditions (P>0.05 PFR+Hypoxia vs. PFR+Normoxia), with no difference between conditions (PFR+Hypoxia vs. PFR+Normoxia P>0.05). MCAv increased similarly from baseline in both conditions (P<0.05, PFR vs Ex1). As intended by design, $P_{ET}CO_2$ was not different throughout both conditions (P>0.05). VE increased similarly during exercise (P<0.05 Ex1 and Ex2 vs. baseline) and PFR (P<0.05 PFR+Normoxia and PFR+Hypoxia vs. Ex1). However, the increase was greater during hypoxia than normoxia (P<0.001 PFR+Normoxia vs. PFR+Hypoxia).

7.4.2 Leg cycling and PEI

Cardiorespiratory and cerebrovascular responses to PEI following leg cycling exercise in both hypoxia and normoxia are illustrated in Table 10 and figure 22. Leg cycling increased HR in both hypoxia and normoxia (P<0.05 Ex1 and Ex2 vs. baseline) and remained elevated from baseline during PEI in both conditions (P<0.05 PEI+Normoxia and PEI+Hypoxia vs. baseline). However, the magnitude of the HR response was greater during hypoxia than normoxia (P<0.001 PEI+Normoxia vs. PEI+Hypoxia). MCAv, MAP, and CVCi were not different between normoxia and

hypoxia at any phase (Table 10). Leg cycling increased MAP and VE in both conditions ($P<0.05$, PEI+Normoxia and PEI+Hypoxia vs. baseline) and remained elevated during PEI ($P<0.05$, PEI+Normoxia and PEI+Hypoxia vs. baseline). Furthermore, CVCi remained unchanged ($P>0.05$) and MCAv tended to increase during exercise and PEI ($P=0.086$). Finally, as intended by design $P_{ET}CO_2$ was not different at any time in both conditions ($P>0.05$).

7.4.3 Control experiments.

SpO_2 was greater during normoxia (97%) than hypoxia (86%) ($P<0.05$). MAP and MCAv were not different between trials, while HR was numerically greater in hypoxia than normoxia but significance was not reached ($P=0.08$).

7.4.4 Comparisons of summative and interactive responses

Figure 15 demonstrates the summative and interactive responses of HR, MAP, MCAv, and VE during PFR+Normoxia and PFR+Hypoxia. DPFR+hypoxia was not greater than the sum of $\underline{\Delta}$Hypoxia and $\underline{\Delta}$PFR+Normoxia ($P>0.05$). Nevertheless, the HR response to $\underline{\Delta}$PFR+Hypoxia was greater than the sum of $\underline{\Delta}$Hypoxia and $\underline{\Delta}$PFR+Normoxia ($P<0.05$). MAP responses to $\underline{\Delta}$PFR+Hypoxia, $\underline{\Delta}$PFR+Normoxia, and the sum of $\underline{\Delta}$Hypoxia and $\underline{\Delta}$PFR+Normoxia were greater than $\underline{\Delta}$Hypoxia.

Figure 16 demonstrates the summative and interactive responses of HR, MAP, MCAv, and VE during PEI+Normoxia and PEI+Hypoxia. Similarly, HR responses to $\underline{\Delta}$PEI+Hypoxia was not greater than the sum of $\underline{\Delta}$Hypoxia and $\underline{\Delta}$PEI+Normoxia ($P>0.05$). Nevertheless, HR response during $\underline{\Delta}$PEI+Hypoxia is greater than $\underline{\Delta}$Hypoxia or $\underline{\Delta}$PEI+Normoxia ($P<0.05$). In contrast, there were no differences in responses in MAP and MCAv ($P>0.05$). While the responses of VE to $\underline{\Delta}$PEI+Hypoxia was not greater than

the sum of ΔHypoxia and ΔPEI+Normoxia ($P>0.05$), VE during ΔPEI+Hypoxia was greater than the response to ΔHypoxia ($P<0.05$).

7.5 Discussion

The aim of the present study was to determine whether hypoxia would augment cardiorespiratory and cerebrovascular responses to muscle metaboreflex activation. The main findings are two-fold. First, hypoxia augmented the HR and VE responses to metaboreflex activation. However, while the HR response was independent of mode of metaboreflex activation (i.e., augmented during both PFR and PEI), VE was dependent upon the mode of metaboreflex activation (i.e., only augmented during PFR). Second, hypoxia failed to augment MAP and MCAv responses to muscle metaboreflex activation and this occurred irrespective of the mode of muscle metaboreflex activation (PEI, PFR).

7.5.1 Exercise in hypoxia

Stimulation of the chemoreflex by sudden reductions in P_{aO2} increases HR, CO, skeletal muscle blood flow and VE (Seals et al., 1991, Joyner and Casey, 2014). In the present study, hypoxia increased HR (+~11 b min^{-1}), VE (8 L min^{-1}), and reduced SpO_2 (~86%) significantly ($P<0.05$). Other studies that investigated hypoxic exercise reported lower SpO_2 (<84% Gujic et al. (2007) and ~79% Wan et al. (2020b)). Nevertheless, the parameters used in this present study are deemed sufficient to potentiate the chemoreflex (Biscoe and Sampson, 1967). Subsequent activation of the sympathetic nervous system was considered as key to regulation of cerebrovascular and cardiorespiratory responses during exercise given the evidence of increases in circulating catecholamines and enhanced vasoconstriction (Clancy et al., 1975, Escourrou et al., 1984, Katayama et al., 2011). However, vagal withdrawal has also been implicated in HR increases during hypoxic exercise. Indeed, cholinergic muscarinic blocker glycopyrrolate reduced HR partially, but β-adrenergic blockade failed to effect HR (Siebenmann et al., 2015a). One proposed mechanism for facilitating vagal withdrawal during hypoxic exercise is the activation of the metaboreflex.

7.5.2 Hypoxia, muscle metaboreflex and HR

Metaboreflex contribution to HR regulation has been a subject of debate. Earlier studies have shown HR returning to baseline values during PEI, even when MAP is elevated, arguing for the contribution of metaboreflex in HR regulation (Rowell and O'Leary, 1990). However, HR increased during PFR, indicative of a role for metaboreflex in HR regulation during exercise (Prodel et al., 2016b). These contrasting results can suggest that the magnitude of may exert differential effects on HR. It is possible that during exercise the mismatch between anaerobic muscle metabolism and lactic acid production is exaggerated following the activation of the metaboreflex in response to contraction. Indeed, it is well established that lactic acid and ischemia markedly potentiate group III/IV skeletal muscle afferents response to contraction (Longhurst and Zelis, 1979, Thimm et al., 1986, Sheriff et al., 1987, Kaufman and Rybicki, 1987, Rotto et al., 1989, Kaufman et al., 1996). Therefore, we hypothesised that hypoxia would enhance the mismatch between oxygen delivery and demand and the rate of anaerobic metabolism and metabolite production, and therefore cardiorespiratory and cerebrovascular responses would be augmented.

In the present study, HR was greater during PFR+Hypoxia than PFR+Normoxia. however, PFR+Hypoxia was not greater than the sum of hypoxia control and PFR+Normoxia. these results suggest a summative effect during the interaction of hypoxia and metaboreflex. Nevertheless, as RPE and HR increased during PFR simultaneously, it is difficult to discount central command contribution (Williamson, 2010). Indeed, increases in HR during PFR + hypoxia was accompanied by further increases in RPE (+3 vs PFR). HR remained elevated following exercise during both normoxia and hypoxia. But the magnitude of elevation during PEI+Hypoxia was larger (+15 b min^{-1}). Interestingly, the magnitude of increase during PEI+Hypoxia is larger than

a 3-min control trial of hypoxia (+11 b min^{-1}) which may suggest an augmentation of the HR response. Nevertheless, the sum of the responses of hypoxia control and PEI+Normoxia was not different than PEI+Hypoxia. These results suggest that hypoxia may induce a summative effect to the HR response during exercise. In contrast, Wan et al. (2020b) reported a hyper-additive effect on HR during exercise in hypoxia. It may be possible that during this present study, chemoreflex and metaboreflex activation may not be sufficient to exert a hyper-additive influence on HR. Wan et al. (2020b) reported a 79% SpO_2 which might explain the contrasting HR responses. Nevertheless, the are differences in the experimental approaches between this study and Wan et al. (2020b) which might elicit different responses. Isometric exercises (Leg extensions) cause large intramuscular force which reduces skeletal muscle blood flow, conversely dynamic exercise (cycling) causes intermittent changes in the intramuscular force hence skeletal muscle blood flow is increased. These distinctive contractile features and alterations in hemdodynamics may also explain why results may differ.

7.5.3 Hypoxia, muscle metaboreflex and blood pressure

During rhythmic hand grip exercise, hypoxia potentiates the muscle sympathetic nerve activity response, but no increases were found during hypoxia at rest (Seals et al., 1991). Indeed, at altitude dynamic exercise increased systolic BP (Vogel et al., 1974, Boushel et al., 2001). This suggest that activation of the chemoreflex by hypoxia enhances the metaboreflex response during exercise. Indeed, Wan et al. (2020) have found that the interaction of hypoxia and metaboreflex exerted a ‘hyper-additive’ influence on MAP. However, no influence on MAP was observed in the present study. One possible explanation may be associated with the level of hypoxia used being not sufficient to affect MAP.

7.5.4 Hypoxia, muscle metaboreflex and ventilation

The role of group III/IV skeletal muscle afferent feedback on the ventilatory response to exercise has been controversial. However, recent observations have demonstrated metaboreflex implications for VE during exercise (Wan et al 2020b). In fact, VE either remained elevated or was augmented during either PEI following cycling exercise or during cycling with PFR (Braz et al., 2014, Prodel et al., 2016b). However, the interaction of hypoxia and the metaboreflex on VE during dynamic exercise remains unknown. Interestingly, Wan et al. (2020a) reported an augmented VE during a normocapnic single-leg extension exercise in hypoxia with lumbar intrathecal fentanyl administration. This suggests a hyper-additive effect on VE exerted by the concurrent activation of the metaboreflex and chemoreflex. Our results show that hypoxia augmented VE to metaboreflex activation during PFR. However, the magnitude of the response of metaboreflex in hypoxia was not different than the sum of metaboreflex and hypoxia. Our results suggest that at this level of hypoxia, chemoreflex activation exerted a summative effect on VE during exercise. The difference in hypoxia levels exerted in this study (SpO_2 87% vs 79%) and different exercise mode (dynamic cycling vs leg extension) may explain the differences in VE responses in both studies.

7.5.5 Hypoxia, muscle metaboreflex and CBF

Braz et al. (2014) and Prodel et al. (2016b) have demonstrated an influence of III/IV skeletal muscle afferent feedback on CBF regulation. MCAv increases during PEI and PFR when reductions in $P_{ET}CO_2$, secondary to hyperpnoea, are avoided (Prodel et al., 2016b). It is possible that concurrent chemoreflex and metaboreflex interaction may potentiate MCAv responses. Given that hypoxia sensitises III/IV skeletal muscle afferents we hypothesised that hypoxia would augment MCAv response during isocapnic hypoxia. However, in the present study, the increases in MCAv during both normoxia and hypoxia

were similar. This indicates that concurrent metaboreflex and chemoreflex activation fails to augment MCAv response. It is fair to suggest that at this level of hypoxia is sufficient III/IV skeletal muscle afferent sensitisation to exert influence on CBF. Nevertheless, our results confirm previous observations that when $P_{ET}CO_2$ is controlled, a metaboreflex contribution to MCAv can be observed.

7.5.6 Experimental considerations

There are several considerations associated with this experiment. MCAv was used to quantify cerebral perfusion, and given that hypoxia induce cerebral vasodilation (Wilson et al., 2011), the magnitude of MCAv response may have been underestimated. But this would have affected both hypoxic conditions equally and it would unlikely that would affect the findings of this study. Secondly, hypoxia administered in this study would be sufficient to initiate the chemoreflex (Biscoe and Sampson, 1967). Nevertheless, the level of hypoxia is considered moderate to other study that have investigated the interaction of metaboreflex and hypoxia.

In conclusion, the results of this study suggest that during moderate isocapnic normobaric hypoxia augmented HR and VE responses to metaboreflex activation but not MCAv or MAP. However, HR and VE response during metaboreflex activation and hypoxia was not greater than the sum of hypoxia control and metaboreflex activation+ Normoxia

Table 8 Cardiorespiratory and cerebrovascular responses to exercise with partial flow restriction (PFR) in both hypoxia and normoxia.

	Experimental Phase				P Value		
	BL	**Ex1**	**PFR**	**Ex2**	**Phase**	**Cond.**	**Int.**
HR (b min^{-1})					**<0.001**	0.056	**<0.001**
Normoxia	70 ± 11	102 ± 5*	110 ± 6*†	103 ± 6			
Hypoxia	70 ± 11	102 ± 3*	122 ± 5*†‡	106 ±4			
MAP (mmHg)					**<0.001**	0.585	0.348
Normoxia	86 ± 4	91 ± 2	99 ± 8	94 ± 5			
Hypoxia	87 ± 6	90 ± 5	104 ± 7	95 ± 5			
VE (L min^{-1})					**<0.001**	**0.008**	**0.002**
Normoxia	14 ± 5*†	21 ± 9	30 ± 13*†	30 ± 11			
Hypoxia	14 ± 8	25 ± 6	43 ± 11*‡	31 ±12*‡			
MCAv (cm s^{-1})					**<0.001**	0.664	0.512
Normoxia	54 ± 9	55 ± 8	58 ± 11	53 ± 7			
Hypoxia	52 ± 8	52 ± 7	59 ± 10	52 ± 7			
CVCi (cms^{-1}/mmHg)					**<0.001**	0.323	0.947
Normoxia	0.62 ± 0.11	0.60 ± 0.08	0.58 ± 0.12	0.57 ± 0.08			
Hypoxia	0.60 ± 0.09	0.58 ± 0.09	0.57 ± 0.12	0.55 ± 0.09			
$P_{ET}CO_2$ (mmHg)					0.2	0.49	0.561
Normoxia	38 ± 3	39 ± 2	39 ± 2	38 ± 4			
Hypoxia	37 ± 5	37 ± 8	37 ± 11	38 ±11			

MCAv, middle cerebral artery mean flow velocity; MAP, mean arterial pressure; HR, heart rate; $P_{ET}CO_2$, partial pressure of end-tidal CO_2; VE, minute ventilation; CVCi, cerebrovascular conductance. Values are mean ± SD. P values represent two-way repeated ANOVA results. * P<0.05 vs. baseline, † P<0.05 vs. exercise, ‡ P<0.05 vs. hypoxia.

Table 9 Cardiorespiratory and cerebrovascular responses to post exercise ischemia (PEI) following exercise in both hypoxia and normoxia.

	Experimental Phase			**P Value**		
	BL	**Ex**	**PEI**	**Phase**	**Cond.**	**Int.**
HR (b min^{-1})				**<0.001**	**<0.001**	**<0.001**
Normoxia	69 ± 12	104 ± 5*	78 ± 16*†			
Hypoxia	69 ± 13	103 ± 6*	93 ± 17*†‡			
MAP (mmHg)				**<0.001**	0.456	0.753
Normoxia	86 ± 4	91 ± 4	95 ± 6			
Hypoxia	86 ± 4	92 ± 5	95 ± 13			
VE (L min^{-1})				**<0.001**	**0.011**	0.162
Normoxia	14 ± 4	33 ± 13	24 ± 6			
Hypoxia	15 ± 10	35 ± 13	38 ± 17			
MCAv (cm s^{-1})				0.086	0.95	0.394
Normoxia	52 ± 8	55 ± 7	54 ± 9			
Hypoxia	52 ± 8	54 ± 8	55 ± 9			
CVCi (cm s^{-1}/mmHg)				0.829	0.856	0.791
Normoxia	0.61 ± 0.09	0.60 ± 0.10	0.58 ± 0.10			
Hypoxia	0.60 ± 0.09	0.58 ± 0.10	0.58 ± 0.10			
$P_{ET}CO_2$ (mmHg)				0.902	0.769	0.589
Normoxia	36 ± 3	37 ± 3	37 ± 3			
Hypoxia	36 ± 3	38 ± 3	37 ± 4			

MCAv, middle cerebral artery mean flow velocity; MAP, mean arterial pressure; HR, heart rate; $P_{ET}CO_2$, partial pressure of end-tidal CO_2; V_E, minute ventilation; CVCi, cerebrovascular conductance. Values are mean ± SD. P values represent two-way

repeated ANOVA results. * P<0.05 vs. baseline, † P<0.05 vs. exercise, ‡ P<0.05 vs. hypoxia.

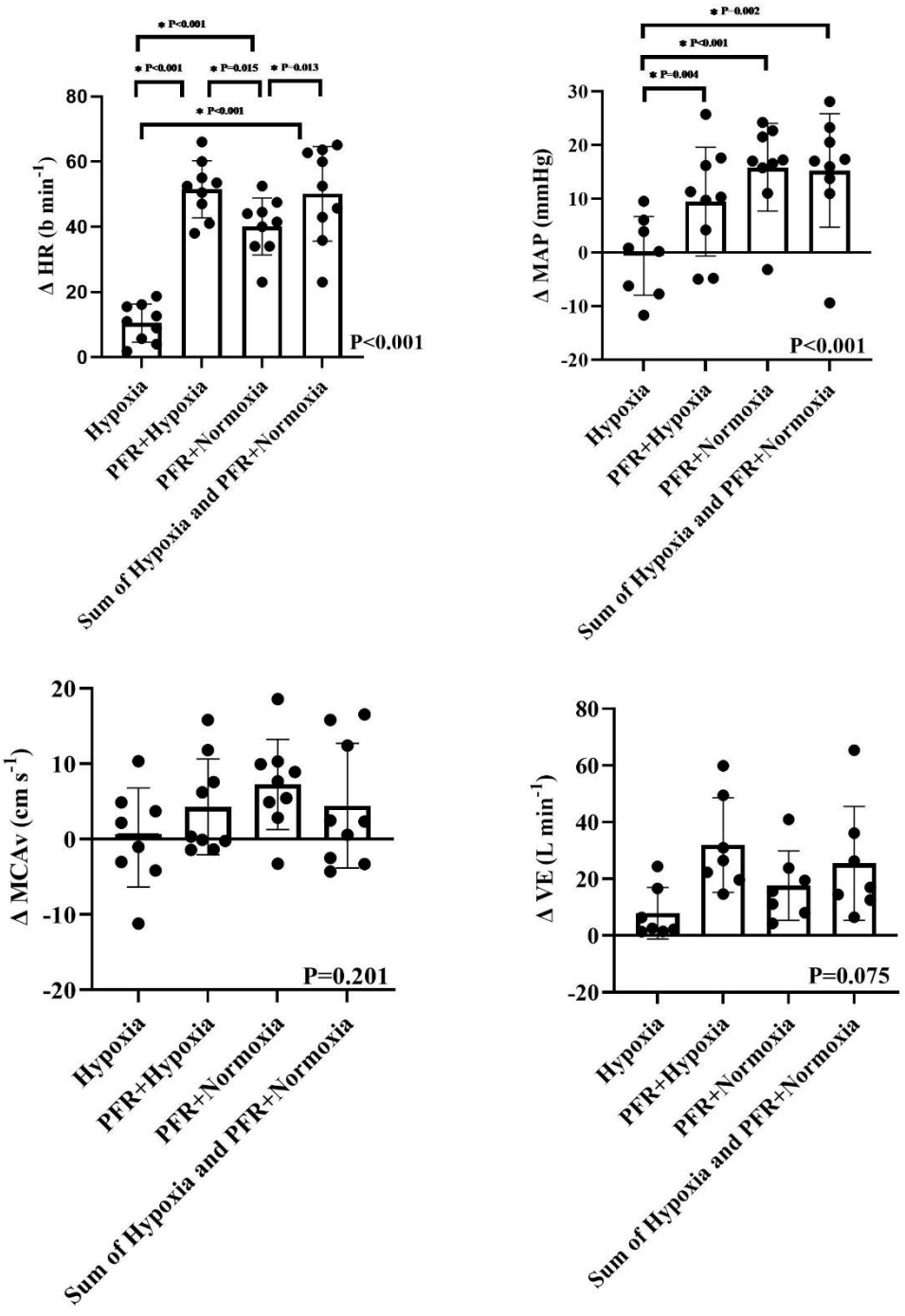


Figure 19 Comparisons of ΔHR, ΔMAP, ΔMCAv, and ΔVE responses to exercise with partial flow restriction (PFR) in normoxia and hypoxia. * represents P<0.05 Horizontal bars show mean and SD

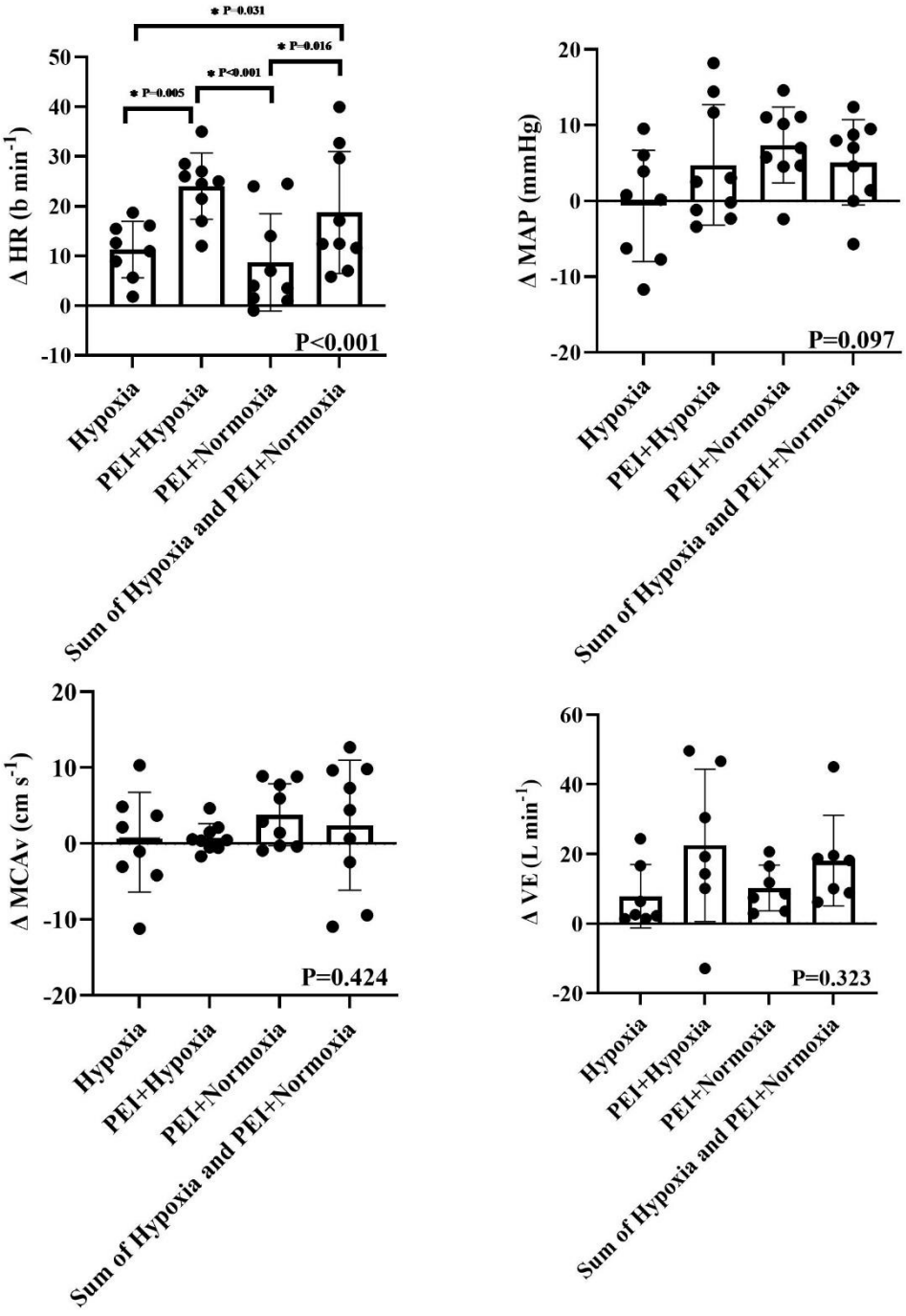


Figure 20 Comparisons of ΔHR, ΔMAP, ΔMCAv, and ΔVE responses to post exercise ischemia (PEI) following leg cycling exercise in normoxia and hypoxia. * represents P<0.05 Horizontal bars show mean and SD.

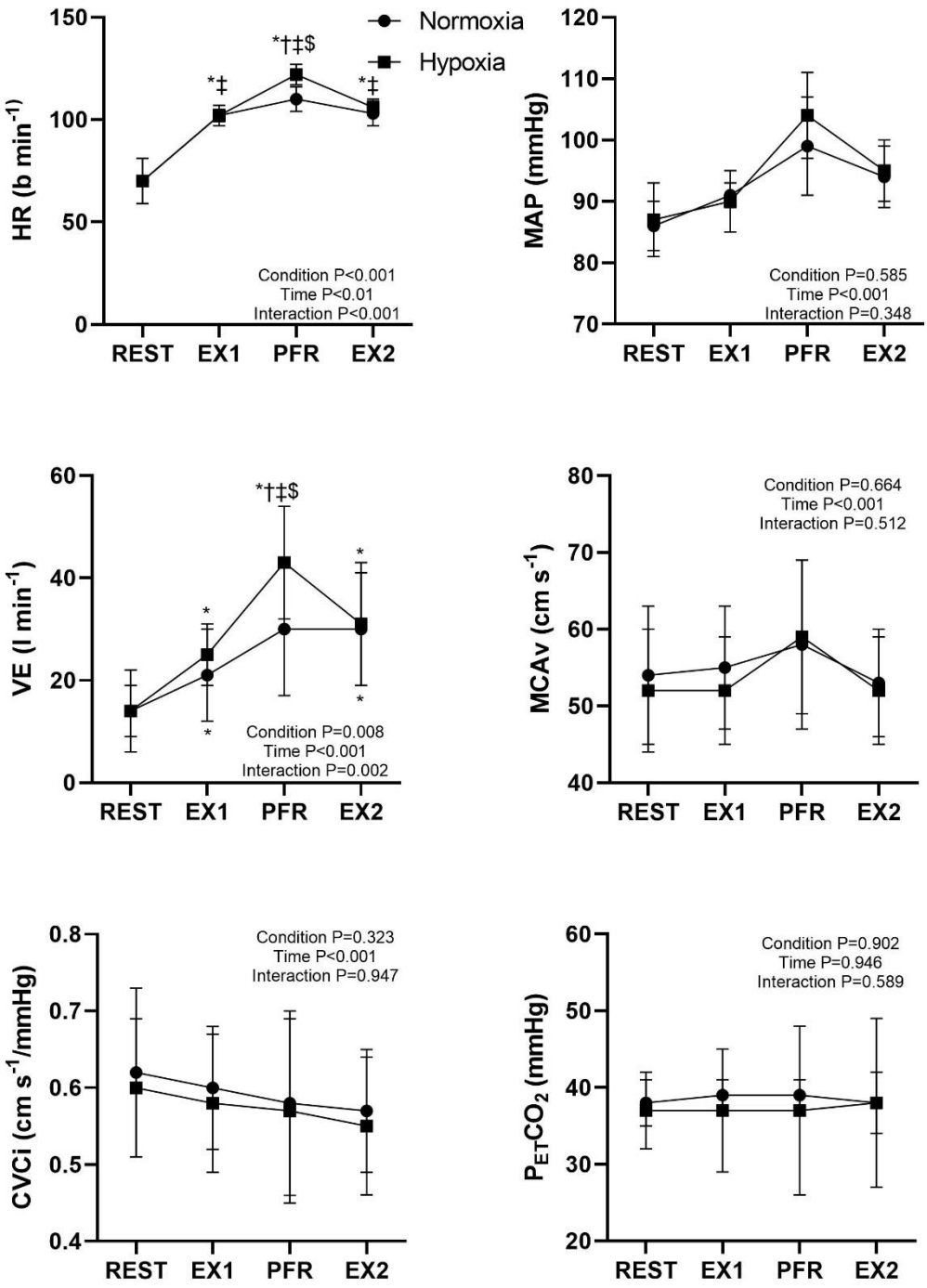


Figure 21 Cardiorespiratory and cerebrovascular responses to exercise with partial flow restriction (PFR) in both hypoxia and normoxia

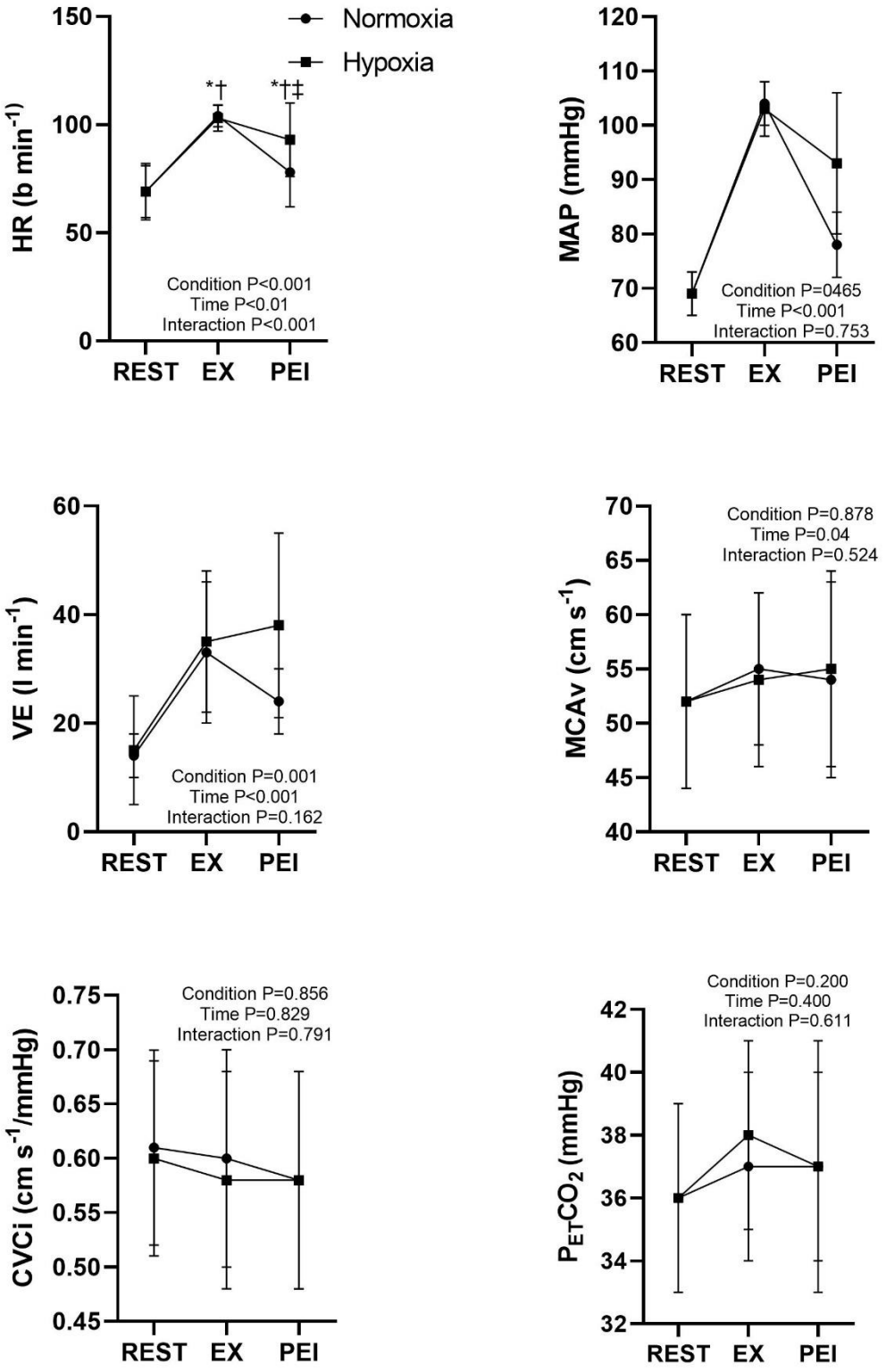


Figure 22 Cardiorespiratory and cerebrovascular variables during static hand grip exercise (IHG) and post-exercise (PEI) during hypoxic and normoxic conditions.

CHAPTER 8: GENERAL CONCLUSIONS

Instances of local ischaemia or systemic hypoxemia (e.g., high altitude or diving) trigger complex neural mechanisms that causes redistribution of oxygenated blood delivery to critical organs and active tissues. The first experimental chapter (Chapter 4) sought to discern the effects of trigeminal nerve stimulation on CBF from secondary factors associated with diving such as apnoea and changes in P_{aCO2}. To achieve this, six trials attempted isolate the effects of trigeminal nerve stimulation (simulated by facial cooling) from other secondary physiological factors such as the accumulation of CO_2 and the pressor response. In thirty-seven young healthy male and females, facial cooling failed to change MCAv in either poikilocapnic or isocapnic conditions. However, when facial cooling was accompanied by a concurrent BH (~30 s) both MCAv (Δ23%) and ICA_Q (Δ26%) increased from baseline. Interestingly, the increases in MCAv and ICA_Q were similar during a breath-hold regardless of facial cooling. There are several interpretations for these findings: 1) secondary factors associated with diving (e.g., CO_2 and pressor response) are more important than trigeminal nerve stimulation in modulating the cerebral perfusion response during diving; 2) there is no contribution of trigeminal nerve stimulation to cerebral perfusion during the diving response. However, a potential limitation of this study is the use of TCD for CBF assessment without direct measurement of MCA diameter. Nevertheless, duplex Doppler ultrasound measurements of ICA diameter and velocity were used to quantify ICAQ. But whether the MCAv and ICAQ responses observed are representative of changes in perfusion in other regions in the brain remains unknown.

The second experimental study (Chapter 5) explored the contribution of ventilation to increases in cerebral perfusion during moderate acute isocapnic hypoxia. The aim was to discern the effects of hypoxia (changes in arterial oxygenation), from the possible secondary effect of increases in ventilation, on increases in cerebral perfusion.

Four experimental trials were performed where participants breathed spontaneously in normoxia, breathed spontaneously in hypoxia, normoxia with ventilation matched to hypoxia (hyperpnoea), and hypoxia with ventilation matched to normoxia (hypopnoea). In eleven young healthy individuals, hypoxia increased in MCAv, but remained unchanged during hyperpnea (ventilation matched to hypoxia). Interestingly, increases in MCAv were similar during both hypoxic trials regardless of levels of ventilation. These findings suggest that ventilation failed to alter MCAv beyond that elicited by reduced oxygen saturation. As discussed above, a possible limitation of this study is concerned with CBF assessment. In this experiment no diameter measurements were quantified and MCAv was used as a surrogate to estimate CBF. Given the known strength and weakness of this assessment tool (Willie et al., 2011c), it is possible that the increases of perfusion quantify was underestimated due to the vasodilatory effect of acute hypoxia on CBF (Willie et al., 2011c). Therefore, future studies should explore whether ultrasonography or magnetic resonance imaging can help quantify the magnitude of influence on CBF.

The third experimental study (Chapter 6) explored the influence of systemic and local stimuli on cardiorespiratory and cerebrovascular responses. The aim was to determine the effect of hypoxia on cardiorespiratory and cerebrovascular to muscle metaboreflex activation. Participants completed three trials of 1) leg cycling with PFR 2) leg cycling followed by PEI 3) static hand grip exercise followed by PEI in conditions of poikilocapnic normoxia and hypoxia (FiO_2=13.5%). SpO_2 was significantly higher during normoxia (97 ±1%) than hypoxia (87% ±3%). MAP was either augmented (PFR) or maintained (PEI) during metaboreflex activation implying the success of the design. Metaboreflex activation during leg cycling (PFR) or following cycling (PEI) either increased or maintained above baseline HR, MAP, MCAv, and VE. However, no differences in HR, MAP, MCAv, and VE between conditions. Nevertheless, findings of this experiment extend the work of Fisher et al. (2013) in elucidating the effect preceding

exercise type and mode of activation on cardiovascular responses. Indeed, HR was increased or remained elevated during PFR and PEI, supporting the role cardiac parasympathetic activity withdrawal in this response. However, there are several limitations that are concerned with the results of the study. Firstly, the level of the hypoxia administered may not be sufficient to influence cardiorespiratory or cerebrovascular responses to metaboreflex activation. Although, HR increased suggesting chemoreflex activation, the level of hypoxia is considered moderate (FiO_2= 13.5%) compared to other studies (FiO_2=<10.5%). It may be possible that participants have adapted to hypoxia application due to the duration (~3.5 hr) of hypoxia and was not applied specifically during metaboreflex activation. Furthermore, poikilocapnic conditions of the study may restrict CBF responses due to increases in $P_{ET}CO_2$ following exercise hyperpnoea (Prodel et al., 2016b).

To address these limitations, experimental study 4 (Chapter 7) examined the interaction between a local (metaboreflex) and systemic (chemoreflex: Hypoxia=FiO_2=10.5%) stimuli on cardiorespiratory and cerebrovascular responses. Four trials of isocapnic leg cycling exercise were performed in which the metaboreflex was activated during exercise in normoxia (PFR+Normoxia); post exercise in normoxia (PEI+Normoxia); during exercise in hypoxia (PFR+Hypoxia); and post exercise in hypoxia (PEI+Hypoxia). Hypoxia augmented HR and VE during PFR. However, the responses were not different to the sum of metaboreflex and hypoxia only. A summative influence can best describe the interaction of hypoxia and metaboreflex on cardiorespiratory and cerebrovascular responses. Since metaboreflex activation modes were constant but hypoxia levels are variable (FiO_2:13.5% vs 10.5%) it is fair to suggest that hypoxia may have potentiated the chemoreflex and metaboreflex response. Therefore, the final experiment of the thesis demonstrated that with a larger hypoxic

stimulus the interaction of metaboreflex and hypoxia can augment cardiorespiratory responses to metaboreflex activation.

Several limitations that need to be addressed in the future. First given that hypoxia causes cerebral vasodilation, it is possible that the MCAv response may have been underestimated. Also, as discussed above TCD use in insonation is limited and flow measurements might provide more accurate assessment of CBF. Another limitation to this study is the difficulty in assessing skeletal muscle afferents, therefore the use of an intrathecal fentanyl might provide more accurate assessment of the role of the metaboreflex.

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APPENDICES

Appendix A Consent Form



Study Title:

The information given on this form is **strictly confidential.**

Please put your initials in

the boxes if answer is yes

Have you read and understood the participant information leaflet on

'Brain blood flow responses to the diving reflex'

☐

Have you had an opportunity to discuss the study and ask any questions

to members of the research team either on the telephone or during today's visit?

☐

Are you satisfied with the explanations that you have been given?

☐

Do you understand that you are able to withdraw from the study

- at any time.
- without need to give a reason.

☐

☐

Participant

I declare that the information given on this form is true to the best of my knowledge. The details of this study have been explained to me and I agree to take part in the study titled *'Brain blood flow responses to the diving reflex'*

Signature ……..... **Date** …….

Name (print) ……

Research Staff

I confirm that I have explained the nature of the study to the participant in terms that they understand, outlining both risks and benefits. I confirm that they have given their consent freely to partake in this study.

Signature ……..……..... **Date** ……...

Name (print) ……

Appendix B General health questionnaire

The University of Birmingham

School of Sport, Exercise and Rehabilitation Sciences

General Health Questionnaire

Name: .....

Address: .....

.....

.....

Phone: .....

Name of the responsible investigator for the study:

Please answer the following questions. If you have any doubts or difficulty with the questions, please ask the investigator for guidance. These questions are to determine whether the proposed study is appropriate for you. Your answers will be kept strictly confidential.

.	You are.....	Male	Female

.	What is your exact date of birth? Day..... Month.....Year..... So your age is..... years		
.	When did you last see your doctor? In the: Last week..... Last month..... Last six months..... Year..... More than a year.....		
.	Are you currently taking any medication?	YES	NO
.	Has your doctor ever advised you not to take vigorous exercise?	YES	NO
.	Has your doctor ever said you have “heart trouble”?	YES	NO
.	Has your doctor ever said you have high blood pressure?	YES	NO
.	Have you ever taken medication for blood pressure or your heart?	YES	NO
.	Do you feel pain in your chest when you undertake physical activity?	YES	NO
0.	In the last month have you had pains in your chest when not doing any physical activity?	YES	NO
1.	Has your doctor (or anyone else) said that you have a raised blood cholesterol?	YES	NO
2.	Have you had a cold or feverish illness in the last month?	YES	NO

3.	Do you ever lose balance because of dizziness, or do you ever lose consciousness?	YES	NO
4.	a) Do you suffer from back pain b) if so, does it ever prevent you from exercising?	YES YES	NO NO
5.	Do you suffer from asthma?	YES	NO
6.	Do you have any joint or bone problems which may be made worse by exercise?	YES	NO
7.	Has your doctor ever said you have diabetes?	YES	NO
8.	Have you ever had heat stroke?	YES	NO
9.	Have you ever had viral hepatitis?	YES	NO
0.	If you are female, to your knowledge, are you pregnant?	YES	NO
1.	Do you have kidney or gut dysfunction?	YES	NO
2.	Do you know of any reason, not mentioned above, why you should not exercise?	YES	NO
3.	Are you accustomed to vigorous exercise (an hour or so a week)?	YES	NO

I have completed the questionnaire to the best of my knowledge and any questions I had have been answered to my full satisfaction.

Signed: .....

Date: .....

Appendix C Altitude health questionnaire

The University of Birmingham

School of Sport, Exercise and Rehabilitation Sciences

General Health Questionnaire

Name: .....

Address: .....

.....

.....

Phone: .....

Name of the responsible investigator for the study:

Please answer the following questions. If you have any doubts or difficulty with the questions, please ask the investigator for guidance. These questions are to determine whether the proposed study is appropriate for you. Your answers will be kept strictly confidential.

.	You are.....	Male	Female

.	What is your exact date of birth? Day..... Month.....Year..... So your age is..... years		
.	When did you last see your doctor? In the: Last week..... Last month..... Last six months..... Year..... More than a year.....		
.	Are you currently taking any medication?	YES	NO
.	Has your doctor ever advised you not to take vigorous exercise?	YES	NO
.	Has your doctor ever said you have “heart trouble”?	YES	NO
.	Has your doctor ever said you have high blood pressure?	YES	NO
.	Have you ever taken medication for blood pressure or your heart?	YES	NO
.	Do you feel pain in your chest when you undertake physical activity?	YES	NO
0.	In the last month have you had pains in your chest when not doing any physical activity?	YES	NO
1.	Has your doctor (or anyone else) said that you have a raised blood cholesterol?	YES	NO
2.	Have you had a cold or feverish illness in the last month?	YES	NO

3.	Do you ever lose balance because of dizziness, or do you ever lose consciousness?	YES	NO
4.	a) Do you suffer from back pain b) if so, does it ever prevent you from exercising?	YES YES	NO NO
5.	Do you suffer from asthma?	YES	NO
6.	Do you have any joint or bone problems which may be made worse by exercise?	YES	NO
7.	Has your doctor ever said you have diabetes?	YES	NO
8.	Have you ever had heat stroke?	YES	NO
9.	Have you ever had viral hepatitis?	YES	NO
0.	If you are female, to your knowledge, are you pregnant?	YES	NO
1.	Do you have kidney or gut dysfunction?	YES	NO
2.	Do you know of any reason, not mentioned above, why you should not exercise?	YES	NO
3.	Are you accustomed to vigorous exercise (an hour or so a week)?	YES	NO

BACKGROUND QUESTIONNAIRE

Have you ever travelled to high altitude (>3,000m)? Yes / No

If so, please give details

What is the highest altitude you have ever been to? ……..m

Have you travelled to high altitude (>3,000m) in the past 6 months? Yes / No

Have you previously had to change your plans at high altitude because of sickness? Yes / No

If so, please give details

Have you previously been diagnosed with acute mountain sickness (AMS), high altitude pulmonary oedema (HAPE) or high altitude cerebral oedema (HACE)? Yes / No

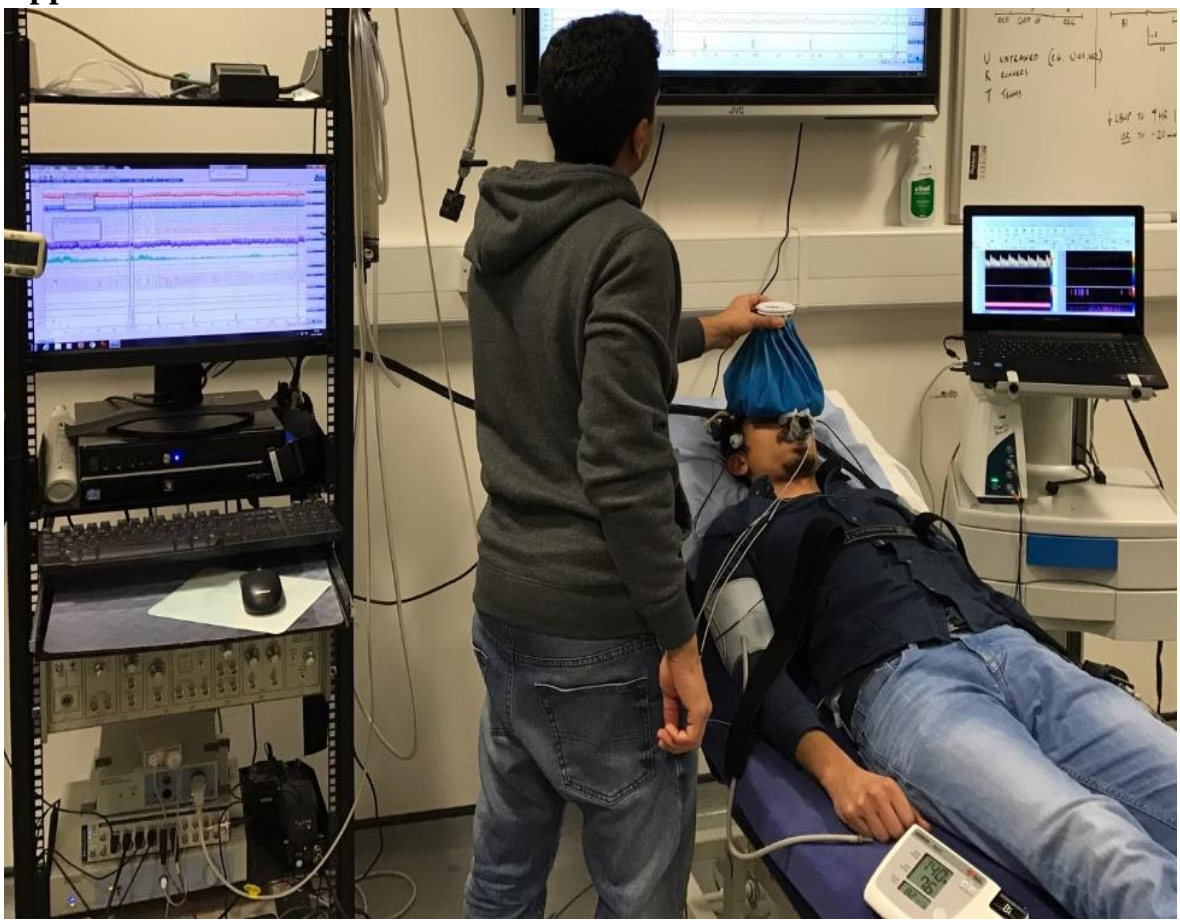
If so, please give details

I have completed the questionnaire to the best of my knowledge and any questions I had have been answered to my full satisfaction.

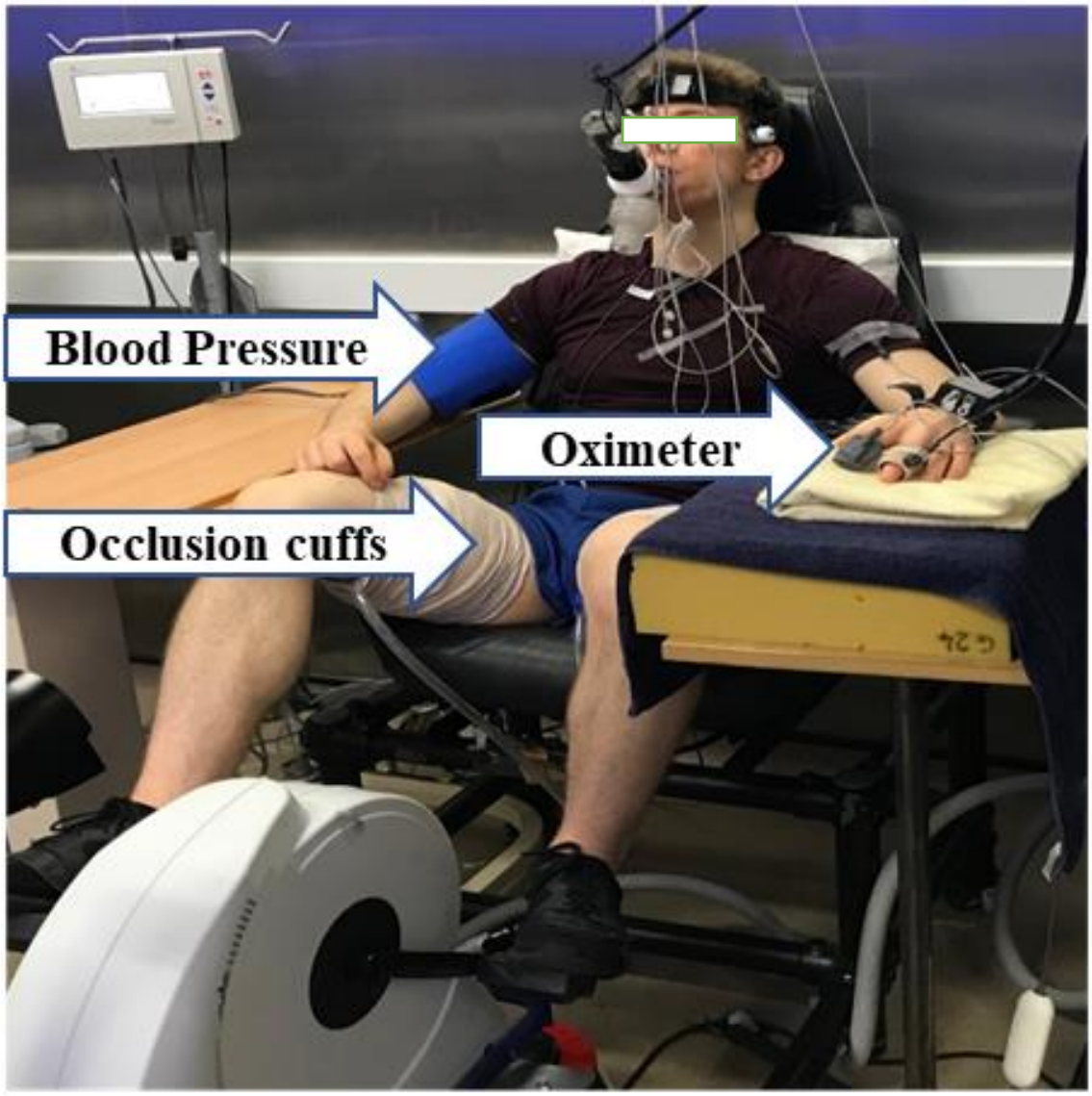
Signed: .....

Date: .....

Appendix D Cold face test.



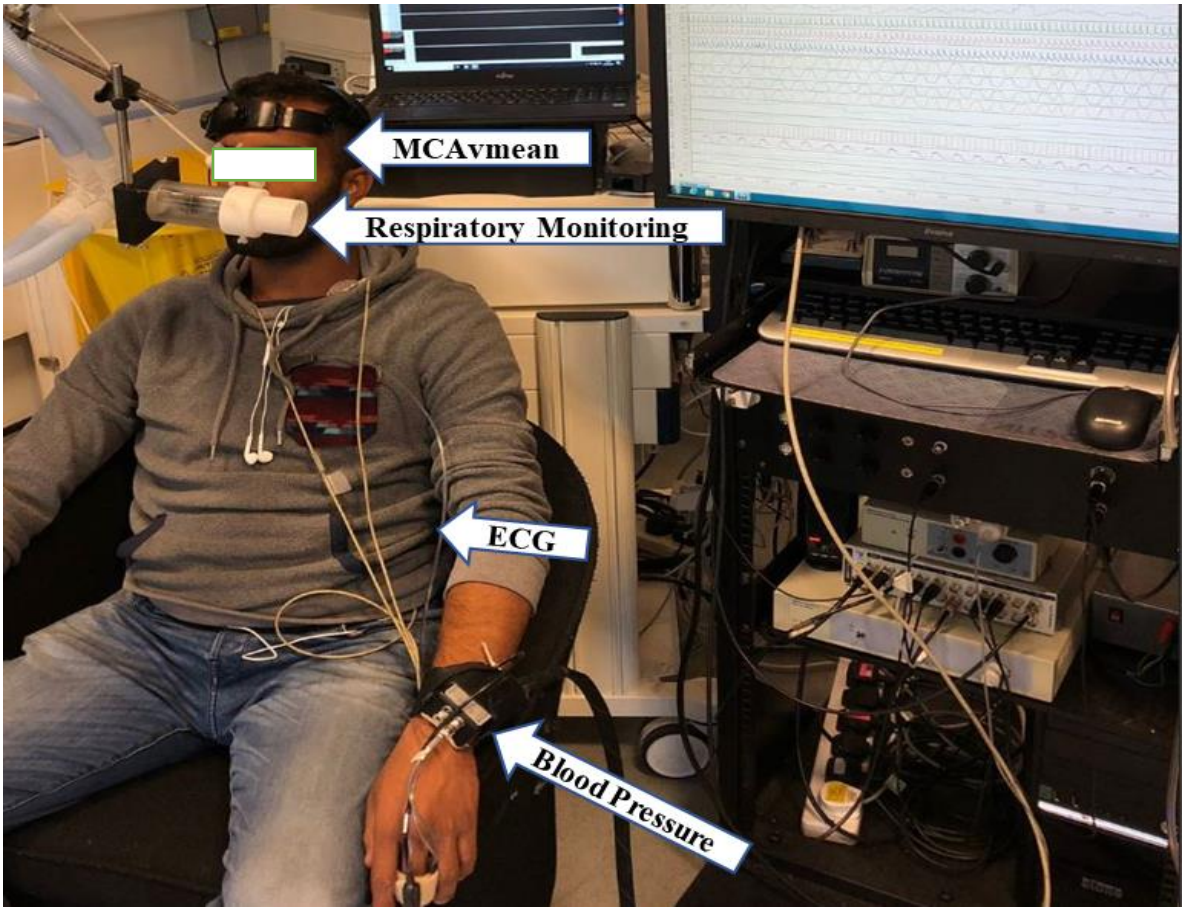
Appendix E Cycling exercise with metaboreflex activation experimental setup.



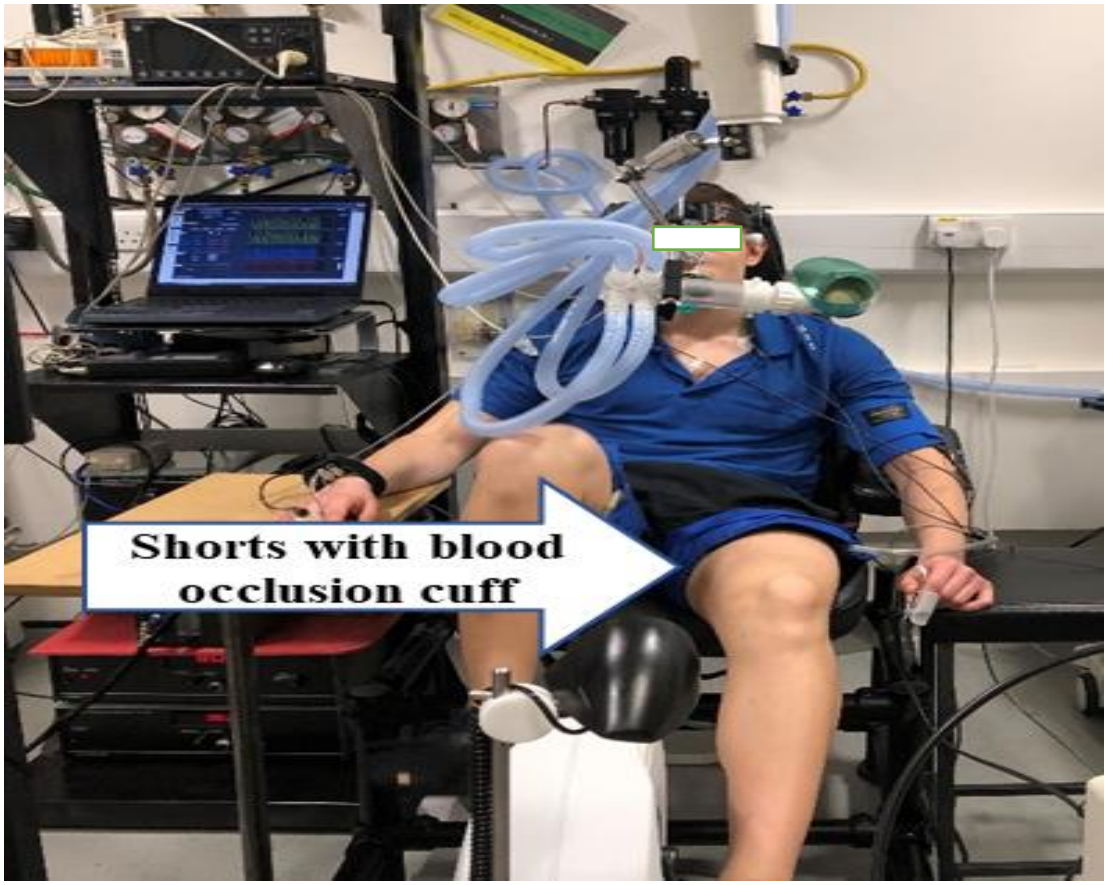
Appendix F Handgrip exercise.



Appendix G Dynamic end-tidal forcing system setup. Chapter 5 used the dynamic end tidal forcing system to examine middle cerebral artery velocity mean (MCAv) responses during 4 isocapnic trials. Electrocardiogrphy (ECG) was used to establish heart rate.



Appendix H Metaboreflex activation during exercise.



Appendix I Duplex ultrasound internal carotid artery (ICA) insonation.

