The undersigned certify that they have read, and recommend to the College of Graduate Studies for acceptance, a thesis entitled:

**INVESTIGATING THE USE OF REMOTE ISCHEMIC PRECONDITIONING TO ATTENUATE THE DECLINE IN VASCULAR FUNCTION DURING HYPOXIA**

submitted by Mathew Rieger in partial fulfilment of the requirements of the degree of Master of Science.

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Abstract

Application of repeated short duration bouts of ischemia to the limbs, termed remote ischemic preconditioning (RIPC), is a novel technique that may have protective effects on vascular function during hypoxic exposures. In separate parallel-design studies, at sea-level (SL; n=16), and after 8-12 days at high-altitude (HA; n=12; White Mountain, 3800m), participants underwent either a sham protocol or one session of 4x5 minutes of dual-thigh cuff occlusion with 5-minutes recovery. Brachial artery flow-mediated dilation (FMD; ultrasound), pulmonary artery systolic pressure (PASP; echocardiography), and internal carotid artery flow (ICA; ultrasound) were measured at SL in normoxia and isocapnic hypoxia [end-tidal PO₂ (PETO₂) maintained to 50mmHg], and during normal breathing at HA. The hypoxic ventilatory response (HVR) was measured at each location. All measures at SL and HA were obtained at baseline (BL), 1 hour, 24 hours, and 48 hours post-RIPC or sham. At SL, RIPC produced no changes in FMD, PASP, ICA flow, end-tidal gases or HVR in normoxia or hypoxia. At HA, although HVR increased 24 hours post RIPC compared to BL (2.05±1.4 vs. 3.21±1.2 L·min⁻¹·%SaO₂⁻¹, p<0.01), there were no significant differences in FMD, PASP, ICA flow, resting end-tidal gases. Accordingly, a single session of RIPC is insufficient to evoke changes in peripheral, pulmonary, and cerebral vascular function in healthy adults. Although chemosensitivity may increase following RIPC at HA, this did not confer any vascular changes. Under the current testing situations, for healthy and asymptomatic participants, the utility of a single RIPC session seems unremarkable during acute and chronic hypoxia.
Lay Summary

Remote ischemic preconditioning (RIPC) is a new technique characterised by repeated periods of occlusion and reperfusion of blood flow through a limb using a cuff or a tourniquet, with the goal of upregulating various anti-inflammatory and anti-oxidative pathways throughout the body. Clinical benefits of RIPC for protection against heart attack, stroke, and reperfusion injury have been well-documented, however the utility of RIPC in high-altitude settings remains unclear. We investigated the use of one single session of RIPC on attenuating previously established signs of vascular dysfunction observed during high-altitude and hypoxic exposures, and found no benefit in terms of reduced pulmonary artery pressure, vascular reactivity in the brachial artery, and improved cerebral blood flow; however, ventilatory sensitivity to hypoxia was increased 24 hours after treatment. Accordingly, the utility of one single 4x5 minute session of RIPC on healthy participants during high altitude travel or hypoxic exposure seems unremarkable.
Preface

Chapter 1. This chapter was written for the sole purpose of publication in this thesis. I wrote Chapter 1, while receiving extensive feedback from Prof. Ainslie through several editing processes.

Chapter 2. This chapter is a modified manuscript that has been accepted for publication in *Experimental Physiology*. Any changes made were strictly for the purpose of adhering to the format of this thesis document. Prof. Ainslie and I designed the experiment. Data collection was completed with assistance of Ryan Hoiland, Joshua Tremblay, as well as Drs. Anthony Bain, Daniela Flück, Michael Stembridge. Dr. James Anholm and Dr. Prajan Subedi provided useful discussions with respect to conception of the experiment. Data collection was completed at the University of British Columbia – Okanagan Campus, as well as at the Barcroft Research Station, White Mountain, California. I completed the analysis and wrote the manuscript, Prof. Ainslie and Ryan Hoiland provided extensive feedback and critically reviewed the manuscript for content and data interpretation, and all co-authors reviewed and edited the manuscript before final submission. Ethical approval was granted through the University of British Columbia Clinical Ethic Research Board (CREB #: H15-01513).

Chapter 3. I wrote chapter 3 and received extensive feedback from Prof. Ainslie prior to finalization.
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Acknowledgements

None of this would have been possible without the guidance, support, and patience from Prof. Phil Ainslie. He has provided me countless opportunities for personal and academic growth throughout my Master’s degree, and has taught me to develop a skill-set and a mind-set that will foster both enjoyment and success in future academic pursuits. The experiences you have given me, the connections you have helped me build, and the lessons you have taught me, are all things I will carry as I navigate through the years to come.

Thank you to my committee members Drs. Neil Eves, Chris McNeil and Brad Monteleone, as well as Dr. Ali McManus- it’s a privilege to work with all of you. The rapid acceleration of the research program here at UBCO is largely a result of your hard work and valuable commitment to training, and as students we are all incredibly lucky to have your support.

To my fellow members of the Ainslie Lab team, as well as everyone involved with the Centre for Heart, Lung and Vascular Health- thank you for making my time at UBCO such a positive experience. I feel extremely fortunate to work with, and hang out with such an awesome group of people day in and day out.

Finally, thank you to my parents, Dennis and Lisa Rieger for continually and unconditionally supporting me through 28 years of good and bad decisions. Everything I am today is because of my family, and their love and support is the keystone that has allowed me to pursue my most unconventional endeavours.
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<th>Definition</th>
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<tr>
<td>AMS</td>
<td>Acute mountain sickness</td>
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<tr>
<td>BL</td>
<td>Baseline</td>
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<td>CO₂</td>
<td>Carbon dioxide</td>
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<td>CMS</td>
<td>Chronic mountain sickness</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
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<tr>
<td>CO</td>
<td>Carbon monoxide</td>
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<td>CBF</td>
<td>Cerebral blood flow</td>
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<td>COX</td>
<td>Cyclooxygenase</td>
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<td>EDHF</td>
<td>Endothelial derived hyperpolarizing factor</td>
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<tr>
<td>FiO₂</td>
<td>Fraction inspired oxygen</td>
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<td>FMD</td>
<td>Flow-mediated dilation</td>
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<td>GTN</td>
<td>Glyceryl-trinitrate</td>
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<td>HA</td>
<td>High altitude</td>
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<td>HAPE</td>
<td>High altitude pulmonary edema</td>
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<td>HAPH</td>
<td>High altitude pulmonary hypertension</td>
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<td>HIF</td>
<td>Hypoxia inducible factor</td>
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<td>HPV</td>
<td>Hypoxic pulmonary vasoconstriction</td>
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<tr>
<td>HR</td>
<td>Heart rate</td>
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<td>HVR</td>
<td>Hypoxic ventilatory response</td>
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<tr>
<td>ICA</td>
<td>Internal carotid artery</td>
</tr>
<tr>
<td>IPC</td>
<td>Ischemic preconditioning</td>
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<tr>
<td>L-NMMA</td>
<td>N-methylargenine</td>
</tr>
<tr>
<td>MSNA</td>
<td>Muscle sympathetic nerve activity</td>
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<td>MCA</td>
<td>Middle cerebral artery</td>
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<tr>
<td>MI</td>
<td>Myocardial infarction</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<td>NOS</td>
<td>Nitric oxide synthase</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<tr>
<td>O₂</td>
<td>Oxygen</td>
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<tr>
<td>PAP</td>
<td>Pulmonary artery pressure</td>
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<tr>
<td>PASP</td>
<td>Pulmonary artery systolic pressure</td>
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<tr>
<td>PCO₂</td>
<td>Partial pressure of carbon dioxide</td>
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<tr>
<td>PaCO₂</td>
<td>Arterial partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>PO₂</td>
<td>Partial pressure of oxygen</td>
</tr>
<tr>
<td>PAO₂</td>
<td>Alveolar partial pressure of oxygen</td>
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<td>PaO₂</td>
<td>Arterial partial pressure of oxygen</td>
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<tr>
<td>PETO₂</td>
<td>End tidal partial pressure of oxygen</td>
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<tr>
<td>PaCO₂</td>
<td>Arterial partial pressure of carbon dioxide</td>
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<tr>
<td>PVR</td>
<td>Pulmonary vascular resistance</td>
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<td>qICA</td>
<td>Internal carotid artery flow</td>
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<tr>
<td>RBC</td>
<td>Red blood cell</td>
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<tr>
<td>RIPC</td>
<td>Remote ischemic preconditioning</td>
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<tr>
<td>RIC</td>
<td>Remote ischemic conditioning</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SaO₂</td>
<td>Arterial oxygen saturation</td>
</tr>
<tr>
<td>ScO₂</td>
<td>Calculated arterial oxygen saturation</td>
</tr>
<tr>
<td>SL</td>
<td>Sea level</td>
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<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>SpO₂</td>
<td>Peripheral oxygen saturation</td>
</tr>
<tr>
<td>SRAUC</td>
<td>Shear rate area under the curve</td>
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<tr>
<td>VE</td>
<td>Ventilation</td>
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1 Introduction

1.1. Overview
The application of brief, repeated rounds of ischemia to an organ or tissue - called ischemic preconditioning (IPC) - protects the targeted tissue against subsequent ischemia-related injuries. Mechanisms of action are poorly understood; however, these short, non-lethal periods of ischemia appear to upregulate various vasoactive, anti-inflammatory and anti-oxidative pathways to defend against future, larger ischemic events. Recently, it has been discovered that these protective effects are observed globally throughout the body, and can be mediated through conditioning tissue distant to the targeted organ or system, through remote ischemic preconditioning (RIPC). Cyclic periods of occlusion and reperfusion of blood flow through an entire arm or leg, for example, can have protective effects on the heart, lung, kidneys, and brain.

Hypoxic exposure, or ascent to high altitude (HA), is associated with alterations in global vascular function and responses throughout the body. Many of the mechanisms and pathways proposed to play a role in regulating vascular responses to hypoxia are also active with RIPC. These observations suggest that RIPC may act in a way to precondition not only against ischemia-related injuries, but also may alter hypoxic vascular responses as well. The aim of this review is therefore three-fold. First, it provides review of the literature pertaining to normal vascular function in the periphery, the lungs, and the brain, at rest as well as during hypoxia. Second, this thesis reviews recent advancements examining the utility of RIPC in health and disease, and how this treatment affects the tone and function of these vascular beds. Finally, this review examines the current literature pertaining to RIPC treatment prior hypoxic exposure, and identifies how RIPC might be beneficial to attenuate some of the risks associated with high altitude travel.
1.2. Peripheral vascular function

1.2.1. The endothelium

The endothelium, which lines the entire vascular system, was once considered only as a simple barrier between the blood and the vessel wall. In 1980, Robert Furchgott and John Zawadski discovered that an intact endothelium was required for arterial smooth muscle relaxation by acetylcholine (Furchgott & Zawadzki, 1980). Thereafter, it quickly became understood that the endothelium is in fact a dynamic organ crucial for the health, function, and maintenance of homeostasis within the entirety of the cardiovascular system (Galley & Webster, 2004). As a semi-permeable barrier, the endothelium regulates the transfer of molecules into and out of the circulatory system, as well as exhibits a number of paracrine, autocrine, and endocrine functions (Widlansky et al., 2003). The global role of the endothelium is beyond the scope of this review, but among the molecules secreted by endothelial cells are pro-coagulants, antithrombotics, matrix products, inflammatory mediators, growth factors, and vasoactive factors [reviewed in: (Deanfield et al., 2007)]. This review will mainly focus on endothelial function as it relates to in-vivo vascular tone.

1.2.2. Molecular signals derived by the endothelium

The endothelium responds to both pharmacological and physical stimuli and is responsible for the release of both vasodilatory and vasoconstrictive factors (Sandoo et al., 2010). The resultant balance of endothelial agonistic and antagonistic signalling on the smooth muscle cells (SMCs) is a key factor in maintaining vascular health and homeostasis in humans (Widlansky et al., 2003). Nitric oxide (NO) is a powerful vasodilator (Palmer et al., 1987) that is formed under the influence of enzyme nitric oxide synthase (NOS). Within the endothelium, NO is synthesized during conversion of L-arginine to L-citrulline by endothelial NOS (eNOS) (Tousoulis et al., 2012), and it is released predominantly in response to increases in shear stress, the pulling force of blood flow along the endothelial lining (Harrison et al., 2006). Then, NO diffuses into vascular smooth muscle cells, where it promotes increased intracellular levels of cyclic guanosine-3,5-monophosphate (cGMP) and the resultant cascade initiates smooth muscle relaxation and vessel dilation (Palmer
et al., 1988). While NO is primarily recognized as a vasodilator, it exerts a key role in vascular health through additional anti-inflammatory, antiplatelet, and antithrombotic pathways. Impaired bioavailability of NO, a consequence of either impaired NO synthesis, or excessive oxidative NO degradation, is a central feature of endothelial dysfunction, and often presents as a precursor to hypertension, hypercholesteremia, or atherosclerosis (Jin & Loscalzo, 2010).

While NO is the most potent vasodilator released by the endothelium, a recent meta-analysis found that when the NO-blockade, L-NMMA, was administered, brachial artery flow-mediated dilation (FMD) was only reduced by an average of ~50% (Green et al., 2014). This finding suggests that smooth muscle relaxation is still largely contingent on alternative dilatory pathways in addition to the NO system. The endothelium is responsible for secretion of a number of different vasoactive molecules, likely to provide a form of overlap and redundancy between vasodilatory mechanisms. Two additional families of vasodilators are synthesized by the endothelium, the cyclooxygenase (COX)-catalyzed prostaglandins (Lavallée et al., 2001), and the endothelial-derived hyperpolarizing factors (EDHFs). Prostaglandins cross the intercellular space and elicit a cAMP-dependent hyperpolarization of the vascular SMCs, while EDHFs cause vasodilation by stimulating K$^+$ or Na$^+$/K$^+$-ATPase (Sandoo et al., 2010). A number of EDHFs have been identified, including carbon monoxide (CO), hydrogen sulphide (H$_2$S), hydrogen peroxide (H$_2$O$_2$), and C-natriuretic peptide (CNP) (Félétou & Vanhoutte, 2009).

1.2.3. Assessing vascular function

It is possible to measure the responsiveness of a blood vessel, by quantifying changes in flow or changes in vessel diameter in response to different stimuli. A greater magnitude of response is normally associated with increased health or function of the endothelial system, and as reactivity of the blood vessels diminishes we begin to see signs of vascular dysfunction (Durand & Gutterman, 2013). When investigating the cause of vascular dysfunction, we can look at either endothelial-dependent, or endothelial-independent contributors to the dilatory response of the blood vessel. Endothelial-dependent dilation refers to factors pertaining directly to the endothelium, and impairments are usually related to bioavailability and synthesis/release of vasoactive molecules. In contrast, endothelial-independent dilation refers to the function of the
SMCs, and the ability of the muscular lining to relax. Not all tests of vascular reactivity are able to differentiate between the two, but it is important to consider where the source of dysfunction originates when evaluating the function of the arteries. Tests of vascular reactivity are described in more detail in the appendix.

1.2.4. Clinical relevance

Cardiovascular disease (CVD) is currently the leading global cause of morbidity and mortality, and markers of vascular dysfunction often present early in the progression of the disease-state (Gokce et al., 2003; Lloyd-Jones et al., 2006). Dysfunction at both the micro- and macrocirculatory levels have been proposed to play a role in the advancement in CVD (Langham & Wehrli, 2011), and the screening endothelial function has been previously used as a risk predictor in otherwise asymptomatic individuals (Naghavi et al., 2003; Green et al., 2011). Endothelial dysfunction comes often as a result of an imbalance between NO production and consumption, and can lead to a cascade of events directly triggering the deposition of atherosclerotic plaque on the arterial wall. Under these conditions, a pathogenic state favourable to platelet and leukocyte adhesion is created. Cytokines become activated, which increases vessel wall permeability to inflammation mediators and oxidized lipoproteins, and this ultimately results in damage to the arterial wall and smooth muscle cell proliferation, with subsequent atherosclerotic plaque build-up (Rudic et al., 1998).

Ultimately, the health of the cardiovascular system is largely dependent on the health of the arteries, and any dysfunction of the vascular system can be reflected by large, proportionate increases in cardiovascular risk and mortality. In a meta-analysis of studies investigating flow-mediated dilation as a predictor of prognosis for future cardiovascular outcomes, Green et al (2011) reported that a 1% increase in FMD was associated with a 9% reduction in relative risk of future cardiovascular events. As such, it is important to have accessible, proper assessments of endothelial-independent and endothelial-dependent vascular responses for early detection of dysfunction, diagnosis, monitoring, and even prevention of CVD. Endothelial responses can be tested at different levels of the vascular tree, which allows for the study of both resistance and conduit vessels. For example, assessment of the microcirculation allows for investigation into the
role of the endothelium in the resistance vessels that determine blood pressure and blood flow, whereas tests of the conduit vessels provide insight into the processes that lead to plaque formation in atherosclerotic-prone arteries (Barac et al., 2007).

1.2.5. Hypoxia and vascular function

Given that >140 million people live at altitudes >2500m (Moore et al., 1998), and millions more are exposed to hypoxia through air travel and activities such as skiing and trekking on a daily basis, it is important understand how the endothelium may contribute to the risk and progression of cardiovascular disease in a hypoxic environment. Flow-mediated dilation is a highly sensitive measurement of endothelial function, and day-to-day variation in measured responsiveness of the endothelium varies greatly based on a multitude of factors. Acutely, FMD responses can vary based on behavioural factors such as diet (Heiss et al., 2007; Volek et al., 2009), previous exercise (Dawson et al., 2013), and caffeine and alcohol intake (Papamichael et al., 2005; Hijmering et al., 2007); as well as physiological factors such as level of oxidative stress (Harris et al., 2009), sympathetic nerve activity (Lewis et al., 2014), hormonal factors (Torgrimson et al., 2007), altered blood flow patterns (Tremblay et al., 2016), diurnal variation (Kim et al., 2015). All of these factors are interrelated and contribute to large within-subject variation of FMD scores, making it difficult to perform well-controlled, repeated measures studies in the field or at high-altitude. High-altitude is associated with elevations in sympathetic nerve activity (Hijmering et al., 2002; Atkinson et al., 2015), oxidative stress (Bailey et al., 2009), and disrupted arterial shear patterns (Tremblay et al., 2016), all of which might contribute to an impairment in FMD. Despite this potential impairment, observations from studies evaluating endothelial function at high altitude are not entirely uniform. Severity of hypoxia, duration of exposure, and concomitant exercise (e.g., trekking vs. passive ascent) all likely contribute to the presence and magnitude of dysfunction observed in FMD. For example, acute, passive hypoxic exposures have reported both impairments (Frøbert et al., 2008; Lewis et al., 2014; Bakker et al., 2015a) and preservation (Tremblay et al. 2016; Tymko et al. 2016; Bruno et al. 2016; Iglesias et al. 2015) of endothelial function compared to normoxic controls. Multi-day treks to high altitude have reported impaired FMD (Lewis et al., 2014; Bakker et al., 2015a), although these studies were also conducted after a longer duration of hypoxic exposure. Methodological and physiological considerations that could possibly explain
the differences in findings will be explained in detail in: Section 3.2.2 RIPC and peripheral vascular function.

1.3. Pulmonary vascular function

When evaluating pulmonary vascular function during hypoxic challenges, it is also highly important to consider the state of the pulmonary vasculature, as the capillaries and resistance vessels within the lung are highly responsive to changes in inspired oxygen tension. Pulmonary vascular resistance (PVR) increases upon ascent to high altitude or with exposure to normobaric hypoxia. For example, hypoxic pulmonary vasoconstriction (HPV) can be detected with elevations as low as 1600m or with reductions of \( F_iO_2 \) (fraction of inspired oxygen) to 0.18 and lower (Swenson et al., 1994; Smith et al., 2012). This HPV is not uniform throughout the lung- rather, it thought to be an adaptive response in order to restrict blood flow to poorly ventilated areas, in favour of optimizing ventilation-perfusion matching. While the partial pressure of alveolar \( O_2 \) (PAO\(_2\)) sets the threshold for initiating HPV, bronchial \( PO_2 \) and mixed-venous \( PO_2 \) may also contribute to vasoconstriction in the lung in an additive fashion (Swenson, 2013). The magnitude of HPV is extremely variable between individuals and can vary by a factor of five-fold (Grünig et al., 2000). Reasons for these differences are still poorly understood. Pulmonary artery pressure (PAP) begins to rise within the first minute of exposure to hypoxia (Morrell et al., 1995; Dorrington et al., 1997) and remains elevated as long as the hypoxic stimulus is present. Multiple temporal components contribute to the rise in PAP; following the rapid-onset initial increase is a second phase of elevation, starting at around 20 minutes and plateauing at ~2 hours (Talbot et al., 2005). The initial increase in PVR and PAP upon exposure to hypoxia may be attributed to intrinsic calcium-dependent smooth muscle contraction (Aaronson et al., 2002), whereas the subsequent intensification of PAP is likely the product numerous modulating influences, as discussed next.

1.3.1. Moderators of acute hypoxic pulmonary vasoconstriction

The mechanisms underpinning HPV are multifaceted and complex, and work through numerous direct and indirect pathways. Hypoxia can act directly on the smooth muscle cells surrounding the pulmonary vasculature, through inhibition of potassium channels, leading to membrane
depolarization and subsequent entry of extracellular calcium into the cells. This influx of calcium triggers further release of calcium from the sarcoplasmic reticulum and subsequent smooth muscle contraction (Sylvester et al., 2012). Furthermore, the contractile elements of the SMCs are more sensitive to calcium under hypoxic conditions, aided by a hypoxia-induced elevation in Rho-kinase activity (Weigand et al., 2011). It has been suggested that a key signal for this component of HPV is the hypoxia-mediated increase in mitochondrial ROS generation (Schumacker, 2011), and indeed it has been shown that elevated ROS and reduced NO-species availability is strongly related to the rise in PAP during hypoxia (Bailey et al., 2010).

On top of directly facilitating contraction of the pulmonary SMCs, hypoxia works through a number of indirect pathways to influence pulmonary vascular tone. Pulmonary vascular tone is largely influenced by pulmonary endothelial cells, which produce a balance of vasoconstrictors (endothelin) and vasodilators (NO, prostacyclin, endothelin-2, carbon monoxide) that act on the vascular SMC’s to modulate PVR (Swenson, 2013). When exposed to extreme hypoxia (3% O₂), isolated pulmonary arterial endothelial cells increase production of hydrogen peroxide (Irwin et al., 2009), suggesting these cells may generate ROS and act as a trigger for HPV. Hypoxia is also sensed by pulmonary capillary endothelial cells, where they respond with membrane depolarization. It has been proposed that this depolarization is propagated, through connexion-40 gap junctions, back to the upstream resistance vessels where they initiate HPV (Wang et al., 2012). Additionally, red blood cells play a role in regulating pulmonary vascular resistance. As hematocrit is increased in hypoxia, viscosity of the blood is increased, mediated by an increase in red blood cell concentration. Furthermore, hypoxic red blood cells are a source of ROS that will enhance HPV within the lung (Kiefmann et al., 2008).

It is also important to consider the role of the hypoxic ventilatory response (HVR) in moderating HPV. While elevations in the HVR may indirectly reduce HPV through improved alveolar ventilation and hence maintenance of a higher PAO₂ (Luks et al. 2017), studies investigating peripheral chemoreceptor responsiveness and hypoxic pulmonary vasoconstriction have been mixed. Hoiland et al. (2015) observed no correlation between isocapnic or poikilocapnic HVR measured at sea level, and PASP measured at 5050 m, suggesting that chemoreceptor responsiveness has little predictive value for pulmonary pressures at high
altitude. In contrast, Albert and Swenson (2014) found a significant inverse relationship between the poikilocapnic HVR and PASP when participants breathed four different hypoxic gas mixtures. Possible explanations for these differences could include changes in acid-base balance upon ascent to high altitude, the confounding effects of large intra-individual daily changes in the HVR (Zhang & Robbins, 2000), or the concurrent use of Diamox during ascent during the former (Hoiland) study, which may influence the degree of vascular remodelling (Pichon et al., 2012). Despite this uncertainty of the relationship between HVR and PASP in otherwise healthy individuals, it appears that HVR is blunted in individuals susceptible to HAPE (Hackett et al., 1988; Hohenhaus et al., 1995). Additionally, the subsequent hypocapnia and alkalosis from elevations in ventilation will also attenuate pulmonary vasoconstriction, independent of FIO₂ (Balanos et al., 2003; Ketabchi et al., 2009).

1.3.2. Delayed elevations in pulmonary pressure with hypoxia

The reversibility of acute HPV can be easily tested with administration of hyperoxia, and in the early stages of hypoxia a rapid and full reversal of PAP is observed when supplemental oxygen is given. As early as 8 hours into the hypoxic exposure, the rise in pressure cannot be quickly and fully reversed with return to normoxia, demonstrating the presence of delayed pulmonary vascular responses are not as transient as those contributing to acute HPV (Dorrington et al., 1997). Activation of hypoxia-sensitive inflammatory and proliferative pathways leads to remodelling of the pulmonary arteries and arterioles, contributing to a sustained increase in pulmonary vascular resistance. Mechanisms for this remodelling of the arteries are complex and poorly understood [reviewed in: (Stenmark et al., 2006)], and processes are thought to differ based on site and vessel size within the pulmonary circulation. Upregulation of hypoxia inducible factor 1α (HIF-1α) and its downstream derivatives has been targeted as a key stimulus for sustained PAP elevations, as HIF-1α transcribes for multiple processes that contribute to cellular proliferation and migration, and upregulation of extracellular matrix proteins. Furthermore, in HIF-1α deficient mice, the acute HPV response is largely attenuated when compared to wild-type mice (Shimoda et al., 2001), and in humans with Chuvash polycythemia, a genetic condition involving augmented HIF expression, HPV is significantly elevated (Stenmark et al., 2006). Together, these findings suggest HIF-1α is also important to the contractile response of SMCs to hypoxia.
1.3.3. Implications at high altitude

Ascent to high altitude is characterised by a progressive increase in PAP in all humans and mammalian species (Swenson, 2013). While HPV may offer a survival advantage in cases of pneumonia or pneumothorax by limiting shunt-induced hypoxemia, the physiological advantage to an adult travelling or sojourning at high altitude seems questionable. Natives of the Tibetan Plateau, the most highly adapted human population to high altitude, exhibit the smallest increases in PAP in response to progressive increases in elevation (Beall et al., 2001), reinforcing the notion that HPV is not a beneficial adaptation to survival in hypoxic environments.

1.3.4. Acute high altitude

Upon ascent to altitudes >2500m, individuals pose a risk of developing high altitude pulmonary edema (HAPE), and this risk increases progressively with further ascent. HAPE presents after an excessive rise in pulmonary vascular resistance in response to hypoxia, leading to increased microvascular pressures and subsequent damage to the alveolar capillary barrier (Bartsch, 2005). Large proteins and erythrocytes can leak into the alveolar space, and the build-up of fluid causes severe dyspnea and hypoxemia (Swenson & Bärtsch, 2012). Individuals who are HAPE-susceptible present with reduced measures of NO-bioavailability during hypoxia, and increased production of endothelin, suggesting pulmonary vascular dysfunction plays a large role in development of the condition (Bailey et al., 2010). This response is further augmented in those who have lower hypoxic ventilatory responsiveness, and an exaggerated level of hypoxic pulmonary vasoconstriction. Furthermore, many high altitude activities (e.g. trekking and mountaineering) involve extended periods of strenuous exercise, which further exacerbates increases in PAP and risk of developing HAPE (Naeije et al., 2010).

1.3.5. Chronic high altitude

Individuals at high altitudes have an increased risk of developing high altitude pulmonary hypertension (HAPH), secondary to prolonged HPV and vascular remodelling of the pulmonary arterioles (Heath et al., 1990, 1990; Maggiorini & Léon-Velarde, 2003). This remodelling includes
endothelial dysfunction, smooth muscle cell proliferation, and adventitial thickening (Durmowicz & Stenmark, 1999; Moudgil et al., 2005). Direct biochemical mechanisms underlying the pathogenesis of HAPH are poorly understood, although it is likely that a reduction in NO production plays a significant role (Beall et al., 2001) in its development. Over time, HAPH can lead to the congenital failure of the right heart; this is especially evident when combined with polycythemia normally observed in chronic mountain sickness (Naeije and Vanderpool 2013).

1.4. Cerebral Vasculature

1.4.1. Regulation of cerebral blood flow

The metabolic cost of maintaining neuronal function is extremely high, yet the brain has almost no capacity for intracellular energy storage. Thus, regulation of cerebral blood flow (CBF) is tightly linked to metabolic need, and disruptions to brain blood flow carry almost immediate consequences (Van Lieshout et al., 2003). For this reason, it is important to continue to develop our understanding of regulatory mechanisms in the intra-cranial and extra-cranial arteries in order to monitor, identify, and even prevent cerebrovascular casualties. It is known that CBF is regulated by a network of integrated systems, with global flow primarily being moderated by arterial pressure of CO₂ (and subsequent extravascular pH), blood oxygen content, blood pressure and sympathetic nerve activity. Regionally, activated brain areas can increase or decrease flow in a feed-forward fashion in order to match blood supply to local metabolic demand, as specialized astrocyte cells directly couple neuronal activation to upstream vascular tone. The focus of this section is not to provide an in-depth review of the integrative regulation of CBF [see: (Willie et al., 2016) for detailed review]. Rather, the goal is to provide a framework of how vascular function within the cerebral vessels is a key component to our health and survival at low and high-altitudes.

1.4.1.1. Regulation by CO₂

Cerebral vasculature is unique from the peripheral arteries (Ainslie et al., 2005), as the entire cerebrovascular tree is highly sensitive to changes in arterial pressure of CO₂ (P₅CO₂). Hypercapnia causes rapid increases in CBF, whereas hypocapnia causes a subsequent reduction in
flow, and the magnitude of reactivity to altered P_aCO_2 directions happens in a relatively linear fashion in both directions (Skow et al., 2013). Globally, the magnitude of flow reactivity to hypercapnia is nearly double that of hypocapnia (Willie et al., 2012). While CO_2 is a contributor to the regulation of CBF, related adjustments in flow are ultimately a product of a cerebral drive to maintain pH at a constant level. Local alterations in CBF are mediated via extravascular pH, independent of intraluminal CO_2 (Kontos et al. 1977).

1.4.1.2. Regulation by O_2

Reductions in the partial pressure of inspired and arterial oxygen elicit compensatory increases in CBF; however, this only occurs when P_aO_2 is reduced below ~50mmHg (Severinghaus et al., 1966a; Willie et al., 2012, 2014). The magnitude of this response depends on the severity of the hypoxic stimulus, as hypoxemia will cause little change in CBF until P_aO_2 reaches a threshold on the steep portion of the oxygen-dissociation curve, suggesting arterial O_2 content, rather than P_aO_2, is the key regulating factor. Hypoxic cerebral vasodilation is multifactorial and involves overlapping mechanisms to create protective redundancies, however it is likely that deoxyhemoglobin plays a primary role in regulating CBF during hypoxia through release of NO and ATP, and increased deoxyhemoglobin nitrite reductase activity [for detailed review see: (Hoiland et al., 2016)].

1.4.1.3. Other moderating factors

Static and dynamic changes in cerebral perfusion pressure are transmitted into changes in CBF in a primarily pressure-passive manner (Lucas et al., 2010; Tzeng et al., 2010; Tan, 2012; Tzeng & Ainslie, 2014). While the cerebral vasculature does exhibit a capacity to respond to changes in pressure, this response only serves to dampen flow responses rather than to maintain flow at a constant set-point. The cerebral vasculature appears to be more sensitive to reductions in blood pressure than increases, as suggested by observations of larger changes (reductions) in flow in the hypotensive range when compared to hypertensive interventions (Numan et al., 2014). Cerebral-specific sympathetic nervous activity is thought to play a role in buffering surges in pressure,
thereby protecting the microvasculature against pressure-induced microvascular damage (Cassaglia et al., 2008).

Local alterations in cerebral blood flow can be directly modified by neuronal activity, in a process referred to as neurovascular coupling. Neurological processes within the brain carry a high metabolic demand, driven primarily by the maintenance of ionic gradients related to action and resting potentials. Increases in neuronal firing are paired with increased metabolic demand of the tissue (Attwell & Laughlin, 2001), and this is met with rapid increases blood flow (and subsequent O₂ and glucose delivery) to the activated region [reviewed in: (Attwell et al., 2010). Astrocyte cells are thought to play a feed-forward role, directly linking synaptic activity upstream microvascular beds, although specific in vivo mechanisms of neurovascular coupling within humans are still poorly understood.

1.4.2. Cerebral blood flow at high altitude

Ascent to high altitude represents an excellent model to examine integrative regulation of cerebral blood flow. Over the first 1-2 weeks, rapid ventilatory and hematological adjustments are continuously taking place, and the cerebral vasculature must adjust to antagonistic stimuli for both dilation and constriction of the vessels. The initial stimulus to increase CBF upon ascent to HA is the reduction in PₐO₂, but as ventilatory compensation and acclimatization occurs, PₐCO₂ begins to fall, promoting respiratory alkalosis. The resultant hypocapnia opposes the hypoxemic rise in CBF, and after a peak in CBF 1-3 days after initial HA exposure, CBF begins to fall (Severinghaus et al., 1966a; Huang et al., 1987; Baumgartner et al., 1994; Frøbert et al., 2008; Willie et al., 2011a). This reduction is further supported by a gradual increase in PₐO₂ as ventilation progressively rises, and also through a gradual elevation in blood viscosity as hematocrit begins to rise. Thus, global cerebral blood flow at high altitude depends on time and severity of altitude exposure, and is moderated extensively by individual responses dictating: a) hypoxic cerebral reactivity b) the hypoxic ventilatory response, c) hypocapnic cerebral reactivity and d) the hypercapnic ventilatory response upon acclimatization. In addition to these processes, cerebrovascular tone and function can be influenced by numerous other hypoxia-induced systemic changes, including increased sympathetic nerve activity, altered NO and adenosine availability,
and increased oxidative stress (Ainslie & Subudhi, 2014). Further research needs to be performed in order to determine how each of these factors plays into cerebrovascular function at high altitude.

1.4.2.1. Acute mountain sickness

Early studies suggested that increased CBF may play a role in the pathogenesis of acute mountain sickness [AMS] (Baumgartner et al., 1994); however, more recent findings fail to demonstrate a correlation between increased flow in the major cerebral arteries, and Lake Louise AMS score [reviewed in: (Ainslie & Subudhi, 2014)], suggesting that development of AMS is not a direct function of arterial dilation per se. More recently, a longitudinal study using 3D pseudo-continuous arterial spin labelling observed increases in global cerebral blood flow in AMS-positive participants compared to those without AMS, despite no differences in the cross-sectional areas of the internal carotid, middle cerebral, and basilar arteries (Liu et al., 2017). These findings implicate increased flow in the smaller cerebral arterioles in the pathogenesis of AMS, which may be influenced by metabolic changes and the local cerebrospinal fluid environment. Alternatively, increased cerebral blood volume has been put forward as a primary trigger for development of high altitude headache, as arterial and venous dilation and subsequent reduction of vessel tone may lead to activation of the afferents of trigeminal vascular system (Goadsby et al., 2009), surpassing the threshold for pain sensation (Lawley et al., 2015). Additionally, headache may be caused by, or exacerbated by distension of pain sensitive structures such as the arteries, veins, or meninges (Davis & Hackett, 2017). In extreme cases, high altitude headache may progress into the development of high altitude cerebral edema (HACE), which is proposed to be an extension to the continuum of acute mountain sickness. The mechanisms underpinning the pathogenesis of HACE are poorly understood, but involve damage to the blood-brain barrier and an uncompensated elevation in brain volume, leading to an increase in intracranial pressure. This disruption to the blood-brain barrier is likely mediated by hypoxic increases in ROS, inflammatory molecules, cytokines, and vascular endothelial growth factor (Davis & Hackett, 2017).
1.5. Remote ischemic preconditioning

1.5.1. Background

Remote ischemic preconditioning (RIPC) is a new technique characterized by brief, alternating periods of occlusion and reperfusion of blood flow to a limb, with the goal of protecting the body against subsequent ischemia-related injuries. Multiple pathways are responsible for the induced protective state and are dependent on the amount of time that has passed since the application of the occlusive stimulus. Early protection starts immediately and lasts 12-16 hours, and is proposed to be a product of a combination of alterations in ion-channel permeability, post-translational protein modification, and the secretion of various autocoids. A second protective window appears

![Diagram showing the temporal and mechanistic components of remote ischemic preconditioning.](image)

**Figure 1.1. Approximated temporal and mechanistic components of remote ischemic preconditioning.** Two distinct windows of protection appear after RIPC, separated by a period of no protection. The early phase appears to be governed by altered ionic channel function and the post-translational modification of proteins, while the delayed phase is a response to altered gene expression and *de novo* protein synthesis. Changes in autonomic function are observed during both periods.
18-24 hours after preconditioning and lasts 48-72 hours, and is a result of differential gene expression and de novo synthesis of proteins, leading to an upregulation of a number of antioxidative and anti-inflammatory pathways. Optimal duration and intensity of RIPC treatment has yet to be elucidated, but most treatment protocols involve use of a blood pressure or a tourniquet over 1-2 limbs, with 3-5 repetitions of equally-weighted 5-10 minute occlusive and reperfusion periods (Hausenloy & Yellon, 2016). There is extensive laboratory evidence of the protective effects of RIPC, spanning across many different organs including the heart (Murry et al., 1986; Kharbanda et al., 2002; D’Ascenzo et al., 2014), lung (Li et al., 2001; Xia et al., 2003; Waldow et al., 2005), kidneys (R.F. et al., 2011; Candilio et al., 2012; Er et al., 2012), and brain (Figure 1.2). The use of RIPC has been trialed in numerous clinical populations, yielding both positive (Weih et al., 1999; Walsh et al., 2010) and neutral (Hausenloy et al., 2015; Meybohm et al., 2015) results. The utility of RIPC has only begun to gain the attention of clinical researchers in the past decade, so long-term results from large-scale trials have yet to be determined; however, RIPC remains a promising technique that may offer a cheap, non-invasive approach to providing additional systemic protection against ischemic and hypoxia-related injuries.

1.5.1.1. RIPC and the heart

The initial landmark study to report an effect of preconditioning was performed in 1986, where Charles Murry discovered that four 5-minute periods of direct circumflex artery occlusion in canines, followed by a sustained 40-minute occlusion, yielded a 75% reduction in infarct size when compared to a group that underwent only the 40-minute occlusion period (Murry et al., 1986). Here, Murry and coworkers speculated this local protective effect may have been due to reduced ATP depletion and/or reduced catabolite accumulation during the sustained occlusion. A few years later, in a similar model, it was discovered that myocardial territories other than those directly supplied by the occluded portion of the circumflex artery were also protected (Przyklenk et al., 1993). In 2002, similar myocardial protective effects were reported with repeated hind-limb occlusions in the porcine model, a finding which aroused interest to subsequent clinical trials in humans (Kharbanda et al., 2002). At this point, it was clear that myocardial protective effects could be initiated by remote ischemic preconditioning of tissues at a distance from the heart.
The utility of RIPC in humans in clinical settings still remains controversial. Although RIPC appears to be most beneficial for the reduction of myocardial infarct size, the complications associated with identifying at-risk populations and implementing daily or bi-daily pre-emptive RIPC treatments seems impractical. This practical consideration has opened up the possibility of using the same RIPC methodology as an immediate treatment after the ischemic event, creating a distinction between pre- and post- treatment under the methodological umbrella of remote ischemic conditioning (RIC). When employed in patients after ST-segment elevation myocardial infarction, either on the ambulance or at the hospital prior to percutaneous coronary intervention, 

**Brain**
- neural protection after cardiac arrest
- cerebrovascular protection after subarachnoid haemorrhage
- reduced severity of stroke

**Heart**
- smaller infarct size
- reduced perioperative injury
- improved myocardial salvage

**Lungs**
- improved outcomes after resection surgery
- improved oxygenation after coronary artery bypass graft
- reduced alveolar injury

**Liver**
- increased graft proliferation
- reduced ischemia-reperfusion injury

**Kidney**
- reduced acute kidney injury

**Figure 1.2. Examples of organs that have been successful targets of RIPC treatment.** In both human and animal models, remote ischemic preconditioning (RIPC) of a limb can have protective effects observed through many different organs throughout the body.
thrombolysis, or reperfusion, remote ischemic conditioning has been successfully and repeatedly demonstrated to reduce MI size, increase myocardial salvage, and reduce all-cause mortality reviewed in: (Hausenloy & Yellon, 2016)]. In addition, RIPC treatment has also been explored as an option to reduce perioperative myocardial injury after coronary artery bypass graft, as there are limited pharmacological options available to reduce reperfusion injury. Indeed, several studies have reported beneficial effects of RIPC in reducing markers of myocardial injury after cardiac surgery; however, a substantial number of studies have also reported neutral findings. Several meta-analysis have detected a cardio-protective effect of RIPC in reducing perioperative myocardial injury (D’Ascenzo et al. 2014; D’Ascenzo et al. 2012; Yasin et al. 2014; Healy and Walsh 2017); however, this RIPC intervention failed to be confirmed by a reduction in major adverse cardiovascular events after cardiac surgery in three large, multicenter randomized clinical trials (Hong et al., 2014; Hausenloy et al., 2015; Meybohm et al., 2015). Failure to report a protective effect of RIPC is likely due to a number of reasons, but possibly includes the masking effects of the powerful anaesthetics, and the concurrent use of pre-existing cardioprotective strategies during surgeries.

1.5.1.2. RIPC and the lung

The benefits of remote ischemic preconditioning have been extended to improved function of the lung in both animal and human models. Three 5-minute periods of iliac artery occlusion and reperfusion attenuated the rise in PVR and PAP after modelling off-pump coronary artery bypass surgery in sheep (Xia et al., 2003). This led to an improvement in arterial oxygenation and gas exchange, evidenced by improved P$_a$O$_2$ and P$_a$O$_2$/F$_i$O$_2$, although the authors did not attempt to identify any mechanistic link between the RIPC treatment and preservation of lung function. In humans, cardiac patients were reported to have reduced alveolar injury and improved arterial oxygenation after valve replacement surgery when treated with 2x3 minutes of aortic cross-clamping with reperfusion (Li et al., 2001). The post-operative rise in mean PAP, measured by Swan-Ganz catheter, was attenuated by the IPC procedure, and this protection was related to a reduced number of neutrophils in the pulmonary venous blood, as well as reduced oxygen free-radical formation (Li et al., 2001). Additionally, Waldow and colleagues found that when pigs hind-limbs were preconditioned prior to ischemia-reperfusion injury of the lung, the subsequent
attenuation of pulmonary function and development of pulmonary hypertension were abolished (Waldow et al., 2005). Interleukin-1β and macrophage counts were reduced in the preconditioned group; however, interleukin-6 was unaffected, suggesting that RIPC may not protect against all indices of inflammation in the lung.

1.5.1.3. RIPC and the brain

The emerging prospect of RIPC offering myocardial protection against ischemia had led to explorative research into protective effects that may be observed in other organs than the heart. Recently, the brain has become a primary target for RIPC research, with hopes of minimizing damage before or after a period of cerebral ischemia (Pan et al., 2016). When the cerebral circulation is arrested, irreversible tissue damage occurs by a host of mechanisms including free radical production, inflammation, excitotoxicity, ionic perturbations, and cell death (Bramlett & Dietrich, 2004).

In 2006, Dave et al. used bilateral hindlimb ischemia in rats 48 hours prior to asphyxial cardiac arrest, a model of global ischemia. They found significant neuronal protection when compared to a sham group, described by the number of normal neurons counted in the hippocampus. This study was followed up using a focal model of ischemia (middle cerebral artery occlusion). Here, the authors found that unilateral hindlimb ischemia resulted in a similar level of protection (Ren et al., 2008). Since then, remote ischemic neuroprotection has been widely observed in multiple species; however, mechanisms for this protection are still poorly understood (Meller & Simon, 2015).

Human studies of RIC-induced neuroprotection are limited to a few, select patient populations. For example, in patients undergoing carotid endarectomy, pretreatment with 10 minutes of supersystolic compression sequentially delivered to each thigh significantly reduced saccadic latency, a surrogate for mild brain injury (Walsh et al., 2010). In another study, 3 five-minute occlusions of the upper arm resulted in reductions in neuron specific enolase, reduced S110B release, and a more rapid recovery after decompression of cervical spondylotic myelopathy (Hu et al., 2010). In patients with subarachnoid haemorrhage, 3-4 sessions of post-conditioning was used in the 2-12 days after the initial event, a period when risk of vasospasm is highest (Gonzalez et al.,
2013). The findings revealed that transient vasodilation was observed, and lactate/pyruvate ratio and glycerol were both reduced suggesting cerebrovascular and metabolic protection after RIC (Gonzalez et al., 2013).

Currently, large clinical studies investigating neuroprotective effects of RIC after stroke or RIPC in populations at risk for stroke are limited. Despite this, there exists a possibility for a link between remote conditioning and stroke outcomes, evidenced by early studies that found an association between previous incidence of transient ischemic attack (TIA) and a reduced severity in subsequent stroke (Weih et al., 1999). Such findings indicate that previous non-lethal ischemic episodes may offer a degree of protection against future insults. In a well-controlled study consisting of 68 patients with intracranial arterial stenosis, 30 patients underwent standard medical management, while 38 had standard management plus twice-daily treatments of 5 repeats of five minute periods of bilateral upper limb RIPC treatment (Meng et al., 2012). In the control group, incidence of recurrent stroke was 26.7% after 300 days, compared to 7.9% in the RIPC group. Brain perfusion status, measured with SPECT and transcranial Doppler, was also significantly greater in the RIPC group. Together, these findings suggest that RIPC, when combined with standard medical care, may be a feasible strategy to improve patient outcomes in populations vulnerable to neurological damage.

1.5.1.4. RIPC and peripheral vascular function

In addition to attenuating myocardial and neurological damage after ischemia, RIPC may demonstrate a generalized effect on the endothelium (Thijssen et al., 2016). Ischemic preconditioning of the upper arm increases blood flow in distant sites such as the contralateral arm (Enko et al., 2011) and the heart (Shimizu et al., 2007; Zhou et al., 2007). However, conflicting results (Kharbanda et al., 2002; Loukogeorgakis et al., 2005; Moro et al., 2011; Bailey et al., 2012) make it difficult to interpret whether a single session of RIPC acutely effects baseline endothelial function in healthy individuals. There is, however, evidence to suggest that in cases where endothelial function is transiently impaired, RIPC may improve endothelial dilation. For example, despite no differences in FMD response before/immediately after preconditioning of the lower limbs, the reduction in brachial FMD following a subsequent bout of strenuous running exercise
was abolished with RIPC (Bailey et al. 2012). Furthermore, in patients with acute myocardial infarction, RIPC of the upper arm yielded no difference in FMD compared to sham controls when tested at baseline; however, FMD was significantly higher than the sham group immediately after PCI, and this elevation was sustained for seven days (Manchurov et al., 2014). Finally, RIPC of one arm prevents the reduction in FMD after ischemia reperfusion injury in the contralateral arm (Kharbanda et al., 2002; Loukogeorgakis et al., 2005). Together, these findings suggest that RIPC treatment may be beneficial in attenuating acute reductions in FMD. Whether this protection translates into improved long-term outcomes, has not yet been investigated.

There is also evidence to suggest that RIPC may have utility for improving endothelial function when repeated over multiple, consecutive days. Understanding the role of chronic use of RIPC could be of large importance, given the strong relation between peripheral vascular function and cardiovascular events (Green et al., 2011). A study implementing 28 days of IPC of the upper limb in healthy adults provided evidence for increases in circulating endothelial progenitor cells and vascular endothelial growth factor, in addition to increased nitric oxide production (Kimura et al., 2007). Jones et al. (2014) investigated the effects of 7 days of upper limb IPC on both the treated arm and the contralateral arm, and observed an increase in brachial artery FMD on both sides that was sustained for 8 days after the intervention. It is important to note however, that this study did not employ a non-treatment control group. To follow up on this study, the same group then tested the hypothesis that this improvement in endothelial function was a function of the delayed phase of RIPC (appearing after 24 hours, lasting 2-3 days), by administering upper arm preconditioning 3 times per week over a period of 8 weeks (Jones et al., 2014b). When compared to controls, FMD was elevated to a greater extent at both 2 weeks and 8 weeks, suggesting that mechanisms of the late phase of RIPC protection (e.g., upregulation of the NO and cyclooxygenase-2 pathways, etc.) play an important role in the endothelial responses. Similar findings have been repeated in patients scheduled for coronary artery bypass graft surgery, where 20 days of tri-daily IPC improved brachial artery FMD, increased expression of nitric oxide synthase mRNA, and increased levels of endothelial progenitor cells (Liang et al., 2015).

In summary, although it is unclear whether a single session of RIPC improves vascular function in healthy individuals, RIPC treatment is likely beneficial to those who have transient impairments
in endothelial function. Furthermore, the use of repeated sessions of RIPC appears to benefit vascular function in both healthy and at-risk populations. Ultimately, more research is needed to identify if these transient improvements in endothelial function translate into improvements in long-term outcomes; however, these findings, combined with the aforementioned reduction in second occurrence of stroke with RIPC (Meng et al. 2012; Meng et al. 2015) suggest that strategic application of repeated RIPC treatments has promising clinical utility.

1.5.2. RIPC and high-altitude travel

Recently, the use of RIPC has gained traction as a potential prophylactic intervention for those travelling to high altitude (Berger et al. 2015). Pathways upregulated to enhance vascular function and protect against ischemia-related injuries may also serve to provide protection against the cardiovascular consequences of a hypoxic environment.

Hypoxic pulmonary vasoconstriction is affected by a number of active pathways upregulated by RIPC, such as those regulating the release of NO and adenosine (Auchampach & Gross, 1993; Yellon & Downey, 2003), HIF-1α (Cai et al., 2013), as well as anti-inflammatory and anti-oxidative pathways ((Bailey et al., 2010; Sylvester et al., 2012). Foster and colleagues (2011) employed a randomized crossover study to test the effects of RIPC (of the lower limbs, 4x5 minutes, 1 hour prior) on pulmonary vascular (and exercise) responses to normobaric hypoxia (F\textsubscript{1}O\textsubscript{2} =13%). When compared to controls, the elevation in PASP was significantly attenuated in the RIPC group. Despite this reduction in PASP observed with RIPC, there was no change in estimated pulmonary vascular resistance, peripheral oxygen saturation, or exercise time trial performance during hypoxia. In a later study by the same group, participants were preconditioned for 5 consecutive days prior to passive ascent to high altitude (3560m), followed by an exercise time trial consisting of a 12.1km run to a summit station at 4342m. Approximately 30 minutes after completing the exercise trial, pulse oximetry and echocardiographic measurements were obtained while participants waited at the summit station. Compared to controls, there was a small, but significant reduction in PASP (2.1 mmHg reduction, P=0.035) after RIPC, as well as an impressive 5% improvement in O\textsubscript{2} saturation [80.3±8.7 RIPC vs. 75.3±9.6 control] (Foster et al., 2014a). A vasodilatory effect of RIPC in the pulmonary vasculature may in part explain this
increase in peripheral oxygenation (Olfert et al., 2011); however, this could also be a product of increased alveolar ventilation. Ventilatory measurements were unfortunately not recorded in the aforementioned studies, so it is therefore not possible to determine if a potential increase in the hypoxic ventilatory response contributed to their findings.

To our knowledge, the only other published study to date investigating the effects of RIPC during hypoxia or high-altitude exposure was performed by Berger and colleagues in 2015. To test the hypothesis that RIPC would improve Lake Louise score and AMS-C score during a hypoxic

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**Figure 1.3. Pulmonary and physical responses to IPC prior to ascent to high altitude.** The percentage change from placebo condition for selected variables, after 5 consecutive days of dual thigh 4x5 minute RIPC treatment. Measurements were taken after rapid ascent to 4342 m. Bars represent mean and 95% confidence intervals. Significant decreases were seen in pulmonary artery systolic pressure, mean pulmonary artery pressure, and an exercise course completion time, while significant increases were seen in O₂ saturation. From Foster et al. HAMB 2014, no permission needed.
exposure, they applied four cycles of lower limb ischemia to participants immediately prior to 18 hours of normobaric hypoxia. The authors found that after 5 hours, Lake Louise score and AMS-C scores were significantly reduced in the RIPC group compared to controls. They also tested for markers of oxidative stress: ascorbate free radical, EPR signal intensity and oxidized SH groups, and found that these were also reduced at 5 hours compared to controls (Berger et al. 2015). At 18 hours, however, when AMS were markedly elevated there were no differences between the groups in any of the symptom scores or measures of oxidative stress. These comparable findings may have been a result of the 18 hour mark falling between the early and late phases of protection of RIPC, a well-described window of no protection (Schoemaker & van Heijningen, 2000; Wang et al., 2001); however, it could also be speculated that RIPC only delays the onset of AMS rather than providing a bi-phasic protection. It is also important to consider the study design itself; the physically assertive nature of thigh cuff-occlusion makes it impossible to truly blind subjects, a

Table 1.0. Studies investigating the use of RIPC during hypoxia or high-altitude travel

<table>
<thead>
<tr>
<th>Study</th>
<th>Method of RIPC</th>
<th>Population</th>
<th>Hypoxic Stimulus</th>
<th>Key Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foster et al., 2011, Respiratory physiology &amp; neurobiology</td>
<td>4x5 minute single thigh occlusion</td>
<td>8 amateur cyclists, age 39±9.7 years</td>
<td>90 minutes FiO2 of 13%</td>
<td>72.8% attenuation in the rise in PASP, no change in saturation, BP, HR</td>
</tr>
<tr>
<td>Foster et al., 2014, High altitude medicine &amp; biology</td>
<td>4x5 minute single thigh occlusion, 5 consecutive day prior to ascent</td>
<td>12 adults, age 42±14 years</td>
<td>Passive ascent to 3560m followed by a run to 4352 m</td>
<td>2.1 mmHg reduction in PASP (P=0.035), 6.5mmHg reduction in mPAP (P=0.005), 5.0% increase in O₂ saturation, no change in heart rate or cardiac output</td>
</tr>
<tr>
<td>Berger et al., 2015 Physiological reports</td>
<td>4x5 minute dual thigh occlusion</td>
<td>14 adults age 24 ± 1 years</td>
<td>18 hours in a normobaric chamber, FiO₂ 12%</td>
<td>Reduced plasma ROS and reduced Lake Louise AMS-C test scores at 5 hours, but not at 18 hours</td>
</tr>
</tbody>
</table>
key design feature when reporting subjective symptom scores. To counter this, the group performed statistical analysis by comparing their results to historic controls from a separate study using the same exposure protocol; however, this also could mean calculated differences in the responses were simply a product of innate differences between two distinct study populations. While this study provided preliminary work to show that RIPC may influence some of the mechanisms responsible for the pathogenesis of AMS, more work needs to be over longer-duration exposures to more accurately represent ascent/exposure profiles experienced by high altitude sojourners.

Presently, only three studies have explored the utility of RIPC during high-altitude or hypoxic exposures. Under these conditions, RIPC has been successful in attenuating the rise in pulmonary artery pressures, and limiting symptoms of acute mountain sickness during the early phase of protection.

1.5.2.1 Mechanisms of RIPC relating to high altitude vascular dysfunction

To our knowledge, published research investigating how RIPC might benefit those travelling to high altitude is limited to the aforementioned studies. There is, however, a large degree of overlap between signalling pathways responsible for the global vascular responses to hypoxia and those upregulated or modified with RIPC. The generation of NO and nitrite is increased with RIPC (Bolli et al., 1997; Rassaf et al., 2014), likely as a result of shear-stress mediated increase in circulating eNOS secondary to reactive hyperemia. However, after RIPC, the increases in NO may have secondary adverse effects via K⁺-channel modification, ROS formation, and mitochondrial depolarization (Lim et al., 2010; Koch et al., 2014; Hausenloy & Yellon, 2016; Aulakh et al., 2017). ROS also contribute to the formation of oxidant-dependant proinflammatory mediators, accelerating the inflammatory process. This paradoxical situation, where ROS act as both a critical determinant of cell death, and as a signalling mediator of cellular protection, is a key feature of RIPC.

It has been touted that ROS may be involved in the pathophysiology of AMS (Bailey et al., 2009). Despite this, results using antioxidants for AMS prevention are questionable (Bailey & Davies,
There is, however, stronger evidence to suggest that increased ROS have a contributory role in the development of HACE, through promotion of capillary leak, and downregulation of Na+/K+ ATPase, causing cellular swelling and cytotoxic edema (Luks et al., 2017b). Reduced NO availability and increased ROS also contribute to the rise in pulmonary pressures with hypoxia, leaving room for speculation that RIPC’s opposing effects on these pathways might benefit the pulmonary vascular responses. Furthermore, the anti-inflammatory effects of RIPC might attenuate the vascular remodelling contributing to the sustained increase in pulmonary pressures with hypoxia. Increases in NO availability and reductions in inflammation could potentially attenuate the development and progression of HAPE.

Proposed impairments in the peripheral vasculature during hypoxia may be attributable to reductions in NO bioavailability, inflammation, and oxidative stress; however, hypoxic increases in sympathetic nerve activity also play a key role in mediating a reduction in FMD (Lewis et al., 2014). In addition to the humoral pathways activated with limb ischemia, RIPC also works through neurogenic pathways, described in detail in: (Meller & Simon, 2015). The protective effect of RIPC against the decline in FMD after ischemia-reperfusion injury is abolished with autonomic ganglion blockade, trimetaphan (Loukogeorgakis et al., 2005), providing evidence for the importance of alterations in autonomic function after RIPC treatment. Lambert et al. observed that when compared to a non-RIPC control, RIPC treatment of the forearm twenty minutes before an experimental ischemia-reperfusion injury to the leg resulted in a reduction in muscle sympathetic nerve activity (mSNA) during leg ischemia as well as in the early period of reperfusion (Lambert et al., 2016). In addition, glutathione- an erythrocyte marker of oxidative stress was also reduced, and finger reactive hyperemia was subsequently enhanced (Lambert et al., 2016). Furthermore, it has been shown that RIPC of the upper arm improves functional sympatholysis in the distal forearm (Horiuchi et al., 2015). Together, these findings suggest that a reduction in sympathetic nerve activity with RIPC, as well as an increase in the sympatholytic response may attenuate the sympathetic constraint on vascular function during hypoxia.
1.6 Purpose and Hypothesis

A large amount of research exists investigating the utility of RIPC in clinical and sub-clinical populations; however, the avenues in which RIPC of the limbs might be used during hypoxia or high-altitude travel have only been minimally explored. The purpose of the following study was therefore to determine the role of a single session of RIPC on arterial function and tone in the vasculature of the lungs, the brain, and the periphery, as well ventilatory responsiveness, during both acute and chronic hypoxic exposures.

It was hypothesized that:

1) RIPC would attenuate the rise in PASP observed during hypoxia;

2) Compared to controls, RIPC would improve FMD of the brachial artery during hypoxia.

3) It was further reasoned, based on reports of improved peripheral oxygenation at high altitude (Foster et al., 2014a), that the hypoxic ventilatory response after RIPC would also increase.
2 One session of remote ischemic preconditioning does not improve vascular function in acute normobaric and chronic hypobaric hypoxia

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2.1. Background

Remote ischemic preconditioning (RIPC) is a non-invasive procedure that has substantial effects on protecting various organs in the body against ischemia-related injuries. In humans and animal models alike, cyclic 5-10 minute periods of occlusion and reperfusion of blood flow through a limb, with total treatment times ranging from 40-60 minutes produces significant protection against ischemia in the heart (Murry et al., 1986), lungs (Kinoshita, 2015), kidneys (Wever et al., 2011), liver (Yan et al., 2015) and brain (Koch et al., 2011). The exact mechanisms responsible for these effects remain unclear, but are likely a result of activation of various anti-inflammatory and anti-oxidative pathways [for review see: (Koch et al., 2014)].

Recent findings suggest that RIPC may also play a role in protection from hypoxic and altitude-related injuries (Berger et al. 2015). For example, in athletes, one 4x5 minute RIPC treatment in the lower limb attenuates the rise in pulmonary artery systolic pressure (PASP) normally seen after 90 minutes of normobaric hypoxia (Foster et al., 2011), and 5 days of consecutive treatment has shown similar pulmonary vascular protective effects when conducted prior to travel to high altitude (Foster et al., 2014a). Together, these findings suggest that preconditioning may offer a degree of protection against the onset of high altitude pulmonary edema. In addition, RIPC has also been linked to reduced oxidative stress and lower symptoms of acute mountain sickness after acute exposure to normobaric hypoxia (Berger et al. 2015); however, these findings were only observed transiently (0-16 hours). After a brief period of no observable protection, a second delayed window of protection appears after ~18-24 hours and lasts for 1-2 days (Miguel, 2004; Koch et al., 2014). While clinical outcomes as a result of RIPC seem promising (Thielmann et al., 2013), only a very
limited number of studies have investigated RIPC and potential further protection against hypoxia (Foster et al. 2011; Foster et al. 2014; Berger et al. 2015).

High-altitude represents an experimental model that allows for the study of hypoxic adaptation in healthy humans. In addition to marked changes in PASP and cerebral blood flow (Stembridge et al., 2014; Willie et al., 2014), ascent to high altitude is typically associated with a decline in vascular function, as demonstrated by impaired endothelial-dependent flow-mediated dilation (FMD) (Lewis et al., 2014; Bakker et al., 2015b) and endothelial-independent dilation (Lewis et al., 2014). Such impairment in vascular function may further compromise the body’s ability to tolerate the various stresses associated with hypoxia. Increased sympathetic nerve activity (SNA) and disturbed blood flow may contribute to the hypoxia-associated reduction in FMD (Lewis et al., 2014; Tremblay et al., 2016). Additionally, the impairment may be attributed to an increase in oxidative stress, which may interfere with the intracellular signalling processes required for smooth muscle relaxation (Munzel et al., 2004). Remote ischemic preconditioning reduces oxidative stress after ischemic injuries (Chen et al., 2015), and these same activated pathways may also attenuate the decline in vascular function at high altitude. Furthermore, RIPC has been demonstrated to preserve FMD immediately after strenuous exercise (Bailey et al., 2012), a period that is normally associated with elevations in SNA and a temporary impairment in vascular function (Atkinson et al., 2015; Tymko et al., 2016b).

Therefore, the primary aim of this study was to explore the potential protective benefits of one single session of RIPC (4 x 5 min) on integrative vascular function during both acute and chronic exposure to hypoxia. The previously observed reduction in PASP and improved haemoglobin saturation at altitude in response to RIPC (Foster et al., 2014a) could potentially be explained by a larger hypoxic ventilatory response (HVR), although this possibility has not yet been investigated. Therefore, a secondary objective of this study was to examine if RIPC evokes any changes in peripheral chemosensitivity to hypoxia. Finally, based on reports that RIPC offers two distinct protective windows (Koch et al., 2014) - from 0-12 hours, and again from 18-72 hours - measurements were repeated immediately (1 hour), 24 hours and 48 hours after the initial RIPC treatment. We hypothesized that RIPC of the lower limbs would reduce the pulmonary artery pressure increase normally observed in hypoxia (Swenson, 2013), as well as attenuate the hypoxic
impairment in FMD (Lewis et al., 2014). We also reasoned that the RIPC intervention would increase the HVR, thereby explaining the previously reported elevations in peripheral oxygen saturation (Foster et al., 2014a). Given that >80 million of people live above 2500 meters and many more travel to altitude per year, with 10-85% of these getting some form of altitude illness (Hackett et al., 1976; Maggiorini et al., 1990), determining the impact of RIPC may provide a simple and inexpensive strategy to alleviate high altitude related illnesses.

2.2. Methods

This experiment was conducted in two separate parts, with one protocol taking place near sea-level (Kelowna, Canada; 344 m), and the second protocol starting after 8-10 days at high altitude (Barcroft Station, White Mountain, California; 3800 m). This study was a part of a series of experiments that took place over the course of a two-week research expedition to Barcroft Station, starting with rapid ascent to high altitude (3800 m, <6-hour drive) on day one. There was no overlap between participation in this study and participation in other investigations relative to carry over effects of drugs and/or exercise, and the questions addressed in this paper are dealt with exclusively within this study alone. During the entirety of their stay at Barcroft Station, participants had access to regular meals and fluids ad lib. All participants abstained from vigorous exercise, caffeine and alcohol for 12 hours prior to testing, and were asked to consume a light snack two hours before coming to the laboratory.

2.2.1. Participants

All participants were free of overt cardiovascular, respiratory and cerebrovascular disease, were non-diabetic, and not taking any prescription medications (other than oral contraceptives, n=2) at the time of their participation, as determined by a screening questionnaire. Each subject provided written informed consent prior to arrival at the lab for familiarization. Participants were different for the SL and HA components of the study, with select characteristics described below. This study was approved by the University of British Columbia Clinical Research Ethics board and conformed to standards set by the Declaration of Helsinki, except for registration in a database, and the Canadian Government Tri-Council Policy Statement for Integrity in Research.
Participants came into the lab on three consecutive days, where the protocol was performed on the first day (Baseline) and then repeated again at three time points: 1 hour (Day 1), 24 hours (Day 2), and 48 hours (Day 3). The testing protocol is depicted in Figure 2.0. Immediately after BL, subjects were randomly allocated into either dual-thigh RIPC or a Sham treatment, both of which are described below.

For each session (BL, 1 hour, 24 hours, 48 hours), subjects lay supine while being instrumented for the tests (described further in “Experimental Measurements”). After 20 minutes of supine rest normoxic echocardiographic images were obtained. Next, a baseline FMD test of the brachial artery was performed, followed by 5 minutes of rest, where baseline VE (ventilation), SpO₂ (%) (peripheral oxygen saturation), BP (blood pressure) and MCAv (middle cerebral artery blood velocity) were collected. Subjects breathed on an end-tidal air forcing system (described below) where the end-tidal partial pressures of O₂ and CO₂ (P_ETO₂ and P_ETCO₂, respectively) were clamped to match previously measured room air values. Once steady state was achieved, the ultrasonographer began scanning the internal carotid artery (ICA) and obtained at least one minute of satisfactory recordings before the isocapnic hypoxia stage. The sonographer held the image of the ICA in place while P_ETO₂ was subsequently and rapidly dropped to 50 mmHg, while P_ETCO₂

<table>
<thead>
<tr>
<th>Condition</th>
<th>Acute Hypoxia</th>
<th>Chronic Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (females)</td>
<td>8 (3)</td>
<td>8 (3)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>24.1±3.6</td>
<td>24.7±3.7</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>69.1±6.2</td>
<td>71.3±9.6</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>173.3±6.2</td>
<td>175.7±4.6</td>
</tr>
</tbody>
</table>

There were no differences in subject demographics (age, height, weight) between treatment conditions or between acute and chronic hypoxia branches of the study (P>0.05).

2.2.2. Experimental Design

2.2.2.1. Part 1: Sea level (acute hypoxia)
was maintained at room-air values. From the establishment of steady state, ICA images were then obtained continuously over the first 10 minutes of hypoxia. Starting at minute 15, a second brachial artery FMD was performed, followed by echocardiographic image acquisition (for PASP) at minute 30. Therefore, room air and hypoxia measures were collected in every visit. The level of hypoxia (\(P_{ET}O_2 = 50 \text{ mmHg}\)) was selected in order to provide an approximated, comparable hypoxic stimulus to that experienced during the subsequent HA component of the study at Barcroft station (Severinghaus et al., 1966b).

<table>
<thead>
<tr>
<th>Room Air</th>
<th>End-Tidal Forcing</th>
</tr>
</thead>
<tbody>
<tr>
<td>PASP</td>
<td>FMD</td>
</tr>
<tr>
<td></td>
<td>(Q_{ICA})</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>HVR</td>
<td>(Q_{ICA})</td>
</tr>
<tr>
<td>-25 min</td>
<td>T = 0</td>
</tr>
</tbody>
</table>

*Normoxia (\(P_{ET}O_2 \approx 100 \text{ mmHg}\))  Isocapnic Hypoxia (\(P_{ET}O_2 = 50 \text{ mmHg}\))*

**Figure 2.0. Experimental design at sea level.** Schematic of the experimental protocol for Part 1 of the study representing acute hypoxia, performed near sea level (Kelowna, 344m). PASP = pulmonary artery systolic pressure measurement; FMD = flow-mediated dilation measurement, \(Q_{ICA}\)=blood flow through the internal carotid artery; HVR = period of assessment of the hypoxic ventilatory response.

**2.2.2.2. Part 2. High altitude (chronic hypoxia)**

A similar time profile was used to the sea-level study. Subjects were tested on three consecutive days, between the 8th and 12th day of continuous residence at the Barcroft Station (3800m), where the protocol was performed on the first day (BL), and then repeated at the three time points: 1 hour (Day 1), 24 hours (Day 2), and 48 hours (Day 3). The experimental protocol is depicted in Figure 2.1. Immediately after BL, subjects were randomly allocated to either dual thigh- RIPC or a sham treatment which acted as time-based control. Consequent to our limited participant pool at HA,
eight participants were selected to receive RIPC in order to more clearly identify any physiological changes after treatment, and the remaining four served as a time-control subset. Cardiorespiratory changes throughout acclimatization have been well-documented, and after the first week, day-to-day changes in HR, VE, and SPO₂ are minimal (Swenson & Bärtsch, 2014).

While resting in the supine position, subjects were instrumented for testing. After 20 minutes of rest, echocardiographic images (for PASP) were acquired, followed by a FMD test of the brachial artery. Baseline ventilatory measurements were then taken followed by end-tidal forcing, clamping P_{ETO₂} and P_{ETCO₂} to BL values. ICA velocity and diameter were measured for at least 1 minute, followed by a subsequent isocapnic drop in P_{ETO₂} to 45 mmHg. This level of hypoxia was selected in order to provide a significant hypoxic stimulus beyond what was already being experienced by the participants at Barcroft station. Once steady state was achieved, ICA measures were taken for 10 minutes, along with measures of VE, SpO₂%, BP and MCAv.

![Diagram](image)

**Figure 2.1. Experimental design during chronic hypoxia.** Schematic of the experimental protocol for Part 2 of the study, performed at Barcroft Station after 8-12 days at high altitude (White Mountain, 3800m). PASP = time of pulmonary artery systolic pressure measurement; FMD = time of flow-mediated dilation measurement, Q_{ICA} = time of measurement of blood flow through the internal carotid artery; HVR = period of assessment of the hypoxic ventilatory response.
2.2.3. Remote ischemic preconditioning

Participants were seated in a chair with blood pressure cuffs placed around both legs at mid-thigh level. At sea level, an automated rapid inflation system was used to quickly inflate the cuffs to 225 mmHg, whereas at high altitude a manual hand pump was used to increase the cuff pressure. The cuffs remained inflated for 5 minutes, followed by a 5 minute period of deflation allowing for reperfusion of blood flow to the limb. This process was repeated 4 times, for a total treatment time of 40 minutes (Foster et al., 2014a). In the time control and sham conditions, the cuffs were only inflated to <20mmHg. We chose this method of dual-thigh occlusion in order to simulate an easily-reproducible treatment that could be applied on short notice in remote locations, with minimal time and equipment. In practice, it could be used in situations where rapid ascent to altitude is required with short notice, or as a treatment to mitigate risk in those already in a hypoxic setting.

2.2.4. Experimental Measurements

2.2.4.1. Cardiorespiratory

In both studies at sea level and high altitude, cardiorespiratory variables were sampled continuously throughout the protocol at 1KHz via an analogue-to-digital converter (Powerlab, 16/30; ADInstruments, Colorado Springs, CO). A 3-lead electrocardiogram (ADI bioamp ML132) was used to measure heart rate (HR), and beat-to-beat blood pressure was recorded by finger photoplethysmography (Finometer PRO, Finapres Medical Systems, Amsterdam, Netherlands). The Finometer reconstructed brachial waveform was used for calculation of mean arterial pressure (MAP) after back-calibrating to the average of three automated brachial blood pressure cuff measurements made at rest (Tango+; Suntech, Morrisville, NC). Respiratory flow and minute ventilation (\(V_E\)) was measured by a pneumotachograph (HR800L, HansRudolph, Shawnee, KS) connected in series to a bacteriological filter, and a calibrated gas analyzer (ML206, ADInstruments) was used to record the partial pressure of both end-tidal CO\(_2\) and end-tidal O\(_2\), sampled at the level of the mouth. Arterial O\(_2\) saturation was measured continually using pulse oximetry (ADInstruments). All measures, unless otherwise stated, are reported as averages over 1-minute bins. In the acute hypoxia protocol, hypoxic cardio-respiratory measurements are taken
between minutes 25-30 of isocapnic hypoxia. In the chronic hypoxia protocol, hypoxic measurements are taken between minutes 9-10 of isocapnic hypoxia. These time-frames were chosen as the most suitable representations of steady-state for each test.

2.2.4.2. End-tidal forcing

A dynamic end-tidal forcing system was used to control P_{ET}CO_2 and P_{ET}O_2 during the normoxic and isocapnic hypoxic periods of the protocols. This system has previously been described in detail (Tymko et al. 2016; Tymko et al. 2015), and is able to effectively control end-tidal gases independent of ventilation at low and high altitudes. P_{ET}CO_2 was kept constant at resting room air values throughout the two protocols, while P_{ET}O_2 was rapidly dropped during the room-air to hypoxia transition until steady-state was achieved; this was determined as at least three consecutive breaths within 1 mmHg of the desired target.

2.2.4.3. Peripheral chemosensitivity

Following the onset of hypoxia, ventilatory and end-tidal data were averaged into 15-second bins. The HVR was calculated using the peak 15-second bin of ventilation following the transition from normoxia to isocapnic hypoxia on the end-tidal forcing system (described above). This peak 15s bin was compared to a 30s bin of data collected immediately prior to the hypoxic drop, and the HVR was thus calculated using the formula: $HVR = \frac{\Delta VE}{\Delta ScO_2\%}$, with ScO_2 (calculated oxygen saturation) calculated from the end-tidal O_2 trace using the equation described by Severinghaus, (Severinghaus, 1979). We used this equation to calculate saturation over pulse oximetry to more accurately reflect the timing of changes in blood oxygenation.

2.2.4.4. Flow-mediated dilation

Reactive hyperemia flow-mediated dilation was performed according to internationally-recognized guidelines (Thijssen et al., 2011). Participants were lying supine with their left arm extended in a fixed position ~80 degrees perpendicular from their body. All measurements were taken after at least 20 minutes of supine rest in a quiet, dark room. Brachial artery image acquisition
was obtained using a 10 MHz multifrequency linear array probe attached to a high-resolution ultrasound machine (15L4, Terason t3200, Burlington, MA, USA). All images were acquired by the same experienced ultrasonographer (JCT), whom has a between-day coefficient of variation in FMD of 8.3 ± 2.1% (n=10, unpublished data). Following optimal image acquisition, and one-minute of baseline recordings, the forearm was occluded by inflating the cuff to 220-250 mmHg for five-minutes. Recordings of diameter and velocity continued 30-seconds prior to cuff deflation and continuously for three-minutes thereafter. Video recordings were anonymized and stored for later offline analysis using specialized edge-detection software (Woodman et al., 2001; Thijssen et al., 2011). Flow-mediated dilation was calculated as the peak increase in diameter following cuff deflation. The FMD stimulus was calculated as the shear rate area under the curve (SRAUC) from the onset of reactive hyperemia to FMD (Pyke & Tschakovsky, 2007).

2.2.4.5. Measurement of extra- and intra-cranial blood flow

A 10MHz multi-frequency linear array duplex ultrasound (Terason T3200, Teratech, Burlington, MA) was used to measure blood velocity and diameter of the internal carotid artery (ICA). These recordings were acquired using simultaneous B-mode imaging (diameter) and pulse-wave mode (velocity). In order to eliminate recordings of turbulent and retrograde flow, measurements were taken at least 1.5cm distal to the common carotid bifurcation. To avoid any artificial changes in arterial wall brightness/thickness, there was no alteration in B-mode gain upon acquisition of the first ultrasound image. Data were anonymized and later analysed offline using the same edge-detection software as described above, and values are reported as an average over a minimum of 12 consecutive cardiac cycles. Flow-reactivity was calculated using a 30-second bin encompassing the peak response to hypoxia.

Blood velocity through the left middle vertebral artery (MCAv) was recorded using a 2MHz transcranial Doppler ultrasound (Spencer Technologies, Seattle, WA). A specialized headband (model M600 bilateral head frame, Spencer Technologies) was used to secure the probe in place. Insonation was achieved through the trans-temporal window using previously described location and standardization techniques (Willie et al. 2011), and data are reported as the average across selected 30-second bins during each stage.
2.2.4.6. Echocardiography

All echocardiographic images were obtained by the same experienced sonographer (MS) on a commercially available ultrasound machine (Vivid Q (Sea-Level) / E9 (High Altitude), GE, Fairfield, CT). M5-S 1.5-4.6 MHz and 4V 1.5-40 MHz transducers were used to collect echocardiographic images, which were saved for offline analysis (Echopac v.113, GE, Fairfield, CT). Subjects lay in the supine left lateral decubitus position. The modified Bernoulli equation was used to calculate pulmonary artery systolic pressure, where \( \text{PASP} = 4V^2 + 5\text{mmHg} \), where \( V \) equals the peak tricuspid regurgitation velocity and 5 mmHg was added for right atrial pressure where the inferior vena cava collapsed under inspiration (Rudski et al. 2010). Measures of PASP are reported using the average of at least three cardiac cycles.

2.2.5. Statistical Analysis

Based upon previous studies investigating FMD and PASP during hypoxia, sample sizes of 9-12 (Jones et al. 2014; Foster et al. 2014; Lewis et al. 2014) were able to show significant changes. We therefore attempted to have >10 participants in each group; however, the time allocated and subject availability for this study only allowed us to have 8 participants in each group at SL, and a small subset of four time-control participants in the HA trial. For example, during isocapnic hypoxia at SL, if we were to assume a PASP of 30 mmHg, and wished to detect a 10% reduction following RIPC, with a standard deviation of the differences of 2.5 mmHg, a power of 0.8 and alpha of 0.05, we would need 10 participants. Results from this study must therefore be treated as preliminary, rather than confirmatory and further studies employing larger sample sizes are needed.

In Study 1 (Acute hypoxia), a two-way repeated measures ANOVA was used for the cardiovascular measures in each treatment group (Factors: Time & \( \text{O}_2 \)). In addition, a two-way repeated measures ANOVA (Factors: Time & Treatment) was used to identify differences in the HVR and ICA reactivity, as well as the change from normoxia to hypoxia for FMD and PASP.
In study 2 (Chronic hypoxia), independent one-way ANOVAs were used on both the RIPC group and the time-control group (Factor: Time). Upon detection of significant main effects, pairwise comparisons were made using Dunnett’s t-tests. While it has recently become convention to allometrically scale FMD changes (Atkinson & Batterham, 2013) and scale for SRAUC as a covariate (Atkinson & Batterham, 2015), baseline diameter and SRAUC did not differ between groups in this study, and were therefore not corrected for. Furthermore, normality of all main outcome variables (FMD, PASP, qICA, VE, and HVR) was confirmed using a Shapiro-Wilk test. All data were analysed using SPSS (version 24, IBM, Surrey, UK). Results are reported as mean ± standard deviation unless otherwise indicated. Statistical significance was defined as $P<0.05$.

2.3. Results

2.3.1. Part 1: Sea level (acute hypoxia)

2.4.1.1. Cardiovascular responses
Table 2.1 presents cardiovascular and respiratory variables during baseline and during isocapnic hypoxia ($P_{ETO_2}$ clamped to 50 mmHg). There were no significant differences in the magnitudes of responses to hypoxia in HR (RIPC: $P=0.91$; Sham: $P=0.91$), MAP (RIPC: $P=0.51$, Sham: $P=0.37$), or $SpO_2$ (RIPC: $P=0.84$, Sham: $P=0.98$) at any time point in either the RIPC or Sham condition.

2.3.1.2. Pulmonary artery pressures

Pulmonary pressures during normoxia and hypoxia are presented in Table 2.1. One subject was excluded from each group due to a lack of suitable echocardiographic images for measuring tricuspid regurgitation; thus, statistical analyses was based on seven participants in each group. While hypoxia consistently increased PASP during each trial ($P<0.01$ for both RIPC and Sham), the magnitude of this rise did not differ between groups or trials (Figure 2.2., $P=0.60$).

2.3.1.3. Flow-mediated dilation
Brachial artery flow-mediated dilation (%) responses during normoxia and hypoxia are shown in Table 2.1. There were no differences in baseline diameter (RIPC: P=0.67, Sham: P=0.83) or SRAUC (RIPC: P=0.40, Sham: P=0.99), and subsequently no changes in FMD response at any time in either condition. The FMD (% dilation) is presented in Table 2.1.

2.3.1.4. ICA flow and intra-cranial velocity

Isocapnic hypoxia resulted in a comparable increase in blood flow through the ICA (P<0.01 for both RIPC & Sham) and blood velocity in the MCA (P<0.01 for both RIPC & Sham) in both conditions; although neither response differed between RIPC and Sham at any point through the protocol (Table 2.1. for MCA & Figure 2.2. for ICA).
Table 2.1. Acute Hypoxia

<table>
<thead>
<tr>
<th></th>
<th>BL</th>
<th>1 hour</th>
<th>24 hours</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FMD (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>normoxia</td>
<td>7.7±±3.0</td>
<td>7.90±2.7</td>
<td>6.78±2.3</td>
<td>7.16±2.2</td>
</tr>
<tr>
<td>hypoxia</td>
<td>7.24±2.9</td>
<td>7.34±2.3</td>
<td>6.24±2.8</td>
<td>6.77±3.4</td>
</tr>
<tr>
<td>Time: P = 0.652, O₂: P = 0.235, Interaction P = 0.856</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PASP (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>normoxia</td>
<td>22.3±±3.6</td>
<td>21.9±±3.2</td>
<td>21.7±±4.9</td>
<td>21.1±±2.5</td>
</tr>
<tr>
<td>hypoxia</td>
<td>25.2±±3.9</td>
<td>25.3±±3.4</td>
<td>24.5±±5.0</td>
<td>24.1±±4.9</td>
</tr>
<tr>
<td>Time: P = 0.999, O₂: P = 0.009, Interaction P = 0.993</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ICA flow (mL/min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>normoxia</td>
<td>242.7±±30.2</td>
<td>232.5±±38.3</td>
<td>237.8±±42.2</td>
<td>235.9±±32.8</td>
</tr>
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<td>hypoxia</td>
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<td>282.6±±49.1</td>
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<td>276.2±±54.2</td>
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<td><strong>MCAv (cm/s)</strong></td>
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<td>56.8±±6.5</td>
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<td>62.4±±4.9</td>
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<tr>
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<td><strong>MAP (mmHg)</strong></td>
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<td>89.9±±8.4</td>
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<td><strong>VE (L/min)</strong></td>
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<td><strong>PETCO₂ (mmHg)</strong></td>
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<tr>
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<td><strong>PETO₂ (mmHg)</strong></td>
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<td>normoxia</td>
<td>95.9±±4.3</td>
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<tr>
<td>hypoxia</td>
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<tr>
<td><strong>SPO₂ (%)</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>normoxia</td>
<td>98.4±±1.0</td>
<td>97.9±±1.2</td>
<td>97.9±±1.0</td>
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</tr>
<tr>
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</table>

Acute hypoxia. Selected cardiovascular and respiratory parameters at baseline, and 1 hour, 24 hours, and 48 hours after dual-thigh RIPC, during room-air breathing and acute hypoxia. End-tidal partial pressure of O₂ was clamped to 50 mmHg under isocapnic conditions in the hypoxia phase. Testing was performed at low-altitude (344m). Data represents group means ± SD. A 2-way ANOVA was used to evaluate statistical differences in each group.
2.3.1.5. Ventilation

Ventilation was elevated during hypoxia (P<0.01 for both RIPC and Sham); however, there were no differences in ventilation across any of the testing times (RIPC: P=0.58, Sham: P=0.89, see Table 2.1). The HVR (Figure 2.2.) was also unaffected by either RIPC or sham treatment (P=0.45).

Figure 2.2. Acute Hypoxia. Selected vascular and respiratory responses to hypoxia at baseline, and 1 hour, 24 hours, and 48 hours after dual-thigh RIPC. A) Absolute increase in pulmonary artery systolic pressure (PASP) from normoxia to 30 minutes of hypoxia. B) Change in brachial artery flow-mediated dilation (FMD) response from normoxia to hypoxia (i.e. normoxia % - hypoxia %) C) Peak flow-reactivity of the internal carotid artery (ICA) upon the transition from normoxia to hypoxia. D) The hypoxic ventilatory response (HVR). Black bars represent means for RIPC, grey bars represent means for Sham. End-tidal partial pressure of O₂ was clamped at 50 mmHg under isocapnic conditions in the hypoxia phase. Testing was to model acute hypoxia, performed at low-altitude (344m). Individual data points are shown, bars represent group means. A 2x4 ANOVA (factors: time, treatment) was used to identify significant differences, P>0.05 for all interactions.
2.3.2. Part 2. Chronic hypoxia (high altitude)

2.3.2.1. Cardiovascular responses

Table 2.2 presents selected resting cardiorespiratory variables after 8-12 days at high altitude, while breathing room air, as well as during isocapnic hypoxia. There were no significant changes within each condition (normoxia vs. hypoxia) in HR, MAP, or SPO₂ in either the RIPC or the Sham group (P>0.05 for all).

2.3.2.2. Pulmonary artery pressures

Reliable echocardiographic images were obtained from 7 participants from the RIPC group and 4 from the time control group. PASP (Figure 2.3.) was unchanged from baseline after treatment in either group (RIPC, P = 0.64 vs. time-control, P=0.88).

2.3.2.3. Flow-mediated dilation

FMD of the brachial artery did not differ from baseline at any point after RIPC (P=0.89) or after time-control (P=0.41) (Figure 2.3.). There were no significant differences in baseline diameter or SRAUC in either group (P>0.05 for all).

2.3.2.4. ICA flow and intra-cranial velocity

Blood flow through the ICA and velocity in the MCA both increased upon the transition from room air to isocapnic hypoxia (P<0.05 for both, see Table 2.2), although the magnitude of this increase did not change after either treatment (RIPC, P=0.51 and time-control, P=0.39).
### Table 2.2. Chronic Hypoxia

<table>
<thead>
<tr>
<th></th>
<th>Room Air (3800m)</th>
<th>RIPC</th>
<th>Time control</th>
<th>P-value</th>
<th>Isocapnic Hypoxia (PETO₂ = 45mmHg)</th>
<th>Room Air (3800m)</th>
<th>RIPC</th>
<th>Time control</th>
<th>P-value</th>
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<tr>
<td></td>
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<td>1 hour</td>
<td>24 hours</td>
<td>48 hours</td>
<td>P-value</td>
<td>Baseline</td>
<td>1 hour</td>
<td>24 hours</td>
<td>48 hours</td>
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<tr>
<td>HR (bpm)</td>
<td>67.4±11.3</td>
<td>68.2±14.0</td>
<td>67.0±13.1</td>
<td>66.4±10.3</td>
<td>0.53</td>
<td>59.3±18.8</td>
<td>58.0±16.0</td>
<td>60.1±16.3</td>
<td>59.9±12.9</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>95.2±4.6</td>
<td>90.5±10.3</td>
<td>93.8±8.2</td>
<td>93.6±6.2</td>
<td>0.63</td>
<td>92.5±6.5</td>
<td>98.7±6.9</td>
<td>89.7±12.6</td>
<td>94.2±10.8</td>
</tr>
<tr>
<td>SPO₂ (%)</td>
<td>90.2±2.1</td>
<td>89.6±1.4</td>
<td>89.2±1.1</td>
<td>89.3±1.9</td>
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<tr>
<td>VE (L/min)</td>
<td>10.8±3.9</td>
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<td>PETO₂ (mmHg)</td>
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<td>PETCO₂ (mmHg)</td>
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<td>28.4±1.9</td>
<td>0.93</td>
<td>30.8±1.9</td>
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<tr>
<td>MCAv (cm/s)</td>
<td>52.7±11.6</td>
<td>53.7±10.7</td>
<td>51.4±8.8</td>
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<td>0.64</td>
<td>61.7±4.0</td>
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<td>62.8±6.8</td>
<td>58.4±7.9</td>
</tr>
<tr>
<td>ICA flow (mL/s)</td>
<td>227.0±46.7</td>
<td>246.8±34.2</td>
<td>248.1±25.2</td>
<td>238.9±25.6</td>
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<td>290.3±31.7</td>
<td>262.0±47.0</td>
<td>521.0±28.4</td>
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</table>

Selected vascular and respiratory parameters at baseline, and 1 hour, 24 hours, and 48 hours after dual-thigh RIPC or time control, after 8-12 days at high altitude (3800m). End-tidal partial pressure of O₂ was clamped to 45 mmHg under isocapnic conditions in the hypoxia phase. P-value represents between-groups significance. * represents difference from baseline (P<0.05).
2.3.2.5. Ventilation

Resting room air ventilation was unaffected by time in either treatment group; however, the hypoxic ventilatory response was higher than baseline after 24 hours treatment with RIPC. For example, HVR at baseline was 2.05±1.4 L·min/% SaO₂, compared to 1 hour (2.44±1.3, p=0.80), 24 hours (3.21±1.2, p=0.04), and 48 hours (2.79±1.5, p=0.21) post RIPC (Figure 2.3.). This increase was driven by an elevation in VE peak (P=0.02) as well VE average (P=0.03) during hypoxia, 24 hours after RIPC treatment (Table 2.2).

Figure 2.3. Chronic hypoxia. Selected vascular and respiratory parameters at baseline, and 1 hour, 24 hours, and 48 hours after dual-thigh RIPC or time control, after 8-12 days at high altitude (3800m). A) Pulmonary artery systolic pressure (PASP), B) Flow-mediated dilation (FMD) of the brachial artery, C) Peak hypoxic flow-reactivity of the internal carotid artery (ICA), D) The hypoxic ventilatory response (HVR). Black bars represent means for RIPC, grey bars represent means for Time control. For C) & D), end-tidal partial pressure of O₂ was clamped to 45 mmHg under isocapnic conditions in the hypoxia phase. Individual data points are shown, bars represent group means. ANOVA was used to determine the effect of time for each condition. *P-value represents difference from baseline.
2.4. Discussion

The aim of this study was to explore the potential protective effects of a single session of 4x5 minutes of RIPC on the peripheral, cerebral, and pulmonary vasculature during exposures to acute and chronic hypoxia. By using tightly controlled hypoxic episodes within the laboratory, as well as prolonged high-altitude exposure in the field, we were able to test early and late protective windows of RIPC in acute and chronic hypoxic models. We hypothesized that our method of RIPC would induce prophylactic vascular benefits during exposure to acute hypoxia, as well as therapeutic benefits during chronic hypoxia. Contrary to our hypotheses, we did not observe any discernable benefits in the form of reduced PASP, improved peripheral vascular function (i.e., FMD), or alterations in cerebral perfusion or cerebrovascular reactivity to hypoxia. We do, however, report an increase in the isocapnic HVR in the late protective window (24 hours) after RIPC treatment at high altitude (3800 m). Although hypoxic chemosensitivity may increase following RIPC at HA, the absence of any accompanying benefits on resting oxygenation or the vasculature suggests that the utility of a single RIPC session seems unremarkable during acute and chronic hypoxia.

2.4.1. Experimental paradigms of RIPC

Previous research investigating the utility of RIPC has been largely focused on clinical populations with the goal of improving outcomes after stroke (Dave et al., 2006; Ren et al., 2008), myocardial infarction (Gho et al., 1996; Costa et al., 2013; Man et al., 2017), and a number of ischemia related injuries [reviewed in: (Tapuria et al., 2008)]. RIPC appears to reduce biomarkers of tissue and neuronal damage after numerous surgical interventions [reviewed in: (Candilio et al., 2012)]; however, two recent large scale clinical trials (with concurrent propofol use) have failed to show an improvement in clinical outcomes when using RIPC prior to coronary artery bypass grafting (Hausenloy et al., 2015; Meybohm et al., 2015).

Despite extensive research into clinical utility, very little research has investigated whether RIPC elicits vascular benefits to those exposed to hypoxia, and if a minimum stimulus is required to elicit any of the potential benefits. For example, RIPC can be structured a number of different
ways, with ischemic episodes ranging from 4-10 minutes, repeated 3-6 times within a treatment, and the volume of total treatments ranging from one or two days, to weeks/months of consecutive daily treatment (Eisen et al., 2004; Meng et al., 2012). We sought to pursue a simple, conservative, and potentially practical form of treatment, using 4 consecutive 5-minute periods of dual-thigh cuff occlusion, interspersed with 5 minutes of reperfusion, for one single forty-minute session. This approach was chosen as it is logistically simple, and would be easy to perform as a field tool in normal trekking circumstances. We took repeated measurements at 3 separate points after the treatment to evaluate differences in previously established early (1 hour-16 hours) and late (18 hours – 2-3 days) protective windows (Koch et al., 2014). While the protective effects of RIPC have commonly been attributed to the upregulation of anti-oxidative and anti-inflammatory pathways, the exact pathways being utilized seem to vary based on the target organ, and the mechanisms of activation are still unclear. Further, how these pathways translate into physiological changes is relatively unknown.

2.4.2. RIPC and peripheral vascular function

In humans, long-term RIPC (daily, 1 month) has been associated with improvements in resting endothelial function (Kimura et al., 2007). This is likely due to RIPC-triggered signalling for the upregulation of the NO system (Gattullo et al., 1999; Kimura et al., 2007; Arroyo-Martínez et al., 2016) as well as the release of vasoactive molecules such as adenosine and bradykinin (Lim & Hausenloy, 2012), which may trigger increases in reactivity of the endothelial lining. Furthermore, recent research suggests that RIPC treatment may reduce sympathetic tone (Lambert et al., 2016), which would likely alter reactivity of the peripheral arteries to changes in shear stress. Improvements in resting FMD in healthy subjects have also been reported after 7 consecutive days of RIPC treatments (Jones et al. 2014), and it has been demonstrated that one session of RIPC offers protection against ischemia-reperfusion injury-induced (Liu et al., 2015), and exercise-induced (Bailey et al., 2012) impairments in FMD. However, in this latter study, and consistent with our findings, it was reported that resting FMD immediately after one treatment was unchanged. Our findings suggest that baseline and hypoxic FMD responses are unaffected by single-session RIPC treatment, although we also report no significant reduction in FMD upon the acute transition from normoxia to hypoxia. Two probable causes may contribute to this finding:
the duration or level of hypoxia was too small to elicit a reduction in FMD (measured after 15 minutes in acute hypoxia); or, the magnitude of reduction in FMD was too small to identify with our sample size. There is emerging evidence that impairment in FMD is not a uniform response to hypoxia and may depend on severity, duration, concomitant exercise (Tymko et al. 2016; Tremblay et al. 2016; Lewis et al. 2014), or the presence of cardiovascular risk factors (Frøbert et al., 2008). In the present study, failure to alter FMD with RIPC could subsequently be explained by the lack of a meaningful vascular impairment in our model of hypoxia.

2.4.3. RIPC and pulmonary vascular tone

It has been previously reported that a single session of RIPC prior to breathing a hypoxic gas mixture for 90 minutes blunts the rise in PASP (Foster et al., 2011). Furthermore, after pre-treatment with 5 consecutive days of RIPC prior to rapid ascent to high altitude (followed by an exercise challenge), the rise in PASP was also mitigated (Foster et al., 2014a). Mechanisms for this attenuation of hypoxic pulmonary vasoconstriction remain speculative and are poorly understood. During the chronic hypoxia (HA) component of our study, the rise in PASP could be at least partially attributed to a degree of pulmonary vascular remodelling, a complex process with proliferative and inflammatory components (Stenmark et al., 2006) that may be affected by RIPC, however the magnitude and time course of remodelling with hypoxia is still unclear. Results from our study suggest there is no change in the amplitude of the PASP response to hypoxia after RIPC in either acute or chronic hypoxia; however, our measures were taken only 30 minutes into the acute hypoxic challenge at point where PASP is still rising (Dorrington et al., 1997). Furthermore, we used a single session versus a 5-day repeated protocol, which may lead to dose dependent effects. Different, overlapping mechanisms may be responsible for the initial rapid increase, and subsequent slow intensification of pulmonary artery pressures (Vejlstrup et al., 1997) in response to hypoxia, and delaying our measurements to 90-120 minutes may have yielded different results. However, at altitude, where subjects were hypoxic for 8-12 days, RIPC failed to elicit any changes in PASP indicating that time of measurement likely does not explain our lack of effect at sea level. At high altitude, all participants exhibited a moderate elevation in PASP that is comparable with other high altitude studies (Antezana et al., 1998). Based on reports of reduced measures of NO-bioavailability and increased production of endothelin-1 (Sartori et al., 1999; Bailey et al., 2010),
we speculate that RIPC may differentially affect those with exaggerated HPV responses who are susceptible to high altitude pulmonary edema, although this hypothesis has yet to be tested.

2.4.4. RIPC and peripheral chemosensitivity

A novel finding of this study is that RIPC augments the HVR during chronic hypoxia (Figure 2.3). We did not attempt to investigate any mechanisms underlying changes caused by RIPC, although this finding broadly supports previous observations by Foster and colleagues (Foster et al., 2014a) that RIPC improves oxygen saturation upon ascent to high altitude. Sensitivity of the carotid body relies on the balance of numerous systemic factors, many of which vary significantly on a daily basis, leading to a large daily intra-individual variation in the hypoxic ventilatory response. Mechanisms underpinning the observed increase in chemosensitivity are purely speculative, but might involve increased release of neurotransmitters such as 5-hydroxytryptamine, or a hypoxia-inducible factor-1 aided improvement in oxygen sensing (Nurse, 2010; Prabhakar & Semenza, 2016).

2.4.5. Methodological considerations

A large amount of uncertainty still exists over the nature and magnitude of the ischemic stimulus required to optimally elicit the desired protective benefits of RIPC. An emerging pattern seems to suggest that there may be a dose-response relationship, i.e. thigh occlusion is more effective than arm occlusion; dual thigh treatment is more effective than single thigh; and more ischemic repeats and more consecutive days of treatment have an additive effect compared to single sessions (Koch et al., 2014). Inevitably, caution must be exercised, since the goal of ischemic preconditioning is to deliver small, non-lethal bouts of ischemia to an area, rather than creating an actual ischemic injury in the distal tissue. If we had utilized a longer duration of ischemia, more repeats, or added consecutive days of treatment we may have observed different results; however, the aim of this study was to explore the efficacy of one simple session of RIPC, with the goal of offering a simple prophylactic treatment to those travelling to high altitude on short notice, or a quick therapeutic and practical aid for those who may be experiencing signs of mountain sickness.
With a treatment such as RIPC, it is difficult to deliver a true “sham” protocol, as it is impossible to blind a subject to the obvious physical sensations associated with cuffs being inflated to 225 mmHg over the thighs. For this reason, naïve subjects were verbally informed that the aim of the study was to explore differences between arterial (200mmHg) and venous (20mmHg) occlusion during their respective trials.

We also acknowledge that the lack of an equally-weighted control group in the high altitude arm; however, with limited time and a small participant pool afforded to the study, we were only able to use four subjects to act as a time control. The most significant changes in acclimatization status occur within the first few days of arrival to high altitude (Swenson & Bärtsch, 2014), and we observed no changes in ventilation or any other physiological parameters in our time control group, suggesting that any acclimatization occurring between days 8-12 over the course of the study had little effect on our findings.

Given the small sample size and lack of a repeated-measures control, these data must be interpreted judiciously and considered more exploratory than confirmatory. For example, power calculations indicate that a much larger sample size (n>250) would have been needed to observe significant changes (>1%) in FMD with a power of 0.8. However, this is not feasible in high altitude research and supports the negligible influence of RIPC on FMD in hypoxia.

2.5. Preliminary Conclusions

Our results indicate that one session of 4x5 minute dual-thigh RIPC treatment seems to have no benefit in terms of pulmonary vascular protection or preserving peripheral endothelial function during acute and chronic hypoxia. Continued exploration of potential protective benefits that may be derived from utilizing different RIPC treatment strategies may be useful. An improved HVR 24 hour after preconditioning during chronic hypoxia suggests that RIPC can influence the hypoxic chemosensitivity of the carotid body. The HVR has been positively related to exercise performance at high (<6100 m) altitudes (Schoene et al., 1984) however, since resting SpO2 was unchanged, and an enhanced peripheral chemosensitivity may augment periodic breathing at altitude [reviewed in: (Ainslie et al., 2013)]- whether these changes are of advantage seems unlikely.
3 Conclusion

The focus of this thesis is to provide a review of current applications of RIPC treatment, as well as an investigation into its potential utility during hypoxic exposures. While there is strong evidence to support that RIPC protects against injuries of ischemia and reperfusion, during hypoxia we found no observable benefit to RIPC treatment in terms of reducing pulmonary artery pressures, improving peripheral endothelial function, or improving arterial oxygenation during acute and chronic hypoxia. A framework for these observations is extended and pertinent methodological considerations are outlined.

3.1. Mechanisms of RIPC

The mechanisms responsible for the protective effects of RIPC are poorly understood and are a matter of debate among scientists across a wide array of disciplines. Conditioning occurs as a sequence of three interrelated events: (1) the initial events in the treated organ or limb in response to the RIC stimulus; (2) transduction of the protective signal to the targeted organ or system; and (3) the events occurring in the target tissue enabling the protective response. It is likely that the observed responses are a product of a unique combination of both neural and hormonal pathways in each targeted system, as a number of different mechanisms have been described in both the early and late phases of protection. Early protection, which appears immediately after preconditioning, lasts ~12-18 hours and is due to alterations in ion channel permeability and post-translational modification of proteins, as well as the release of autacoids such as adenosine, NO, and bradykinin. The delayed phase of protection appears after ~18-24 hours, and lasts until ~72-96 hours after RIPC treatment. This phase occurs as a result of triggered changes in gene expression and de novo protein synthesis. These two phases are separated by a brief window of no protection.

Signal transduction does not appear to be mutually exclusive to either the neural or humoral pathways. In a rat cardiac transplant model, RIPC was found to be protective on the transplanted heart after preconditioning both the donor (Kristiansen et al., 2005), and the recipient (Konstantinov et al., 2005), prior to transplantation. Transplanting plasma from a preconditioned
rabbit has also been shown to elicit protective benefits on the plasma-recipient (Shimizu et al., 2007). However, studies using ganglion blocks (Gho et al., 1996), adenosine receptor blocks (Liem et al., 2002), and B2-receptor antagonists (Schoemaker & van Heijningen, 2000), as well as sectioning techniques (Lim et al., 2010; Donato et al., 2013), have also provided evidence for the importance of neurogenic pathways in RIPC-protection. There is no current consensus unifying the individual neural or humoral components of RIPC from the initial ischemic stimulus to the end-point protective effect, however it appears that there is a large amount of overlap between the two pathways (Przyklenk & Whittaker, 2011; Gopalakrishnan & Saurabh, 2014). For a detailed review of specific molecular pathways contributing to the RIPC protective response, see: (Tapuria et al., 2008). In the current study, measurements were taken 1 hour, 24 hours, and 48 hours after RIPC treatment in order to observe the effects of both the early and the delayed phases of protection. While no attempts were made to determine mechanisms responsible for the results, separation of early and late phase responses would provide broad insight into the activated mechanistic pathways.

### 3.2. RIPC and Vascular Function

#### 3.2.1. RIPC and pulmonary vascular tone

Based on reports of reduced pulmonary artery pressure during acute normobaric and hypobaric hypoxia after RIPC (Foster et al., 2011, 2014b), it was hypothesized that using dual-thigh RIPC would produce a similar attenuation of the rise in PASP during acute isocapnic hypoxia, as well as during a prolonged stay at high altitude. In the current study, RIPC produced no changes in PASP during either model of hypoxia; however, there were a number of methodological differences between our protocols and those by Foster and colleagues that may have contributed the differences in findings. In 2009, Foster et al. reported that RIPC reduced PASP after 90 minutes of hypoxia compared to controls, whereas our measurements were only taken after 30 minutes. The pulmonary vasculature responds to hypoxia with vasoconstriction, and PAP rises progressively over ~2 hours (Dorrington et al., 1997), indicating that our measurements were taken at a point where PAP is still rising. Although the effect of RIPC may have therefore been masked by the limited exposure to hypoxia, we also observed no benefit of RIPC 24-48 hours post-
conditioning during chronic hypoxia at high altitude, a time period where pulmonary pressures were stable and seemingly unaffected. Additionally, Foster et al. (2011) observed no change in estimated pulmonary vascular resistance or cardiac output after RIPC, making it difficult to interpret what may have contributed to the reduction in PASP. When the same group used 5 consecutive days of RIPC prior to rapid ascent to high altitude, PASP was measured after strenuous exercise in the hypoxic environment – here, exercise elevates PASP to an even greater extent than just hypoxia alone. In this study, the difference in PASP between the two groups at this time was only 2mmHg, a difference that likely has very limited clinical benefit. Furthermore, the subjects in both of the studies by Foster et al. were ~15 years older on average than the participants used in the current study. Age is associated with greater magnitude of HPV (Balanos et al., 2015), which may expand the opportunity for observable benefits to prophylactic ischemic conditioning.

It has been previously reported that a single session of RIPC prior to breathing a hypoxic gas mixture for 90 minutes blunts the rise in PASP (Foster et al., 2011). Furthermore, after pre-treatment with 5 consecutive days of RIPC prior to rapid ascent to high altitude (followed by an exercise challenge), the rise in PASP was also mitigated (Foster et al., 2014a). Mechanisms for this attenuation of HPV remain speculative and are poorly understood. Results from our study suggest there is no change in the amplitude of the PASP response to hypoxia after RIPC in either acute or chronic hypoxia; however, our measures were taken only 30 minutes into the acute hypoxic challenge at point where PASP is still rising (Dorrington et al., 1997). Further we used a single session versus a 5-day repeated protocol, which may lead to dose dependent effects. Different, overlapping mechanisms may be responsible for the initial rapid increase, and subsequent slow intensification of pulmonary artery pressures (Vejlstrup et al., 1997) in response to hypoxia, and delaying our measurements to 90-120 minutes may have yielded different results. However, at altitude, where subjects were hypoxic for 8-12 days, RIPC failed to elicit any changes in PASP indicating that time of measurement likely does not explain our lack of effect at sea level. At high altitude, all participants exhibited a moderate elevation in PASP that is comparable with other high altitude studies (Antezana et al., 1998). Pulmonary vascular remodelling, which occurs as early as 8 hours into a hypoxic exposure (Dorrington et al., 1997), may have limited the influence of RIPC, however this study did not attempt to measure the reversibility of pulmonary artery pressures. One can further speculate that RIPC may differentially affect those with
exaggerated HPV responses who are susceptible to high altitude pulmonary edema, although this hypothesis has yet to be tested.

3.2.2. RIPC and peripheral vascular function

A major focus of this study was to determine if RIPC would be beneficial to peripheral vascular function, through attenuating a decline in brachial artery FMD during acute and chronic hypoxia. Consistent with previous research, there was no change in FMD response during rest after a single session of RIPC. Several studies have shown that RIPC does little to enhance FMD in healthy subjects; however, in cases where vascular function is transiently impaired, such as post ischemia reperfusion injury, percutaneous coronary intervention, or strenuous exercise (Bailey et al., 2012; Kharbanda et al., 2002; Manchurov et al., 2014), RIPC has been demonstrated to attenuate or even abolish the subsequent reduction in FMD. Thus, it appears that RIPC of a limb may only improve FMD responses in cases of vascular dysfunction. Findings from the current study suggest that the FMD response during hypoxia is unaffected by RIPC treatment; however, it also appears that in the current testing conditions, vascular function was not impaired by hypoxia. Even during hypoxia, the values we report for FMD % dilation scores are well within population norms for the age demographic, and no statistical differences were found between normoxic and hypoxic values at any point in either of the acute or chronic studies.

While our findings suggest that FMD was not impaired by hypoxia, a number of studies have investigated the role of hypoxia on vascular function with mixed results. Studies have reported reductions in endothelial function at in participants with predetermined risk factors at high altitude, such as those with metabolic syndrome (Frick et al., 2006), elevated cardiovascular risk factors (Frøbert et al., 2008) and those predisposed to HAPE (Berger et al., 2005) or AMS (Bruno et al., 2015). In healthy participants however, the presence and magnitude of arterial dysfunction appears to rely on the duration and severity of hypoxia, as well as the method of ascent to high altitude. FMD appears to be preserved when the ascent to high altitude is conducted passively (Bruno et al., 2015; Tremblay et al., 2016; Tymko et al., 2016), however when the ascent or sojourn includes prolonged physical activity, a period of elevated markers of inflammation and endothelial
activation (Comassi et al., 2015), FMD is reported to be impaired (Lewis et al. 2014; Bakker et al. 2015).

During acute exposure to hypoxia, the level of impairment in vascular function appears to be contingent on the duration and severity of the hypoxic stimulus. Recently, a well-controlled study investigated the role of isocapnic hypoxia on endothelial dysfunction (Lewis et al., 2014) and found that during 30 minutes of hypoxia, FMD was reduced to a larger extent during moderate hypoxia ($P_{ET}O_2 = 55$ mmHg) compared to mild hypoxia ($P_{ET}O_2 = 75$ mmHg), however when reductions in SRAUC were accounted for, these reductions were both attenuated. During a longer 5.5-hour normobaric hypoxia exposure ($F_iO_2 = 0.11$), FMD was progressively reduced at all time points (60, 210, 330 minutes; 27-35%), suggesting that the reduction in FMD during acute hypoxia is also dependent on the duration of exposure (Lewis et al. unpublished data). In the current investigation, findings of preserved FMD during acute hypoxia may have been a consequence of measuring after only 15 minutes of hypoxia; extending our measurements to 30 or 60 minutes may have yielded different results.

Despite the lack of unity among findings relating to vascular function during hypoxia or high altitude exposure, evidence of disruptions in endothelial function via impaired acetylcholine dilation in AMS-susceptible trekkers and reduced FMD in HAPE-S participants compared to healthy controls, combined with observations of reduced FMD in Andeans with CMS compared to those without CMS, suggest that methods to improve vascular function may be one way to reduce the incidence or severity of high-altitude illnesses. While the current study observed no improvements with FMD after RIPC, after 10-14 days at high altitude none of the participants reported any symptoms of AMS or showed an abnormal pulmonary vascular response, leaving room for speculation that other individuals who respond poorly to high altitude exposure may have benefited more from the therapeutic measures. Our findings are further supported by previous reports that a single session of RIPC of a limb is insufficient to evoke vascular benefits to otherwise healthy individuals (Bailey et al., 2012; Kharbanda et al., 2002; Loukogeorgakis et al., 2005). However, repeated treatments of RIPC, when performed over 7 consecutive days or 3x a week, can improve conduit artery FMD (Jones et al. 2014a; Jones et al. 2014b) therefore, adding additional days of treatment to our protocol may have yielded different results.
3.2.3. RIPC and cerebral vascular tone

The current investigation observed no changes in cerebral blood flow (MCA<sub>v</sub> and ICA<sub>q</sub>) during hypoxia after RIPC treatment. The P<sub>A</sub>O<sub>2</sub> and P<sub>A</sub>CO<sub>2</sub> has a key regulatory influence on CBF (Willie et al., 2016), and considering our use of an end-tidal forcing system to keep P<sub>ET</sub>O<sub>2</sub> and P<sub>ET</sub>CO<sub>2</sub> constant throughout the protocols, it is not surprising that no change in CBF was reported. During acute hypoxia, the absence of a change in ventilation during end-tidal forcing after RIPC treatment supports the notion that steady-state CBF would likely be unaltered by acute changes in P<sub>i</sub>O<sub>2</sub> or F<sub>i</sub>O<sub>2</sub>. Furthermore, while increased NO-bioavailability has been cited in response to RIPC treatment, studies using NO donors GTN or sodium nitroprusside have shown no increase in CBF in healthy individuals (White et al., 2000; Rashid et al., 2003), supporting our observation of unaltered cerebral vessel tone. During chronic hypoxia, 24 hours after RIPC, an increase in the HVR was observed; however, P<sub>ET</sub>O<sub>2</sub> was held constant making it difficult to interpret if an increase in oxygen saturation would be observed upon ascent to higher altitude, and if this increase would translate to an alteration of CBF.

3.3. RIPC and peripheral chemosensitivity

In the current study, during acute hypoxia, the HVR, measured as the peak 15 seconds of ventilation in the first minute of exposure to hypoxia, was unchanged after RIPC treatment. However, 24 hours after RIPC during chronic hypoxia, the HVR was significantly elevated from baseline by 1.1 L·min<sup>-1</sup>·%SaO<sub>2</sub><sup>-1</sup> (see Figure 2.3). It appears that no study to date has directly investigated the effect of RIPC treatment on peripheral chemosensitivity. Nevertheless, our findings of an increased HVR broadly support the observation of significant increases in resting SpO<sub>2</sub> after RIPC (Foster et al. 2014).

Despite the current observation of an elevated HVR 24-hours post-RIPC during chronic high altitude, it is important to consider the role of oxidative stress on chemoreceptor function. Reactive oxygen species increase the sensitivity of the carotid body to hypoxia, which suggests that a procedure or treatment (such as RIPC) involving an increase of antioxidant activity might depress
the HVR. However, the HVR is the sum of numerous counteractive pathways activated during a hypoxic exposure, and the maintenance of the HVR throughout most of the protocol indicates that antioxidative pathways upregulated by RIPC may have been counterbalanced by alternative pathways that augment ventilation during hypoxia. When PaO₂ decreases, glomus cells within the carotid bodies have a reduced potassium permeability, causing rapid depolarization of the membranes. In response, these cells fire repetitive action potentials, causing calcium-triggered release of the neurotransmitter dopamine, which then initiates action potentials in the sensory afferents, ultimately signalling for increases in ventilation from the respiratory control center. The aim of this study was not to investigate direct mechanisms of action for RIPC treatments; however one explanation for the observation of enhanced chemosensitivity may involve upregulation of Kir6 channels in the carotid body, enhancing transmission of the hypoxic signalling. In Kir6.2 knockout mice, ischemic protection of the myocardium was abolished, suggesting intact K(ATP) channels may be a target for ischemic conditioning (Gumina et al., 2003). Alternatively, increased release of neurotransmitter 5-hydroxytryptamine has been reported after RIPC (Chao de la Barca et al., 2016) and may augment the HVR, or improvements in oxygen sensing in the carotid body may come secondary to an increase in HIF-1α (Nurse, 2010; Prabhakar & Semenza, 2016).

3.4. Methodological considerations

3.4.1. RIPC dosage

The aim of this study was to explore potential vascular benefits from a brief, single treatment of RIPC, administered in an easy-to-replicate fashion with minimal time or equipment needed. There is evidence to suggest that in larger doses, RIPC does indeed exhibit vascular benefits. For example, by reduced pulmonary pressures upon ascent to high altitude after 5 consecutive days of preconditioning (Foster et al., 2014a), and improved brachial artery FMD after 7 consecutive days of RIPC (Jones et al., 2014). Perhaps if multiple episodes of treatment were employed over a longer time frame, different results might have been evident. It is important to note, however, that there appears to be a dose-response curve when selecting the duration of each ischemic period and the number of ischemic repeats within a single session. For example, rabbits treated with 1-4 five minute periods of ischemia had an average infarct size of 20% of the at risk myocardium after
sustained coronary artery occlusion, but when treated with six or eight cycles the infarct size rose to 42% and 47% of the area at risk, respectively, compared to 60% in the controls (Iliodromitis et al., 1997). In another study in mice, 2 and 5 minute periods of limb occlusion exhibited equal cardioprotective benefits, whereas 10 minute cycles of limb ischemia abolished the protective effects (Johnsen et al., 2016). It remains unclear which biomarkers can be tested for in order to demonstrate that protection from RIPC has been achieved, however, our method of 4×5-minute periods of thigh occlusion appears to fall within the peak duration and number of repeats for ischemic cardioprotection. Despite this, it is unknown whether the activated factors responsible for vascular protection follow the same dose-response curve as those protecting the myocardium.

### 3.4.2. End-tidal forcing

Measures such as pulmonary artery pressure and cerebral blood flow are highly dependent on the \( P_AO_2 \). By using an end-tidal forcing system, we tightly controlled the participants’ \( P_{ET}O_2 \) and \( P_{ET}CO_2 \) throughout the hypoxic protocol, subsequently maintaining a constant \( P_AO_2 \) and \( P_ACO_2 \) throughout the entire duration of the experiment. While no changes were observed in the pulmonary vascular or cerebral vascular response to a controlled level of systemic hypoxia, this model may have masked an altered pulmonary vascular response by keeping \( P_{ET}O_2 \) constant at 50 mmHg, rather than allowing for ventilatory-induced changes in blood oxygen tension. At SL, in order to better replicate a hypoxic stimulus such as ascent high altitude, a select inspired fraction of \( O_2 \) (i.e. 0.12) could have been used; however, with no change in the HVR at SL it is unlikely that our results would have differed.

### 3.4.3. Variability in measures

Flow-mediated dilation of the brachial artery is a highly-sensitive measurement that is subject to variation from a number of daily environmental and physiological factors, as well as the reliability of both the sonographer and the analysis process. The use of specialized edge-detection software minimizes variability within the analysis, and use of an experienced sonographer with rigid methodological scanning techniques can significantly improve the reliability of the data; however, even with tight control of diet (i.e. avoiding foods with antioxidative properties, having subjects
come in 4-6 hours fasted), exercise (no strenuous exercise 12 hours prior), and diurnal variation (testing at the same time every day), there is still a large degree of variation in the measurement of FMD that cannot fully be controlled. Post-hoc power calculations have shown that >250 participants would have been needed to show a significant 1% change in FMD; this former number is of course impractical for exploratory field research of this nature.

In addition to FMD, the HVR also exhibits a large degree of variability between testing days for participants. Daily variations in the HVR are poorly understood, but may be partially explained by daily variations in blood pH and quality of sleep (Terblanche et al. 2004). Zhang and Robbins (2000) tested repeatability of the HVR over six consecutive days and found within subject inter-day variability of 26%. Preliminary visual inspection of the data indicates there are no decisive trends or skewing of results. Further studies employing larger sample sizes are needed to decisively determine the effect of RIPC on these highly sensitive measures.

3.4.4. Acclimatization

In the HA protocol, it is possible that acclimatization to hypoxia may have influenced the results. Acclimatization occurs progressively over the course of hours to months of exposure to hypoxia, and the measured physiological variables and responses may have drifted between the 8th and 12th day of stay at Barcroft Station. Little is known about changes in FMD during sojourn at high altitude, especially with respect to exposure at a constant altitude, however cardiovascular variables such as HR and VE (Peacock, 1998), CBF (Hoiland et al., 2016) and PASP (Kronenberg et al., 1971; Dubowitz & Peacock, 2007) remain relatively constant after 3-6 days of acclimatization. A small time-control subset was employed to successfully demonstrate no significant changes in our measured variables due to acclimatization over the course of the testing period.

3.4.5. Use of a sham intervention

Obvious limitations exist when it comes to delivering a true sham-treatment in place of a procedure involving repeated periods of dual-thigh cuff occlusion. Some studies have delivered the RIPC
while patients were under anaesthetics, and others have even used a dummy limb under a bed sheet to blind the surgeon and/or investigators. In an attempt to blind the participants, we chose to use a commonly-employed method of inflating the cuffs to 5-10 mmHg, instead of 200-225 mmHg. Additionally, naïve participants were informed the rationale of the study was to compare differences between arterial and venous occlusion. The effects of small, repeated venous disturbances on cardiovascular factors are unknown and may have influenced our results.

3.4.6. Experimental Design

The high variability of outcome measures, along with the small number of participants, makes it difficult to decisively evaluate the efficacy of RIPC under the current conditions. At sea level, a larger number of subjects combined with a repeated-measures crossover design would have added more statistical strength to the data; however, the short testing period and length of time required to be confident the treated participants were free from long-lasting effects of RIPC treatment meant that using counter-balanced groups was the strongest option. At high altitude, unless multiple trips separated by a great length of time are considered, a repeated-measures crossover is an unlikely option. While it appears that no formal rules exist when allocating subjects to unequally weighted treatment and control groups, under ideal circumstances we would have equal-sized treatment and sham/time control groups. A more heavily weighted treatment group was employed in order to better identify any changes that may have occurred after RIPC, with a small subset of time controls in order to observe any variation due to acclimatization.

3.5. Future Directions

Much research is still necessary to validate or refute the effectiveness of RIPC on preserving vascular function during hypoxia. Optimal dosage to elicit vascular protection is still unclear, and based on reports of improved normoxic vascular function after repeated days of RIPC (Kimura et al., 2007; Jones et al., 2014a), it may be beneficial to repeat a similar protocol after 2-4 consecutive days of limb conditioning. Additionally, further insight into the efficacy of prophylactic RIPC treatment prior to ascent to high altitude could be obtained by testing participants in the first 1-4
days of high altitude exposure, a period when vascular changes and risk of acute adverse effects is greatest.

Both AMS and HAPE appear to have a consistent, but unexplained trait of susceptibility. Trekkers who were deemed HAPE-prone exhibited 30% less exhaled NO than control subjects during a stay at 4,559 meters (Duplain et al., 2000), and a similar investigation found a strong negative relation between the level of high-altitude pulmonary hypertension and NO-bioavailability (Bailey et al., 2010). Additionally, one study found a significant reduction in FMD in participants who developed AMS when travelling to 3842 meters, compared to participants in the same who did not develop AMS. Collectively, these findings suggest that there may be a role for RIPC - prophylactically or therapeutically - for those who are deemed susceptible to HAPE, pulmonary hypertension, or AMS. At low altitudes, the populations that appear to benefit most from RIPC are those who are at an elevated risk for endothelial injury or dysfunction (ie. ischemia reperfusion injury, CHD, CVD), and it is possible that this same paradigm exists for those who have a history of demonstrating adverse responses to high altitude.
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Appendix

A1. Measurements of peripheral vascular function

A1.1. Flow-mediated dilation

Shear stress acts directly on the endothelium, and is likely the main physiological stimulus for the release of vasoactive factors regulating vascular tone. Using a brief period of limb ischemia, reactive hyperaemic stimulus upon resumption of arterial flow can be harnessed as a trigger for the endothelial-dependent dilation of the arteries. Flow-mediated dilation (FMD) is a common assessment of endothelial function, using a pressure cuff to induce limb ischemia and subsequent reactive hyperemia, while using B-mode and Doppler ultrasonography to simultaneously measure vessel diameter and blood velocity (Corretti et al., 2002). Standardization procedures for performing (Thijssen et al., 2011) and analysing (Atkinson & Batterham, 2013; Woodman et al., 2001) FMD have recently been put forward in order to increase inter-tester as well as between-studies reliability. FMD is commonly performed with the cuff distal to the imaging site on either the femoral, or brachial artery. After acquisition of a baseline image and a 5-minute period of cuff inflation to a suprasystolic pressure (220-250mmHg), rapid cuff deflation results in a surge of blood flow and consequently increases in shear stress, which evoked a vasodilator response. The reactive hyperemia, quantified as the peak blood flow response after rapid cuff deflation, is a measure of downstream microvascular reactivity, and is likely a result of myogenic and local vasodilators in the resistance arteries (Anderson et al., 2011; Crecelius et al., 2013). Conversely, FMD (expressed as either percent dilation, or absolute dilation in mm) is a measure of macrovascular reactivity (Thijssen et al., 2011), and is a product of vasodilator production in a proportional response to increases in shear rate (Pyke & Tschakovsky, 2005). While FMD provides a non-invasive, clinically relevant measure of endothelial function, the measurement is highly dependent on the experience and skill-level of the examiner, as tiny changes in angle or placement of the ultrasound probe can be extrapolated to large, erroneous changes in observed blood flow and vessel diameter. Accuracy and consistency of arterial diameter and blood flow analysis can be
improved greatly with the use of specialized, offline edge-detection software (Woodman et al., 2001).

A1.2. Glyceryl trinitrate induced dilation

In addition to performing FMD of the brachial or femoral arteries, a sublingual dose of glyceryl trinitrate (GTN) can be administered in order to directly trigger arterial vasodilation while imaging with B-mode ultrasound (Maruhashi et al., 2013). Ultrasound measurements of vessel diameter are continuously recorded for 10 minutes after GTN administration, and the peak diameter observed in that period is compared to baseline in order to give an index of maximal dilation. The mechanisms by which GTN causes vasodilation are unclear, although it is thought to act as a NO donor to the smooth muscle cells surrounding the artery, directly promoting maximal relaxation (Corretti et al., 2002). GTN operates independent of the endothelium, and is therefore considered to be an index of smooth muscle function, or endothelial-independent dilation. The ratio of dilation from FMD to GTN can then be calculated to give an estimate of global NO-dependent vasodilator function.

A2. Assessment of the pulmonary vasculature

A2.1. Echocardiographic assessment of pulmonary pressures

Given the invasiveness of right heart catheterization, the skill required to minimize risk of complications, and the cost of instrumentation, direct measurement of pulmonary arterial pressures and resistance should be reserved for critical situations. Alternatively, cardiac echocardiography allows us to perform non-invasive estimations of pulmonary pressures through Doppler-examination of the right heart. Using the peak velocity of the measured tricuspid regurgitant jet, the pressure difference between the right atrium and right ventricle can be estimated with the modified Bernoulli equation: \( P = 4v^2 \), where \( P \) equals the pressure difference, and \( v \) is the peak regurgitant jet velocity (Berger et al., 1985). This pressure difference can be then added to right atrial pressure, which is often assumed to be 5mmHg, or else calculated based on the magnitude of inferior vena cava collapse upon rapid inhalation or “sniff” [described in: (Rudski et al. 2010)].
Right atrial pressure is then added to the calculated peak pressure difference calculated from the Bernoulli equation, to give us an index of systolic pressure of the pulmonary artery (PASP).

A2.2. Quantifying HPV

The attempt to quantify HPV raises the question of which measure serves as the better surrogate, PVR or PASP, since neither of these outcomes directly measure pulmonary vascular smooth muscle tone. PVR varies significantly in response to changes in CO, demonstrated by several-fold increases in CO during exercise without any large change in arterio-venous pressure gradient across the lung (Naeije & Chesler, 2012). For this reason, PVR is not necessarily a good indicator of pulmonary smooth muscle cell activity. Alternatively, PASP is not largely affected by changes in CO, and may provide a better approximation of pulmonary vascular smooth muscle tone.

A3. Methods for measuring cerebral blood flow

With significant advancement in ultrasound and MRI technologies over the past 40 years, non-invasive metrics have become the gold standard for evaluating cerebral blood flow. MRI and PET allow for highly specific measurements within deep vascular beds, but involve astronomical equipment costs, require highly experienced operators, and has zero portability. In contrast, use of Doppler and ultrasound technologies allow us to make cheap, non-invasive, and temporally relevant measurements of vessel diameter and/or blood velocity within select intra- and extra-cranial arteries.

A3.1. Transcranial Doppler of the intracranial arteries

Transcranial Doppler (TDC) ultrasonography can be used to record blood velocity through large intracranial vessels through 3 primary approaches: (1) the trans-temporal approach, where the probe is placed slightly superior to the zygomatic arch; (2) the trans-ocular approach, where the probe is placed over the closed eye; and (3) the foramen magnum approach, where the probe is placed inferior to the occipital protuberance. Through these cranial windows, a Doppler beam is focused on the vessel of interest, and the difference in frequency (ie. Doppler shift) between
incident and reflected beams is used to calculate the velocity of the red blood cells travelling through the vessel. Standardization techniques for locating and confirming vessels within the skull are described in: (Willie et al., 2011b). It is important to consider that TCD ultrasound does provide a measurement of vessel diameter and therefore cannot be used to calculate absolute flow. Changes in flow are indexed through changes in red blood cell velocity, and this relationship only remains linear under physiological conditions where the diameter of the insonated vessel remains constant. In mild hypoxic conditions there is little dilation of the MCA, however at altitudes >5300m the MCA appears to dilate progressively with concurrent increases in severity of hypoxia (Wilson et al., 2011). In this case, for example, measurements of only cerebral blood velocity would subsequently underestimate flow if arterial dilation is not accounted for. Furthermore, changes in P_{aCO_2} greater than approximately 7.5 mmHg above or below baseline will cause vessel dilation or constriction, respectively, as will transient changes in mean arterial pressure (Lewis et al., 2015).

A3.2. Duplex ultrasound of the extra-cranial arteries

Ultrasound images of the internal carotid artery (ICA) and the vertebral artery (VA) can be obtained with duplex ultrasound, a technique which allows for simultaneous measurement of arterial diameter and blood velocity. Absolute measurements of velocity and diameter can subsequently be used to calculate volumetric flow through the each artery, and global cerebral blood flow is represented by the sum of bilateral ICA and MCA flows. Offline analysis of each ultrasound recording is best performed with specialized tracking software (e.g. FMD/BloodFlow Software Version 4.0), using methods described in: (Woodman et al., 2001) adapted for the extra-cranial arteries.