The effects of low altitude on isolated mammalian arteries, intact murine circulation, and as a therapy for myocardial infarction and hindlimb ischemia in mice

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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Abstract

Humans residing at low and moderate altitudes (500 m - 3000 m above sea level) have a lower risk of dying from many cardiovascular diseases in comparison to sea level residents [1-3]. We hypothesized the lowered barometric pressure at these altitudes enhances vasodilation in the systemic vasculature through reduced external compressive forces on arteries. This could promote an anti-artherogenic environment and protect altitude dwellers from cardiovascular diseases. To explore potential mechanisms behind the altitude benefit, we investigated how normoxic low altitude (through small reductions in barometric pressure) affects arterial function *ex vivo* and hemodynamics *in vivo*. We also investigated the value of normoxic low altitude simulation in treating ischemic disorders such as myocardial infarction (MI) and peripheral arterial disease (PAD) in mice.

We studied the effects of normoxic low altitude simulation on isolated resistance arteries of healthy C57BL6 mice (13.3±1 weeks) *ex vivo* using a pressure myograph system enclosed in a hypobaric chamber. Mesenteric arteries (n=14) were exposed to barometric pressures of 754 mmHg, 714 mmHg and 674 mmHg, and we used stepwise manipulation of perfusion pressures or flow rates to assess myogenic tone in the presence or absence of inhibitors of major endogenous vasodilators (i.e., L-NAME and Meclofenamate). We observed clear and immediate increases in vessel diameter at 714 mmHg and 674 mmHg (20.9±9.3% and 28.2±8.6%, respectively) compared to 754 mmHg ($p<0.01$) when perfusion pressure was increased. Flow-mediated vasodilation was enhanced under altitude simulation conditions with and without L-NAME and Meclofenamate. Vascular resistance was reduced significantly at 674 mmHg vs. 754 mmHg (2.14±0.60 mmHg*min/μL vs. 3.21±0.49 mmHg*min/μL, $p<0.05$).

We then catheterized the left ventricle of healthy C57BL6 mice (n=8) and generated pressure volume loops during consecutive acute exposures to 754 mmHg, 714 mmHg, and 674 mmHg to study the *in vivo* hemodynamic effects of normoxic low altitude simulation. We found that total systemic vascular resistance was reduced with normoxic low altitude simulation (10.09±0.15 mmHg*min/μL at 754 mmHg vs. 8.11±1.45 and 8.18±1.24 mmHg*min/μL at 714 mmHg and 674 mmHg, respectively; $p<0.05$). We also observed significant increases in stroke volume and cardiac output from 754 mmHg to 714 mmHg and 674 mmHg ($p<0.05$).

Then, to study the effect of normoxic low altitude simulation on a disease model of myocardial infarction, we performed left-anterior descending artery (LAD) ligation on three-month old C57BL6 mice. Treatment group mice (n=13) were placed in an altitude chamber to recover from surgery for 3-hours daily at 714 mmHg for 1 week, and controls (n=12) were only exposed to 754 mmHg at this time. Echocardiographic evaluation of left ventricular function was performed on Day 1 and Day 8. Ejection fraction improved by 14.2±5.3% in treatment group mice ($p<0.01$ versus Day 1) but did not change for control group mice on Day 8. Cardiac output and stroke volume increased by 11.48±3.9 mL/min and 14.33±8.3 μL respectively in treatment group mice ($p<0.01$ versus Day 1), while control mice showed no significant improvement. Infarct size was significantly smaller in treatment group mice.

Lastly, we performed femoral artery ligation to generate a model of PAD in three-month old C57BL6 mice. Control group mice (n=10) recovered at 754 mmHg (control) for 14 days. Treatment group mice (n=15) were placed in a low altitude simulation chamber (at 714 mmHg) to recover from surgery for 3-hours daily for two weeks. Hindlimb perfusion imaging was performed using a laser doppler line scanner for all mice prior to the surgery, and on days 1, 3, 7 and 14 post-surgery. At two weeks, ischemic reserve was significantly higher in the treatment group mice vs.

the control group mice (0.50±0.13 vs. 0.20±0.06; p=0.01). Treatment group mice also had higher limb function scores and showed better ability to walk at two weeks.

We conclude that normoxic low altitude simulation through reduced barometric pressure noticeably increases vascular diameter in isolated vasculature and improves cardiac output and stroke volume *in vivo* in healthy mice. Furthermore, we found that normoxic low altitude simulation can be used to improve cardiac function and reduce infarct size after an MI and improve ischemic limb blood flow in mouse models of PAD. We conclude that these changes are mediated by a reduction in the compressive forces experienced by arteries when barometric pressure is reduced at low altitudes and not hypoxia mediated mechanisms.

Preface

This dissertation contains my original work, which is currently in the process of peer-review publication.

Chapters 2 and 3 of this dissertation have been combined to form a larger manuscript which is currently in-press at PLOS One as SHAHID Anmol, PATEL B Vaibhav, MORTON S Jude, STENSON H Trevor, DAVIDGE T Sandra, OUDIT Y Gavin, MCMURTRY M Sean. **Low altitude simulation without hypoxia improves left ventricular function after myocardial infarction by reducing ventricular afterload.** *In press* with PLOS One.

My contribution to this project was conducting experiments to obtain data, analyzing this data, and writing and revising the manuscript. This project received ethics approvals from the University of Alberta Research Ethics Office.

Chapter 4 of this thesis is currently under peer review with Physiological Reports as SHAHID Anmol, STENSON H Trevor, MCMURTRY M Sean. **Low altitude simulation without hypoxia improves limb blood perfusion in mice with hindlimb ischemia.**

My contribution to this project included study design, data acquisition, cleaning and analysis as well as writing and revising the manuscript. This project received ethics approvals from the University of Alberta Research Ethics Office.

NOTE: For the purposes of this dissertation, “normoxic low altitude simulation” and “reductions in barometric pressure” will be used interchangeably. Low altitude simulation has been referred to as “negative pressure” when referring studies whose authors have used this terminology. “Air pressure”, “ambient pressure”, “room pressure”, and “atmospheric pressure” are used in altitude literature interchangeably and may be used when discussing pre-existing literature.

Dedication

This thesis is written in memory of my maternal grandfather Mohammad Sadiq Bhatti who saw me as a curious and inquisitive child and dreamt that I would pursue academia just as he did. I dedicate this thesis to my father Shahid Ali who made countless sacrifices to make sure his children had the opportunities to pursue higher education and live in a country that supports equal opportunities and freedoms for everyone. The support of my loved ones and the company of my pets (Kit, Bam, Bug & Lily) has meant to world to me.

Acknowledgements

I would like to thank to my supervisor, Dr. Michael Sean McMurtry for making this dissertation possible. He has been an incredible and kind mentor, always encouraging and sympathetic to the plights of my graduate career. I also would like to thank my supervisory committee member, Dr. Evangelos Michelakis for his candid and supportive mentorship which has helped me overcome so many adversities during my graduate career. My sincerest thank you to committee member Dr. Sandra Davidge and her lab technician Dr. Morton for always being so approachable and helpful with the *ex vivo* project and much more.

I would like to thank Alois Haromy, Kyoko Hashimoto, and Dr. Trevor Stenson for being so accommodating with my projects and always making the lab environment very warm and welcoming. My fellow students Drs. Yongzhe Hong, Ghazi Alotaibi, and Sola Mansour have been the kindest colleagues and friends I could have asked for and I can't thank them enough.

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Glossary of Terms

AA: Arachidonic acid

Ach: Acetylcholine

ATP: Adenosine triphosphate

cAMP: Cyclic adenosine monophosphate

cGMP: Cyclic guanidine monophosphate

CAD: Coronary artery disease

CCAC: Canadian Council on Animal Care

CHD: Coronary heart disease

CO: Cardiac output

COX-1: Cylooxygenase-1

COX-2: Cylooxygenase-1

CVDs: Cardiovascular diseases

CVP: Central venous pressure

DAG: Diacylglycerol

GTP: Guanosine-5'-triphosphate

HACE: High altitude cerebral edema

HAPE: High altitude pulmonary edema

HIF: Hypoxia inducible factor

HLI: Hindlimb ischemia

HPV: Hypoxic pulmonary vasoconstriction

HR: Heart rate

iNOS: Inducible nitric oxide synthase

IP_3: Insitol triphosphate

IDV: Integrated density value

LAD: Left anterior descending artery

LV: Left ventricular function

MAP: Mean arterial pressure

Mch: Methacholine

MI: Myocardial infarction

MLC: Myosin light chain

MLCK: Myosin light chain kinase

MLCP: Myosin light chain phosphatase

mmHg: millimeters of mercury

MMPs: Matrix metalloproteinases

NO: Nitric oxide

NOS: Nitric oxide synthase

PAD: Peripheral arterial disease

PAP: Pulmonary artery pressure

PASMCs: Pulmonary artery smooth muscle cells

P_B: Barometric pressure

PE: Phenylephrine

PGI_2: Prostaglandin I_2

pO_2: Partial pressure of oxygen

PSS: Physiological salt solution

ROI: Region of interest

SV: Stroke volume

TIMPs: Tissue inhibitors of metalloproteinase

UAPWC: University of Alberta Animal Policy and Welfare Committee

VEFG: Vascular endothelial growth factor

WHO: World Health Organizatio

Chapter 1 General Introduction

1.1 Cardiovascular diseases: leading cause of death and economic health burden globally

Cardiovascular diseases (CVDs) are illnesses of the circulatory system which affect the anatomy and physiology of the heart and blood vessels [5]. CVDs include an assortment of conditions including myocardial infarction (MI), coronary artery disease (CAD), heart failure, arrhythmias, pericardial diseases, aneurysms, peripheral atherosclerotic disease (i.e., peripheral arterial disease (PAD)), cerebrovascular disease, systemic hypertension, venous thromboembolic disease, and pulmonary hypertension [6, 7]. Of these, MI is very common and is associated with significant morbidity and mortality [8]. Most risk for MI is related to exposure to modifiable risk factors, including abnormal lipids, smoking, hypertension, diabetes, obesity, psychosocial factors, low consumption of fruits and vegetables, consumption of alcohol, and low levels of physical activity [9]. However, other exposures may modify the risk. CVDs are the leading cause of morbidity and mortality and have placed a significant burden on our society both regionally and globally.

1.1.1 Prevalence, mortality and economic impact of cardiovascular diseases on a Canadian and global scale

The prevalence of cardiovascular diseases is unmatched by any other illness worldwide. CAD is the leading cause of cardiovascular disease, followed by stroke [8]. In 2003, a third of all deaths in Canada were due to CVDs [10] and this trend has persisted over time with 2.4 million (8.5%) adults diagnosed with ischemic heart disease in Canada alone in 2012 [11]. In 2009, there were 315,000 Canadians reported to be living with a stroke [12]. On a global scale, the prevalence

of CVDs was 422.7 million in 2015, and the age-adjusted prevalence in 2015 was 6304.0 per 100,000 [8]. With the aging population increasing globally, cardiovascular diseases remain a major contributor to the disease burden worldwide [13].

The impact of CVDs on a global scale is just as dire - 31% of all global deaths are a result of these diseases. The World Health Organization (WHO) estimated this to be 17 million people in 2015. CAD and stroke contributed to the largest numbers, 7.4 million and 6.7 million, respectively [14]. The burden of CVDs has been found to be much greater in lower- and middle-income countries and it is estimated that 75% of cardiovascular deaths occur in these countries, likely due to a lack of access to effective and equitable healthcare service [15]. However, even in a developed country like Canada, CVDs have been the second leading cause of death after cancer, accounting for 22.7% of all deaths in 2009 [16]. On a positive note, advances in medicine and increased awareness of cardiovascular disease risk factors have caused the global mortality from CVDs to decline steadily over the last few decades [17]. However, it is pertinent that CVDs are studied rigorously and the trends in prevalence, morbidity, and mortality are constantly updated as these diseases are still a leading cause of death worldwide.

Any discussion of the global burden of CVDs will invite a summary of the economic impact of these diseases. In 2010, the total global expense of CVDs was estimated to be $863 billion USD [18]. This expense accounted for 14% of total health expenditures from 2013 to 2014, more than the cost of any other group of diseases [8]. Canadians spent 22.2 billion in the year 2000 on CVDs, making them the most costly illnesses in Canada [12]. As the aging population increases and the rate of obesity reaches an all-time high, the economic cost of CVDs is expected to grow significantly over the next few decades.[19]

1.2 The epidemiological link between altitude and cardiovascular disease in humans

Epidemiological studies have consistently shown a significant reduction in mortality from coronary artery disease, myocardial infarction, and stroke in individuals living at low and moderate altitudes above sea level (<3000 m) [1, 2, 20, 21]. A major limitation to understanding and discussing existing literature on the effect of altitude on humans lies in the discrepancy of ways that altitude is described and defined in these studies. Therefore, for the purposes of this dissertation, the Bartsch et. al [22] classification of altitude (shown in **Table 1** below) will be used for all discussion.

Altitude (above sea level)	**Classification and Definition**
0-500 m	Near sea level
500 m – 2000 m	Low Altitude–minor impairment of aerobic performance becomes detectable Residents of these altitude show improved outcomes from CVDs.
2000 m – 3000 m	Moderate Altitude—altitude illness starts to occur, and acclimatization becomes increasingly important for performance. Residents of these altitude show improved outcomes from CVDs.
3000 m – 5000 m	High Altitude—altitude illness and acclimatization become clinically relevant; performance considerably impaired
Above 5500 m	Extreme Altitude—prolonged exposure leads to progressive deterioration

Table 1.1 A system of classifying altitude based on meters above sea level and some physiological and health implications on humans. This classification system will be used to discuss any mention of altitude for the purposes of this dissertation. Adapted from Bartsch et al [22].

Mortimer *et al*. [3] reported a link between low and moderate altitudes (between 914-2135 m above sea level) and lower mortality from CAD in 1977, after evaluating the mortality rates of Caucasian men in New Mexico [3]. Although this report was criticized for having some confounding variables [23], a number of studies conducted since have also found strong associations between low and moderate altitudes of residence and lower mortality from CAD and stroke [1, 2, 20]. Interestingly, a study of 1198 Greek subjects recognized a protective effect of living at a altitudes as low as 950 m above sea level after adjustment for differences in various lifestyle variables and cardiovascular risk factors [21].

A notable study of altitude and cardiovascular disease included 1.64 million Europeans living between 259 m and 1960 m included mortality data, social and demographic information, and places of birth and residence [24]. The investigators showed that there was a relative risk reduction of approximately 22% per 1 kilometer of altitude above sea level for MI and ~12% per 1 kilometer for stroke [24]. Residence at low and moderate altitude has also been reported to be associated with lower all-cause mortality in the general American population [25]. **Table 1.2** below summarizes noteworthy epidemiological studies of altitude with respect to CVDs and/ or all-cause mortality in humans, with most studies reporting altitude to have a positive effect on human cardiovascular health.

It is speculated this "altitude benefit" may be due to factors such as a lower body mass index, lower incidence of diabetes, reduced number of smokers, lower blood pressure, lower levels of total plasma cholesterol, lower atherogenic lipoprotein cholesterol (C-LDL), and a higher level of physical activity of low and moderate altitude dwellers [21, 26-32]. Some studies have also reported lower incidence of CVDs in individuals living at high altitudes with lower levels of available oxygen [33, 34]. At these altitudes, adaptations to lower oxygen such as an improved

hematocrit, more angiogenesis and improved cardiac efficacy have been considered as potential mechanisms providing health benefits [35]. Sex-differences have been identified in the cardioprotective effect of altitude, with men seeming to gain more benefit more from residence at low and moderate altitudes reportedly due to overall higher levels of physical activity, more leisure time, and higher likelihood of employment in physically demanding careers. [1-3, 20, 21, 36].

Meanwhile, some studies examining patients with a history of cardiovascular disease report a detrimental effect of altitude on recurrence of cardiovascular events [37, 38], potentially due to elevation of serum cholesterol and hematocrit with altitude [39]. These studies seem to be the minority, with a larger and more robust body of literature suggesting a strong link between low and moderate altitude of residence and protection from death from MI, CAD, and stroke. Although this beneficial effect has been reported in residents of low, moderate, and high altitudes, larger populations reside at low and moderate altitudes (500 m – 3000 m) and thus have been studied more extensively in epidemiology.

Study	**Altitude**	**Outcomes of interest**	**Population**	**Sample size**	**Result**
Al Huthi et al. (2006) [40]	1500-2500 m	Risk factors for CVDs and complications from ACS	Yemeni Patients with history of acute coronary syndrome (30-69 years old)	768	Acute coronary syndrome occurs at younger age in higher altitudes in Yemenis
Al Tahan et al. (1998) [38]	620 m vs. 2000 m	Frequency of thrombotic stroke	Patients with history of stroke	190	Higher rate of thrombotic stroke at 2000 m attributed to a higher hematocrit

Ashouri et al. (1994) [41]	2200 m altitude vs. sea level	Acute myocardial infarction	Patients with history of myocardial infarction	124	Lower incidence of MI & lower rate of complication from MI
Baibas et al. (2005) [42]	950 m vs sea level	Total and coronary mortality	Residents of plains and a mountainous village in Greece	809	Lower total and coronary mortality at altitude – attributed to increased physical activity
Ezzati et al. (2012) [43]	1500 m	Life expectancy, mortality from ischemic heart disease	Death records of US population between 2001-2005	12.1 million	Higher altitudes had longer life expectancies and significantly lower deaths from IHD. No difference found in stroke.
Faeh et al. (2009) [44]	0-1500 m or above	Mortality	40 to 84-year-old German-Swiss residents	1.64 million	Mortality from stroke decreases 12% with every 1000 m increase in altitude and 22% for coronary artery disease
Fabsitz & Feinleib (1980) [20]	0 m to over 1500 m	Mortality from cardiovascular diseases	35 to74 year old residents of 3000 counties in USA	>1 million (exact study population undisclosed)	Altitude is negatively correlated with CHD and cerebrovascular accident mortality
Mortimer et al. (1977) [3]	914-2135 m	Mortality from coronary artery disease	Men and women in New Mexico	State population of 951,000 – 1,016,000 in 1960-1970	Reduced mortality from coronary artery disease in males living about 1200 m
Morton et al. (1964) [45]	1063 m – 3094 m	Mortality from heart disease	1960 population of Colorado	1,325,089	No beneficial association found at living at higher altitudes in Colorado vs. lower altitude in Colorado (*note that 1063 m as a baseline is

					already low altitude)
Ramos et al. (1967) [33]	4330 m	Cause of death	Naturally deceased residents of Cerro de Pasco, Peru	300	No cases of coronary artery disease or myocardial infarction and evidence of atherosclerosis was only found in one cadaver
Virues-Ortega et al. (2009) [34]	0-3000 m or above	CVDs as a cause of death	Seniors (60+)	555	CVDs as a cause of death were less common in higher altitude residents but life expectancy was lower in older residents at higher altitudes
Voors & Johnson (1978) [1]	0-1500 m	Atherosclerotic heart disease mortality	White adult residents in large cities of US	Not available** Very large population data set	There is lower mortality from atherosclerotic heart disease with higher altitudes
Winkelmayer et al. (2012) [46]	76 m – 1828 m or greater	Acute MI, stroke, death	Dialysis patients	984,265	Lower rates of CVD/ death with higher altitude of residence
Wozniak et al. (2012) [47]	1200 m or above vs. sea level	Survival	Heart transplant patients (1990-2008) in US	36,529	Patients living above 600 m had improved survival after transplant – survival even better at 1200 m or above

Table 1.2 A brief summary of several population-based studies investigating the association of altitude with incidence of cardiovascular diseases (CVDs), mortality from CVDs, and/or all-cause mortality. Primary population-based studies (mortality studies, ecologic studies, cohort studies, cross-sectional studies and retrospective studies) have been included whereas any experimental studies or systematic literature reviews have been excluded. All studies shown above observed

chronic exposure to altitude, with study participants being long-time residents of the altitudes investigated.

In interpreting the effects of altitude on humans, it is important to consider the potential effects of changing oxygen levels at varying altitudes. Although the percentage of oxygen in inspired air is constant at different altitudes, there are significant differences in the partial pressure of oxygen (P_{O2}) with increasing altitude as barometric pressure falls. Although sea level barometric pressure is generally considered to be 760 mmHg (= 760 Torr, 1 atm, 1013.25 millibars, or 101.325 kilopascals), it is important to note that temperature, moisture, gravitation/ rotation of the earth (seasonal), and latitude influence barometric pressure significantly [48]. So, at a given elevation, barometric pressure will likely differ between an equatorial area, a sub-tropical area, or a sub-polar area and the partial pressure of oxygen will be similarly affected [49, 50]. Considering the latitude

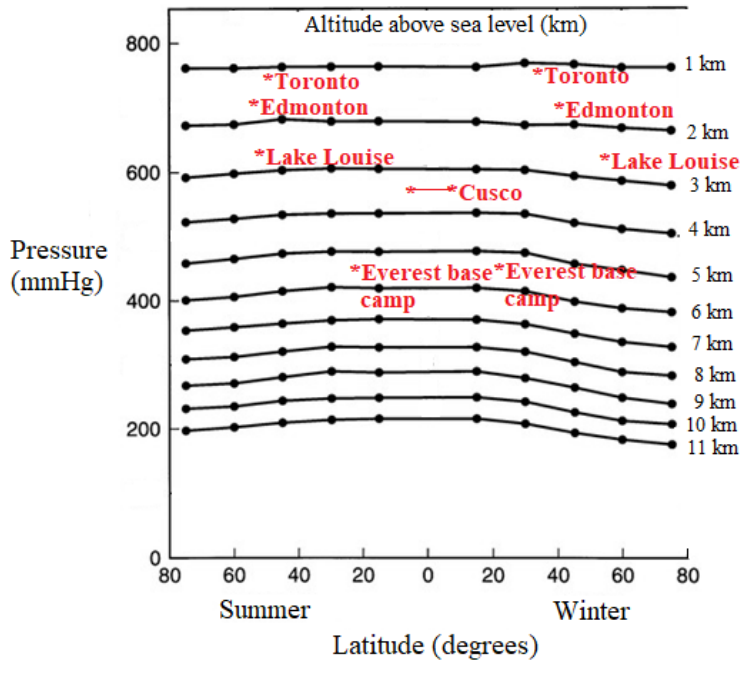

Figure 1.1 Barometric pressure varies with latitude and season and is shown here at altitudes between 0 and 11 kilometers above sea level. The seasonal fluctuation in barometric pressure of various Canadian cities and international areas of high altitude are denoted. At higher latitudes, a marked decrease in barometric pressure during the winter season can be seen. Adapted from West (1996).

and altitude of an area, along with seasonal variation can affect barometric pressure significantly. This is shown in **[Figure 1.1]**, with select high elevation international areas and Canadian cities marked as per the Oxford Atlas of the World [51].

Atmospheric pressure and inspired oxygen are approximately only 50% of their sea level values at 5500 m and only 30% of their value at sea level at extreme altitudes like the summit of Mount Everest (8900 m) [52]. A visual representation of increasing altitude and the corresponding decrease in P_{O2} is shown in **Figure .2** below [53]. Differences in inspired oxygen at various altitudes can create starkly different tissue environments for humans and other animals, with potential hypoxemia leading to important shifts in cardiovascular and pulmonary function [54].

Low and moderate altitudes, despite having a lower level of oxygen available for inspiration in comparison to sea level, may not constitute a significantly hypoxic environment for healthy humans where as high and extreme altitudes do [55-57]. Whether the cardiovascular benefits seen in humans with altitude exposure are a function of lowered levels of inspired oxygen at increasing altitudes is difficult to determine without a careful review of cardiovascular, pulmonary, and cerebral parameters of humans at clearly defined altitudes.

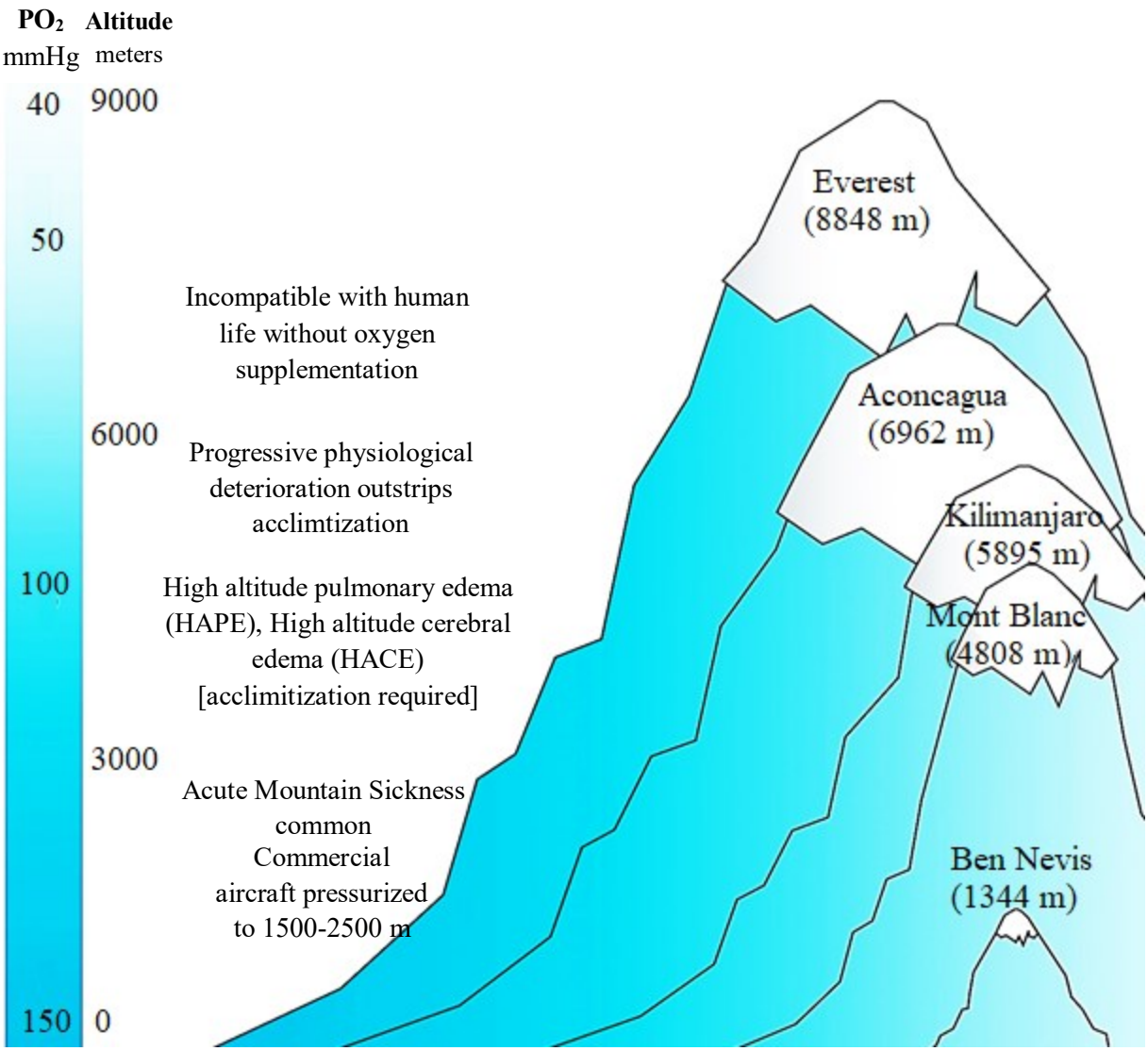

Figure 1.2 The partial pressure of oxygen (P_{O2}) decreases with exposure to increasing altitude (shown in meters above sea level). Common clinical effects of altitude exposure are shown, beginning at approximately 2500 m. Adapted from Wilson et al [53] and Hofmeyr et al [58].

1.3 The effect of altitude on humans

Until the mid-1900s, the effect of altitude on humans had largely been discussed in the context of mountaineering. In 1955, Alberto Hurtado, a Peruvian physician specializing in the study of altitude sickness first brought forward observations that hypertension and CVDs resulting from atherosclerosis (including MI and Coronary thrombosis) were rare in Peruvian natives and Caucasians living at altitudes of 3000 m or above in Peru [59-61]. Hurtado discussed potential mechanisms behind this, and deliberated heavily on a report from Campos et al. (1956) that showed condition of greater vascularity and dilatation of the vascular bed in all organs including the heart at altitude [62]. These initial reports generated significant interest in the cardiovascular benefits of residence at altitude and since then the effects of altitude on the human have been described heavily in the context of both visiting and living at altitude. These works are discussed below, separated into physiological variables along with a brief overview of principles needed to understand them well.

1.3.1. Cardiovascular function and hemodynamics at altitude

To appreciate the effect of altitude on the human heart, systemic circulation, and pulmonary circulation, available literature studying changes in measurable cardiovascular and hematological parameters with exposure to low, moderate, and high altitudes is discussed below.

1.3.1.1 Heart rate, cardiac output & stroke volume at altitude

At ascent to altitudes above 2000 m, acute increases in resting heart rate (HR) have been reported by numerous studies [63-68]. Acute exposure of 2700 m altitude with rapid ascent using

a cable car showed immediate but modest increases in heart rates of healthy subjects (from 69.1±12.6 to 80.4± 15.4 bpm). At high and extreme altitudes (>3000 m), heart rate increases immediately and sharply upon exposure to a larger extent [69]. Acclimatization to altitude begins with sustained short-term altitude exposure and has important implications on further cardiovascular responses to altitude [70]. At 3800 m, it has been reported that sharp increases in HR seen in the first 24 hours of altitude exposure drop back down within 36 hours of exposure (as acclimatization begins) [71]. However, depending on the severity of altitude, the rate of ascent, and individual susceptibility of altitude, acclimatization may take several days [72].

Several studies have attributed increased HR with altitude exposure to enhanced autonomic regulation of the heart in unacclimated individuals [73], with heart rates returning to near normal values after acclimatization occurs [74, 75]. In the autonomic system, the sympathetic nervous system releases norepinephrine (NE) from postganglionic neuron endings, which binds to β_1 adrenergic receptors in the heart to modulate the sympathetic influence on heart rate (chronotropy), contractility (inotropy) and conduction velocity (dromotropy) [76]. Hypoxia, cold exposure, and exercise associated with altitude exposure are thought to increase sympathetic activation acutely, affecting heart rate and other cardiac parameters shown in **Figure 1.** below [77].

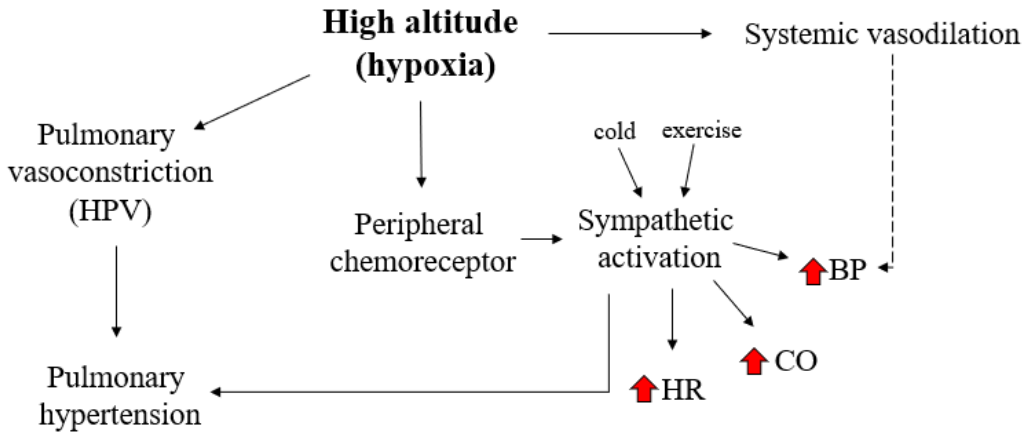

Figure 1.3 A summary of the effects of hypoxia (as seen with significant altitude exposure) on the heart, pulmonary and systemic circulation. Heart rate increases acutely as a result of hypoxia associated with altitude exposure before acclimatization. BP = blood pressure; CO = cardiac output; HPV = hypoxic pulmonary vasoconstriction, HR = heart rate. A dotted arrow denotes inhibition. Adapted from Bartsch & Gibbs (2007).

Others have found that heart rate variability differs between individuals upon ascent to 2400 m from sea level and can be used predict a common illness related to altitude exposure (acute mountain sickness (AMS)) upon further ascent to 4000 m [78]. Sex differences have been identified in heart rate variability with exposure to altitude, with women showing less variability between 3619 m and 5140 m presumably due a lower level of physical fitness in comparison to the men in this particular report [79].

Along with increases in heart rate, cardiac output (CO) has been reported to increase linearly with acute exposure to altitude and speculated to be because of increased sympathetic activity and vagal withdrawal in attempt to meet tissue oxygen demands in the face of a hypoxic environment (as seen with high altitude) [80]. Increased CO with no significant changes in stroke volume (SV) has been reported at altitudes below 4000 m [81], as can be predicted with the mathematical relationship shown in **Equation 1** below.

$$CO = HR \times SV$$

Equation 1 Cardiac output can be described as a product of the heart rate (HR) and stroke volume (SV) at any given time [82].

A study of airplane passengers flying in an aircraft pressurized to 585 mmHg (equivalent to 2152 m) showed that HR changes only slightly under normoxic cabin pressure conditions [83] and makes for an interesting comparison to previous altitude exposure studies. It is argued that cabin pressures in airplanes run low enough to cause hypoxia, however, the majority of passengers do not experience any adverse effects indicative of a hypoxic environment [84]. This suggests that the human cardiovascular system is not significantly affected by minor reductions in P_{O2} (as one would experience in a commercial airliner pressurized to low/moderate altitudes) and perhaps the effects of other altitude related factors such as changes in barometric pressure should be considered.

1.3.1.2 Blood flow at altitude

Very early observational work has shown that exposure to altitude may affect vascular diameter [62], and consequently blood flow in humans. A hypoxia mediated sympathetic activation leading to systemic vasodilation as shown in **Figure 1.2** (above) is often thought to be the mechanism [77] and increased levels of plasma catecholamines upon ascent to high altitudes from sea level support this theory [77, 85]. Systemic blood flow (Q) is increased in conditions of systemic vasodilation, and can be defined as the mathematical relationship between the difference in pressure (ΔP) from one point to another, and the resistance (R) experienced between the two points as shown in **Equation 2** below. By virtue of this mathematical relationship, systemic vasodilation seen with exposure to altitude increases blood flow through the systemic circulation by decreasing resistance in an important way (discussed in section 1.3.1.3. Systemic vascular resistance (SVR) at altitude).

$$Blood\ flow\ (Q) = \frac{\Delta P}{Resistance\ (R)}$$

Equation 2 A reiteration of Ohm's Law to describe blood flow. (ΔP) between the arterial system and the venous system is the major driving force of blood flow through the systemic vasculature. [82]

Poiseuille's equation **(Equation 3** below**)** emphasizes the influence of vasodilation (or increased *r* (radius)) on blood flow upon exposure to altitude. While blood vessel length (L) is unlikely to change with altitude exposure, changes in internal radius and blood viscosity (η)must be considered for their effect on systemic blood flow at altitude.

$$Flow\ (Q) = \frac{\Delta P \pi r^4}{8\eta L}$$

Equation 3 Poiseuelle's equation emphasizes the effect of changing vascular diameter on blood flow. This is discussed in detail in Section 1.3.1.3 below.

An clinical indicator of systemic blood flow, forearm blood flow, measured at sea level and Tibetan residents at 4200 m after breathing supplemental oxygen and after exercise showed that the high altitude resident Tibetans had more than double the forearm blood flow of low altitude residents, resulting in improved oxygen delivery to tissues [86]. Internal carotid arterial flow in Tibetan residents followed a similar pattern of much higher blood flow when compared to residents of lower altitudes. [87] Interestingly, increased epicardial coronary blood flow with acute high altitude exposure has been reported in healthy patients at altitudes up until 4500 m whereas patients with CAD only have preserved coronary reserve only up until 2500 m [65]. It remains to be

explored whether these large increases of blood flow are a function of hypoxia induced vasodilation, or other factors associated with altitude.

1.3.1.3. Systemic vascular resistance (SVR) at altitude

The systemic vascular system must deliver suitable blood flow throughout the body and as such small arteries have developed to have a large influence on systemic blood flow by regulation of their vascular tone through changes in diameter (shown in **Equation 3** above). The vascular endothelium (discussed in section 1.3.2 below) is critical to controlling the constriction and dilation of the blood vessels, heavily impacting the systemic vascular resistance (SVR) one experiences at any given time. This is the resistance to blood flow offered by all the systemic vasculature and is described mathematically as a relationship between the mean arterial pressure (MAP), the central venous pressure (CVP), and cardiac output (CO) in units of $mmHg*min*mL^{-1}$ or $dynes*sec*cm^{-5}$ shown in **Equation 4** [82]. SVR is a quantitative value used to describe left ventricular afterload [88].

$$SVR = \frac{MAP - CVP}{CO}$$

Equation 4 A physiologically relevant reiteration of Equation 2, allowing a calculation of systemic vascular resistance [82].

Not many studies have considered the direct effects of altitude on SVR, and it is noticeable that previous literature has instead focused on the cardiovascular response to decreased oxygen tension at higher altitudes. As shown above (**Figure 1.2**), systemic arteries relax to allow local arterial dilation (i.e., maintain a higher diameter) and enhance blood flow and oxygen supply to

hypoxic tissues [89]. A very early report by Campo & Iglesias (1956) hinted at a reduced total peripheral resistance at high altitudes of over 3000 m, a predictable effect of increased vascular diameter with vasodilation (see **Equation 3**) [62]. A recent study showed a decrease in SVR with increase in altitude (1589.1±191.2 vs. 1187.8±248.7 dynes/sec/cm; p=0.004) from baseline to 150 minutes at a simulated altitude of 4800 m [90]. Taken with studies reporting systemic vasodilation at altitude [91], it may be inferred that exposure to altitude can decrease SVR to a significant extent [81]. However, since studies looking at SVR have usually been conducted in high altitude conditions (<3000 m), it is difficult to predict whether SVR is similarly affected upon exposure to low and moderate altitudes.

1.3.1.4 Blood pressure at altitude

The effect of altitude on blood pressure in humans, although observed by dozens of studies, has not been defined adequately. Studying acute exposure to altitude, a very early report by Smith (1915) comparing blood pressure of healthy humans at sea level to Fort Stanton, New Mexico (1898 m) showed that majority of the subjects at the higher altitude reported a modest 1-10 mmHg increases in their systolic and diastolic blood pressure readings [92]. These findings have since been corroborated by reports at 2950 m, 3618 m, 4600 m, and 5140 m [93] [66].

Other studies, however, have reported small decreases in blood pressure with acute exposure to moderate altitudes, including a study at 3000 m [67]. Interestingly, no significant changes in mean arterial pressure (MAP) have been detected by two separate studies taking MAP measurements at 1700 and 2200 m before final ascent to altitudes over 4000 m. [94, 95]. The rate of ascent to altitude, age, and lifestyle factors may explain the sundry reports of acute exposure to altitude on blood pressure.

Reports of chronic exposure to altitude on blood pressure have also shown mixed results, perhaps also due to differences in lifestyle factors of the high-altitude populations studied. Whereas some have reported increased hypertension in the Tibetan and Ethiopian populations, inhabiting altitudes of 3000 – 4300 m over their lifetime, a large body of literature reports lower blood pressure of high altitude residents, shown in Andean, Nepalese, and Indian populations residing between 2000 – 4000 m [29, 96] [97-100]. A drop of 10 mmHg or more in systolic and diastolic blood pressure was found in Peruvian men born at sea level and residing at 3778 m for 2-15 years [101]. Similarly, in Nepalese residents of moderate altitude (2300 m), systolic blood pressure has been reported as considerably lower than that of low altitude dwellers [102]. Lower blood pressure has been thought to be highly instrumental in the reduced incidence of chronic heart disease in high altitude residents of Asia and South American than among comparable low altitude peoples [54, 103].

1.3.1.5 Blood constituents and volume at altitude

In the late 1800s, French Physician Denis Jourdanet noticed increased viscosity of human blood at high altitudes in Mexico [104]. Another high-altitude physician, Gilbert Viault, hypothesized that this change was due to an increase in the concentration of circulating red cells to allow acclimatization to lower levels of oxygen [105]. Since then, countless reports of increased hemoglobin and red cell counts at high altitudes have substantiated this theory and HIF1α (discussed in section 1.4 below) is now known to play an important role in this response [104, 106]. At high altitudes, HIF1α binds to the erythropoietin gene on chromosome 7, stimulating red cell production [107]. Circulating erythropoietin levels have been found to increase up to 400% within an hour of exposure to high altitude [108]. Interestingly, increased hemoconcentration has

been reported to decrease blood volume by 10% at 2900 – 4000 m and over 25% at altitudes between 4000-5000 m above sea level [109].

Changes in the blood with high altitude exposure have important implications on human health. Excessive production of red blood cells (polycythemia) is common at high altitudes and heightened in individuals with a hypoxic depression in ventilation [110]. Polycythemia can lead to severe thrombotic events, and is a serious consequence of high-altitude exposure [111]. Increased red blood cell concentration has also been implicated in higher recurrence of MI and stroke in patients with a history of cardiovascular events in some epidemiological studies of moderate and high altitudes [37, 112]. As mentioned above (*section 1.2*), low altitudes have generally been associated with positive cardiovascular outcomes. However, a recent study of 1466 subjects residing at low altitude (500-1500 m) in Saudi Arabia reported increased hemoglobin, mean corpuscle volume, and leukocyte counts vs. those living 200 m below sea level [113]. Mixed reports of changes in blood properties at low altitudes makes it challenging to clearly discern the relationship between hematological changes and cardiovascular health without further systematic study.

1.3.2 Endothelial function at altitude

The endothelium produces an array of vasodilators and vasoconstrictors in reaction to neurohumoral and metabolic stimuli [114]. The vascular endothelium regulates vascular smooth muscle cell function through major pathways that utilize nitric oxide, prostacyclins, and endothelium derived hyperpolarizing factor. How much each pathway effects the vasculature is often dependent on the vascular bed in question, however, it is thought that nitric oxide is the major player in larger conduit arteries [115]. Smaller arteries utilize endothelium derived hyperpolarizing

factor to a large extent, and prostacyclin contributes more to control over vascular tone in instances where nitric oxide is decreased (i.e., pathological conditions) [115].

Acting conversely to the vasodilators described above, the endothelium is also responsible for mediating vascular smooth muscle contraction through a variety of endogenous substances. The endothelium can modulate the vascular smooth muscle cell tone of target vessels in response to changing physiological conditions or input from the nervous system. The vascular endothelium can create vasoconstriction through endothelin-1, thromboxane A2, prostaglandin H2, O_2^- anions, and endoperoxides [116]. Of these, endothelin-1 has been recognized as the most powerful vasoconstrictor [117].

In pathological conditions including the presence of cardiovascular risk factors, the endothelium can experience alterations to its structure and function, leading to a vessel that is particularly vulnerable to the rise of atherosclerosis [114]. The endothelium is a particularly sensitive and responsive construct, and as such, altitude can significantly affect the interplay of vasodilators and vasoconstrictors described above, influencing the vasculature to behave in peculiar ways. A study of plasma concentrations of several eicosanoids (i.e., prostaglandins, thromboxane, leukotrines) in peripheral venous blood samples of 10 adults at 4350 m reported that the concentrations of vasodilating substances were found to be increased with respect to vasoconstrictors [118]. The impact of altitude on endogenous endothelial agents such as Nitric Oxide (NO), prostaglandins, and endothelin-1 is discussed below and summarized in **Table 1.3** below. The effects of altitude on endogenous levels of endothelium derived hyperpolarizing factor (EDHF) has not been measured in humans and is not discussed further in this dissertation.

Endothelial factor	Physiological difference	Population studied	Altitude	Reference
Endothelin-1	Increases and is correlated to higher degree of altitude sickness	Healthy subjects	1700 - 5050 m	Morganti et al (1995) [95]
			0 – 5000 m	Cruden et al (1999) [119]
			4559 m	Modesti et al (2006) [120]
			0 m, 3500 – 5129 m	Boos et al (2016) [121]
Endothelium derived hyperpolarizing factor	No direct measurements in humans exposed to altitude			
Nitric oxide	Measured NO decreases with altitude but is higher in individuals who show reduced symptoms/ severity of altitude sickness	Healthy subjects	2300 m	Guzel et al (2000) [122]
			5050 m	Donnelly et al (2011) [123]
		Mixed populations including some residents of high altitudes and pregnant women	0 m – 5500 m Systematic review of 32 studies - Some studies focused on gradual increases in altitude	Beall et al (2012) [124]
		Otherwise healthy with altitude sickness (high altitude pulmonary edema) patients	3400 m	Ahsan et al (2006) [125]
Prostaglandins and metabolites				
PGI2	No direct measurements in humans exposed to altitude			
6-keto-PGF1a	Significant increase at very high altitudes	Healthy subjects	3600 and 6522 m	White et al (2009) [126]
PGE2	Acute increase then decrease after acclimatization	Healthy subjects	4350 m	Richalet et al (1991) [118]

6-keto-PGF1	Acute increase then decrease after acclimatization	Healthy subjects	4350 m	Richalet et al (1991) [118]
PGF 2a	Acute increase then decrease after acclimatization	Healthy subjects	4350 m	Richalet et al (1991) [118]
Thromboxane A2	Significant decrease	Pregnant women	>3000 m	Bashir et al (2015) [127]

Table 1.3 An overview of select studies showing the effects of altitude on endothelial factors. As the studies are conducted at different altitudes and with different populations, it is difficult to determine how altitude exposure may affect each endothelial factor at varying altitudes.

1.3.2.1 Nitric Oxide and altitude

A soluble gas continuously synthesized from the amino acid L-arginine in endothelial cells by nitric oxide synthase (NOS), this vasodilator acts to modulate vascular tone, regulate cell growth, and maintain vascular homeostasis [114]. The nitric oxide vasodilation pathway **(Figure 1.4)** requires that Ca^{2+} levels in the endothelial cell are increased, usually due to downstream effects of agonists or mechanical factors like shear stress [128]. An isoform of nitric oxide synthase (eNOS) is responsible for synthesizing nitric oxide in the endothelium. In inactive conditions, eNOS exists in small invaginations in the cell membrane bound to a protein called caveolin [129]. Detachment from the caveolin and activation of eNOS occurs when levels on intracellular calcium increase [129]. Nitric oxide agonsists such as acetylcholine (Ach), bradykinin, adenosine diphosphate, adenosine triphosphate, substance P, and thrombin [130] are able to cause release of

calcium from the endoplasmic reticulum, influencing the dissociation of eNOS from caveolin [128].

Once Ca^{2+} levels have been increased, NOS produces NO. This gas then diffuses to the vascular smooth muscle cells, relaxing these cells and dilating the vessel [131]. This occurs through the activation of soluble guanylate cyclase to produce 3', 5' monophosphate. Protein kinases that are cGMP-dependent are activated, resulting in a cascade of phosphorylation events that lead to an increase in myosin light chain phosphatase activity [132], causing vascular smooth muscle cell relaxation [131]. This generally occurs due to one of three ways: decreases in concentrations of intracellular free calcium, desensitization to calcium, and thin filament regulation [131]. In the respiratory epithelium, an inducible form of nitric oxide exists (iNOS) and is upregulated in response to many factors like hypoxia and inflammation [133].

Under normal conditions, the production and release of nitric oxide to relax conduit blood vessels is a major anti-atherosclerotic factor, which is mainly exercised by relaxing smooth muscle cells [134]. Dysfunction in the production and release of nitric oxide has been implicated in hypertension, hypercholesteremia, diabetes, and heart failure, and is termed endothelial dysfunction [135]. Impaired NO bioavailability is a hallmark of endothelial dysfunction and can happen due to reduced production of NO by NOS or increased breakdown of produced NO through reactive oxygen species [135]. A balancing act between nitric oxide and endothelin act to maintain vascular tone, and is discussed below in section ***1.3.2.3***. Apart from endothelin-1, several other endothelial factors may affect the bioavailability of nitric oxide as shown in **Figure 1.5**.

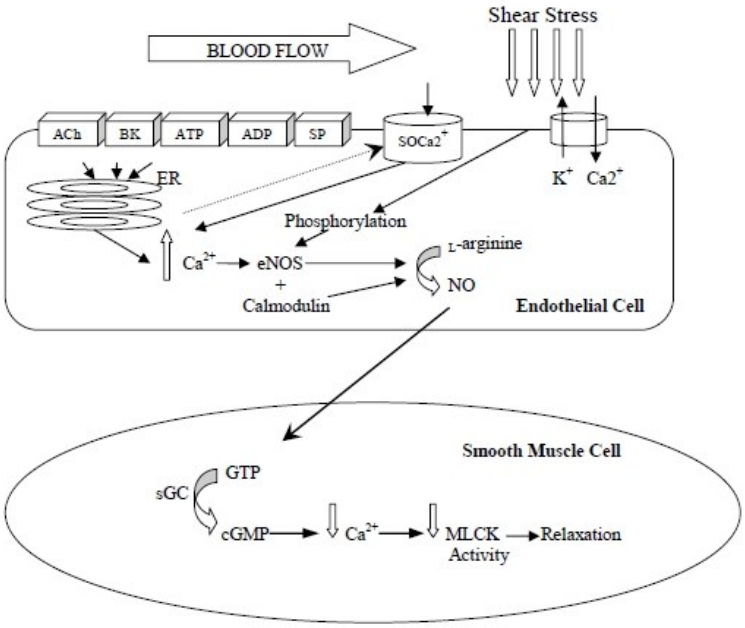

Figure 1.4 An illustration of nitric oxide (NO) and its actions on the vascular smooth muscle cell. Factors such as acetylcholine (Ach), bradykinin (BK), adenosine triphosphate (ATP), adenosine diphosphate (ADP), have the ability to affect calcium release from the endoplasmic reticulum (ER). Store-operated Ca^{2+} channel ($SOCa^{2+}$), soluble guanylyl cyclase (sGC), cyclic guanosine-3', 5-monophosphate (cGMP); myosin light chain kinase (MLCK) are shown in this figure and discussed above. Reprinted as per the reproduction guidelines of The Open Cardiovascular Journal ; Sandoo et al [128].

Nitric oxides can be biologically active and are measurable in blood, broncholavage, saliva and urine [124]. The metabolism of NO also leads to tyrosine nitration in blood and tissues [136]. As such, there are ways to measure changes in nitric oxide experienced by humans at ascent to altitude. An early study reported decreases in exhaled NO (in parts per billion) of healthy subjects ascending to low and moderate altitudes of 1336, 2300, and 3440 m for short periods of time in comparison to measurements taken at sea level [122, 123]. Beall and colleagues conducted a systemic review of 32 studies of visitors to altitude (794-5050 m) revealing a trend of decreased

NO levels in the lung, plasma and red blood cells with acute altitude exposure [137]. NO is a major contributor to vasodilation, and decreased levels of NO with acute altitude exposure may suggest that other vasodilatory mechanisms are at play in altitude induced systemic vasodilation.

Interestingly, it has been reported that higher plasma nitrogen oxide levels were found in individuals resistant to high altitude pulmonary edema at 3400 m in comparison to levels in individuals with high altitude pulmonary edema at this altitude [125]. This suggests that higher NO levels are associated with decreased vulnerability to altitude sickness, implicating NO as a player in acclimatization to altitudes high enough to induce hypoxia.

1.3.2.2 Prostaglandins and altitude

Prostaglandin metabolites (PGE2, PGI2, PGFa, Thromboxane A2) represent an assembly of highly vasoactive substances with direct and indirect actions on vascular smooth muscle cells in response to a variety of stimuli. The importance of prostaglandins lies in their ability to modulate the vascular effects of calcium and produce vasodilatory effects [138]. Of these, PGI_2 (prostacyclin) is recognized as an important vasodilator, and is produced in the endothelial cells when there is agonist binding to receptors on the endothelium or shear stress causing an increase in calcium levels [138]. Arachidonic acid is converted into prostaglandin H2 through cyclooxygenase action (COX1 and COX 2). This results in the formation of PGI_2 and thromboxane A_2 (TXA_2). Interacting with the thromboxane receptor (TP) on the vascular smooth muscle cell, thromboxane A2 causes vasoconstriction. PGI_2 has the capability to bind to the thromboxane receptor (TP) to cause vasoconstriction, or more with more affinity to the prostacyclin (IP) receptor to cause vasodilation [139]. This occurs through the activation of adenylate cyclase to form adenosine monophosphate,

and the use of protein kinase A to increase Ca^{2+} reuptake by the sarcoplasmic reticulum and Ca^{2+} extrusion from the cell [139, 140]. The effects of PGI_2 and TXA_2 on the endothelium is shown **Figure 1.5** below [141].

6-Keto-PGF1a, often used to assess PGI_2 production, has been reported to increase significantly at extreme altitudes (5000 m) versus sea level measurements [126]. A study that exposed healthy subjects to altitude of 4350 m over the course of 8 days reported that subjects experienced acute mountain sickness and hypoxia leading to large increases in PGE2, 6-keto-PGF1a and PGF 2a in the first four days before returning to normal [118]. Furthermore, a study of pregnant women at high altitude showed significant increases in thromboxane A2 and a significant decrease in levels of PGI2 compared to pregnant women at the lower altitudes [127]. Instances of directly measured plasma PGE and PGE2a under stimulated altitudes have only been recorded to date in rat models, reporting increases in these substances at higher altitudes [142]. It has been noted that changes in prostaglandin levels with altitude exposure show associations with the presentation of acute mountain sickness, hypoxic pulmonary vasoconstriction, and hypoxic cerebral edema [118, 142]. There is a gap in knowledge concerning the levels of prostaglandins in the blood that may be affected by altitudes too low to elicit hypoxia in humans.

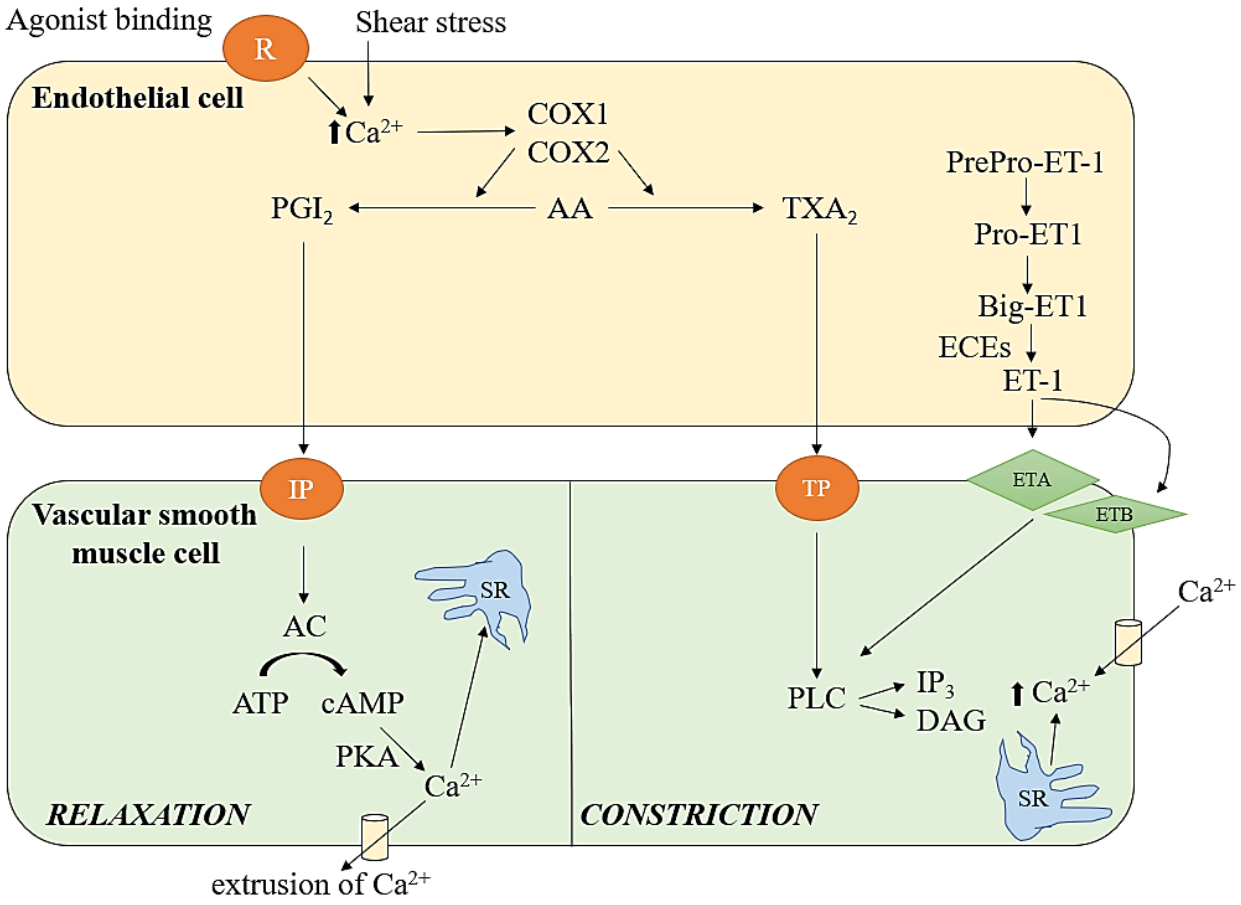

Figure 1.5 An illustration of the production of endothelin-1, prostacyclin, and thromboxane A_2 on the endothelium, which then have direct effects on vascular smooth muscle cells. Ca^{2+}: calcium ion, R: receptor, COX-1: cyclooxygenase-1, COX-2: cyclooxygenase-2, AA: arachidonic acid, ATP: adenosine triphosphate, cAMP: cyclic adenosine monophosphate, PGI_2: prostaglandin-I_2 (prostacyclin), TXA_2: thromboxane-2, ET: endothelin, ET-1: endothelin-1, ECEs: endothelin-converting enzymes, ETA: endothelin receptor A, ETB: endothelin receptor B, PLC: phospholipase C, IP3: inositol triphosphate, DAG: 1, 2-diacylglycerol. Adapted from Oishi et al (2011).

1.3.2.3 Endothelin-1 and altitude

Known as a powerful endothelial vasoconstrictor, endothelin-1 production is stimulated by hormonal factors such as angiotensin and vasopressin [143]. Endothelin-1 is synthesized as its inactive form pre-proET-1, which is then turned into proET-1 before being cleaved into inactive big-ET-1 (shown in **Figure 1.5** above). This version is then converted into the active form of endothelin-1 by endothelin-converting enzymes and matrix metalloproteinases [144, 145]. Once it is released, endothelin-1 is capable of interaction with G-protein-couple receptors such as ETA and ETB, which are located on vascular smooth muscle cells [146]. When the ETA receptor is stimulated, there is an increase in the calcium levels inside of the vascular smooth muscle cell through the downstream actions of phospholipase C (PLC), inositol triphosphate (IP3), and diacylglycerol (DAG) [147]. This is achieved by increasing the influx of Ca^{2+} coming into the vascular smooth muscle cells and increased release of Ca^{2+} from the sarcoplasmic reticulum. The more available calcium then binds to calmodulin, activating myosin light chain kinase phosphorylation and subsequently causing vasoconstriction [148]. It has been discovered that eNOS and ET-1 are distributed throughout the cell cytoplasm as well as with intracellular organelle membranes in the endothelial cells [149] [150]. The proximity in location of the eNOS and endothelin-1 seems to fine tune the effects of endothelin, so that any imbalances in the production of endothelin-1 may be counterbalanced by increased NO production [151].

In an 8-day ascent to Mount Everest, increased plasma endothelin-1 levels (2.7 ± pg/ml vs. 1.8±0.1 pg/ml at sea level) were reported in healthy subjects at low altitudes of 1700 m and above [95]. It was noted that plasma endothelin-1 increased progressively when healthy subjects ascended Mount Everest (an altitude of 5050 m within a period of 8 days) [95]. This work was replicated in healthy subjects climbing Mount Rosa of the Italian Alps not much later [95, 119].

In mountaineering studies, increased plasma levels of endothelin-1 was found to be highly correlated with the degree of pulmonary hypertension and acute mountain sickness observed in subjects exposed to acute hypobaric hypoxia [120]. It was suggested that the level of endothelin-1 release upon high altitude exposure could be correlated with the degree of altitude-related sicknesses experienced [120].

1.3.3. Pulmonary parameters at altitude

A well-defined response to high altitude exposure is hypoxic pulmonary vasoconstriction (HPV), an adaptive mechanism that occurs in response to alveolar oxygen tension dropping below a threshold level [152]. HPV is unique to pulmonary circulation, and serves to constrict pulmonary arteries supplying the hypoxic area of the lung in an attempt to redirect perfusion to lobes with better ventilation so that systemic oxygenation may be optimized [152-154]. Hypoxic conditions activate the HPV response, which may trigger or be accompanied with an increase in pulmonary artery pressure (PAP) reported at high altitudes. The pulmonary artery smooth muscle cells (PASMCs) are instrumental in the HPV response and can elicit HPV even in the absence of endothelium [155]. Endothelial factors, particularly endothelin and thromboxane are implicated in modulation of the HPV response [156, 157]. HPV is retained in isolated lung, lung slices, and pulmonary artery rings studied in an organ bath when exposed to hypoxia [158]. Isolated PASMCs also experience contraction during low oxygen conditions, demonstrating the sensitivity of these cells to oxygen tension [159]. The overall mechanism of HPV, although complicated beyond the scope of this dissertation, can be simplified to an alveolar sensing of low oxygen tension and the subsequent inhibition of specific potassium channels in PASMCs leading to membrane depolarization, opening of voltage gated calcium channels (L-type), and finally vasoconstriction [155, 160].

It is important to note that HPV with exposure to hypoxia is blunted in long-time residents of high altitudes like the Tibetans who have mostly resided at elevations between 3000 - 5000 m permanently [161-164]. In a study comparing Tibetans to the Han Chinese who reside permanently moderate altitudes of approximately 2700 m, Tibetans responded less vigorously to hypoxic challenge (shown in **Figure 1.6** below). Further differences in HPV response have been identified with different levels of hypoxia experienced, as well as sex differences [165].

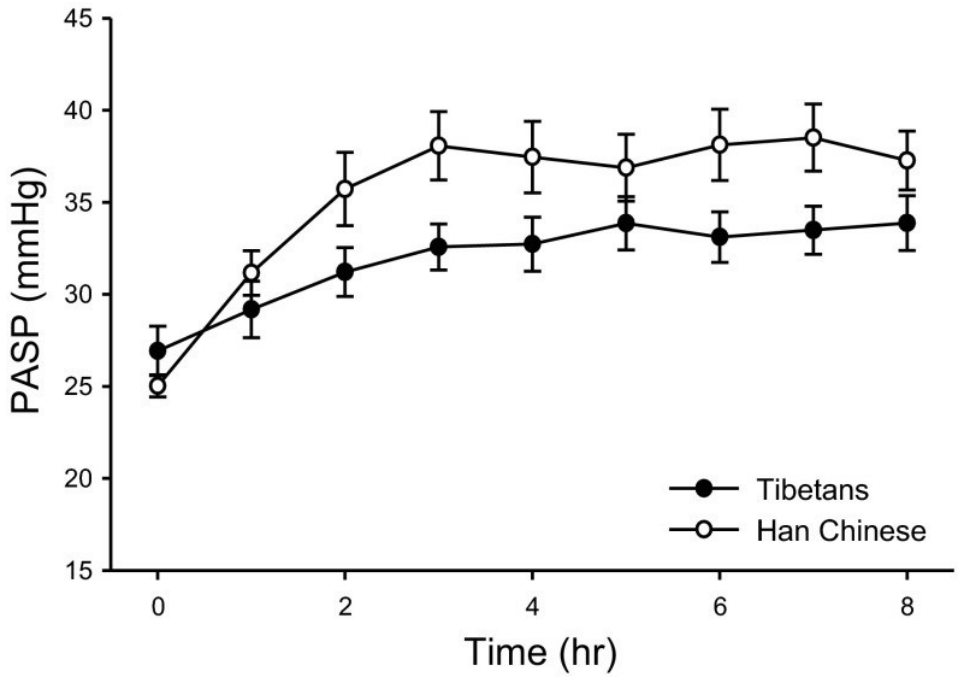

Figure 1.6 Pulmonary artery systolic pressures (mmHg) in response to 8 hours of sustained isocapnic hypoxia (at end tidal P_{O2} 50 mmHg) were lower in healthy Tibetans compared to Han Chinese volunteers. Reprinted as per the creative commons license attributed 3.0 (unported): the Physiological Society from Petousi et al (2014).

HPV has been discussed in the pathophysiology of many altitude related diseases, including acute mountain sickness, and the more severe high-altitude pulmonary edema (HAPE)

[166]. It has been suggested that an uneven HPV response could lead to HAPE, with overperfusion of certain regions of the pulmonary vascular bed and resulting increased capillary pressure, stress failure of the pulmonary capillaries, altered permeability of large proteins, and alveolar fluid leak across the capillary endothelium leading to interstitial and alveolar edema and an inflammatory response [167, 168] [166, 169].

According to an early study, HPV can be detected in humans and other mammals at altitudes as low as 1600-2500 m [170]. Increases in pulmonary vascular resistance are observed at higher altitudes, corresponding to increased pulmonary artery pressure (PAP) as a response to lower alveolar oxygen levels. Pulmonary vascular resistance shows dramatic increases at altitudes nearing 4000 m [90]. Vogel et al have reported an increase of 25 mmHg in PAP at 3300 m, compared a baseline PAP of 15 mmHg at sea level [81, 171]. PAP increases in conditions of low oxygen and can lead to chronic pulmonary hypertension if left unmanaged [158]. Due to the large variety of altitudes investigated in previous studies, it is unclear exactly at which altitudes HPV starts to become a physiological limitation for humans and whether this has implications on cardiovascular health of low and moderate altitude residents.

1.4 Altitude and experimental work in animal models

A major focus of research on altitude has been to study mountaineering and the resulting altitude disorders. As mentioned above, high altitudes (>3000 m) are associated with adverse cardiovascular effects in humans. Many of these effects are due to the inability of humans to acutely adapt to the levels of hypoxia at these altitudes [172]. It is known that increases in altitude have a linear relationship with decreases in the partial pressure of oxygen [54]. At high altitudes, the reduction in the partial pressure of oxygen leads to a hypoxic state in mammals where blood flow is altered and directed to meet demands of essential organs [173].

HIF1α is a master regulator in hypoxic situations, setting off downstream effects (by affecting over 90 genes) aiming to meet the metabolic demand of bodily tissues in these conditions [174]. HIF1α exists as a prolyl that is hydroxylated by prolyl hydroxylase domain protein 2 (PHD2) in its oxygen-dependent degradation domain, binding to the E3 ubiquitin ligase von Hippel-Lindau protein (VHL) for degradation under normoxic conditions [175]. The target genes of HIF1α include vascular endothelial growth factor (VEGF), and glucose transporters GLUT1 and GLUT 4 in addition to other genes that allow cells to depend on glycolysis for energy [176]. The activation of HIF1α is immediate in hypoxic and anoxic conditions, suggesting that HIF1α may modulate aerobic metabolism acutely [175]. At higher altitudes corresponding to lower partial pressures of oxygen, HIF1α is not degraded to the extent it would be in normoxic tissues and becomes bound to HIF1β and the hypoxic response elements, which control target genes and allow the downstream effects of HIF1α to become apparent [174]. This effect is highlighted in **Figure 1.7** below.

Apart from its well-studied activation in hypoxic environments, HIF1α is also known to be activated in non-hypoxic conditions through growth factors, vascular hormones (such as angiotensin II), cytokines and some viral proteins [177-179]. Non-hypoxic activation of HIF1α does not seem to depend on the stabilization of HIF1α, but rather on the increase on HIF1α translation through the PI3 kinase pathway leading to accumulation of HIF1α [180]. It is believed that HIF1α may be activated in non-hypoxic conditions so it's downstream angiogenic influences are utilized in instances such as wound healing [180]. Whether HIF1α is activated in fairly normoxic conditions at lower altitudes has not been investigated.

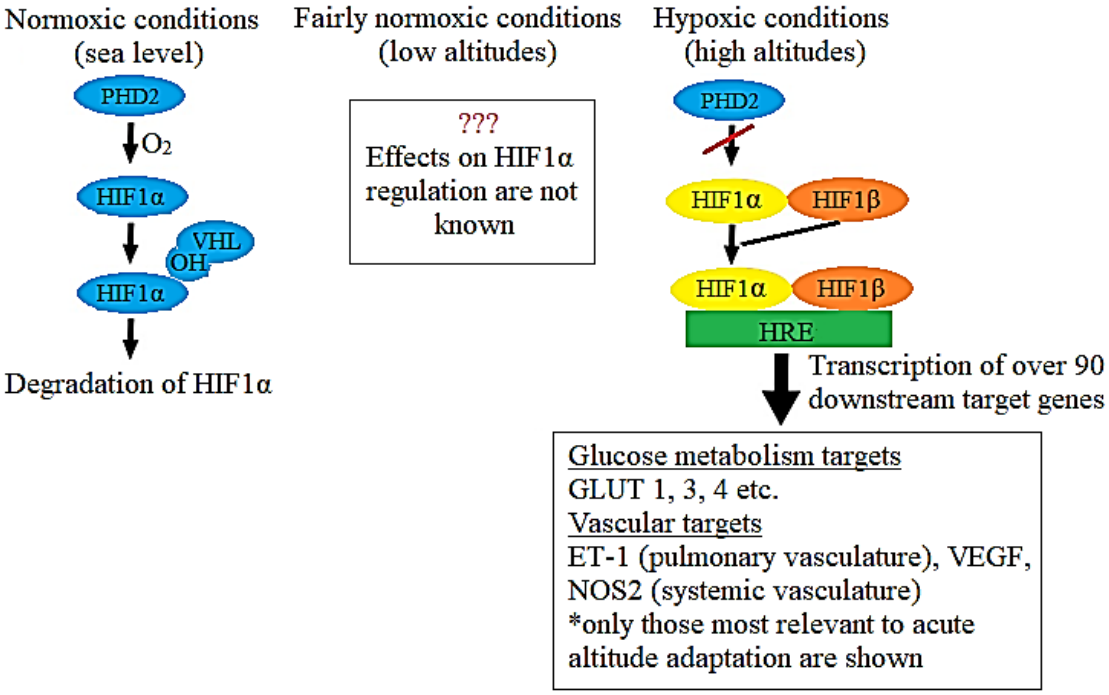

Figure 1.7 Ascent to high altitudes causes mammals to experience hypoxia. HIF1α is a major player in adaptation to hypoxia, acting in low oxygen conditions to activate downstream targets that would allow tissues to meet metabolic demands. HIF1α = hypoxia inducible factor 1 alpha; HIF1β = hypoxia inducible factor 1 beta; VHL = von-Hippel Lindau; OH = hydroxide; HRE = hypoxia response element; GLUT = glucose transporter; ET-1 = endothelin-1; NOS2 = nitric oxide synthase 2; VEGF = vascular endothelial growth factor. Adapted from Hopfl et al (2003).

In laboratory work with animal models, hypobaric hypoxic conditions mimicking high altitudes have been studied as a method to limit infarct size when administered before or after MI, utilizing intermittent hypoxia to attain cardioprotection [181-183]. It is proposed that intermittent exposure to hypobaric hypoxia may provide cardioprotection by way of limiting infarct size, preserving Ca^{2+} homeostasis, regulating calcium/calmodulin-dependent protein kinase II activity, reducing myocardial apoptosis, and increasing vascularization [184]. Many studies have used preconditioning with hypoxia, an experimental method of inducing acclimatization to lower oxygen, to provide cardioprotection in ischemia/reperfusion type injuries [181]. However, the optimal duration of hypoxia and the altitude range of simulation and their effects post-MI remain unclear.

Pre-conditioning of Wistar rats with hypobaric hypoxia simulating an altitude of 5000 m has shown attenuation of infarct size in an ischemia/reperfusion injury model [181]. In a rabbit MI model, 4 weeks of daily exposure to acute hypobaric hypoxia to a simulated altitude of 4000 m reduced infarct size [185]. Similarly, exposing Sprague-Dawley rats after MI to intermittent hypobaric hypoxia corresponding to an altitude of 5000 m above sea level showed enhanced coronary flow, reduced left ventricular dilation, and improved left ventricular function vs. normoxic rats [183]. These reports suggest that hypoxic conditions associated with high altitude simulation are beneficial post-MI. Whether there are additional mechanisms beyond a hypoxic stimulus, and whether altitude simulation post-MI to low and moderate elevations where hypoxia is not substantial might be beneficial, are unknown.

1.5 The effects of altitude on arterial structure

Mammalian vasculature is a structure highly responsive to molecular, physiological, and physical changes. It is often modeled in biomechanics and physics as a cylindrical structure where the diameter, arterial elasticity, and length are variable. An understanding of basic arterial structure is crucial to understanding how the arterial system may be affected by factors such as changing metabolism, exposure to reduced levels of oxygen, and exposure to reduced barometric pressure seen with ascent to altitude.

1.5.1 Arterial structure, function, and remodeling

The arterial wall is a layered structure in which collagen and elastin are the primary load bearing components – the structural and mechanical relationship between these fibers is crucial for proper arterial function [186-188]. Elastic fibers accommodate the pulsatile blood flow and constant stretching of large arteries and collagen fibers allow vessels to remain structurally sound at higher strains [189]. The structure of arteries is prone to constant remodeling as a result of natural processes (i.e., aging, menopause, pregnancy), and pathological conditions, which can have significant implications on risk for cardiovascular events [190].

Evident in the first few decades of life, diffuse intimal thickening precedes further aging related reduction in levels of elastin in these arteries, where remaining elastin elongates and loses some of its elastic recoil properties [188, 191]. Arteries of the upper and lower peripheral vessels, as well as the carotid artery have shown thickening of the intima media with age [192-194] [195-197]. As a result, arteries begin to rely more on collagen for their structural integrity, leading them

to be stiffer and larger [198]. Compliance and stiffness are important properties of an artery and are used to mathematically describe how an artery may behave as a result of age related or pathological remodeling. Compliance (C) is described as:

$$C = \frac{\Delta V}{\Delta P}$$

Equation 5 A measure of compliance indicates how changes in blood volume (V) affect changes in pressure (P) in a given artery. Compliance is the inverse of stiffness [82].

In the aging arteries, compliance is decreased with reduced elasticity or the presence of atherosclerosis [82] **(Figure 1.8).** Menopause is also known to decrease compliance in women [199]. In pathological conditions, endothelial dysfunction decreases compliance, and is very useful in clinical practice as a measure of arterial stiffness and cardiac risk [200]. Transmural pressure, the difference between pressure inside and outside the vessel, can also have important effects on the vascular tone and can often be described with its potential effects on compliance. In vascular smooth muscle cells, contraction increases vascular tone and decreases compliance. VSMC relaxation has the opposite effect [82].

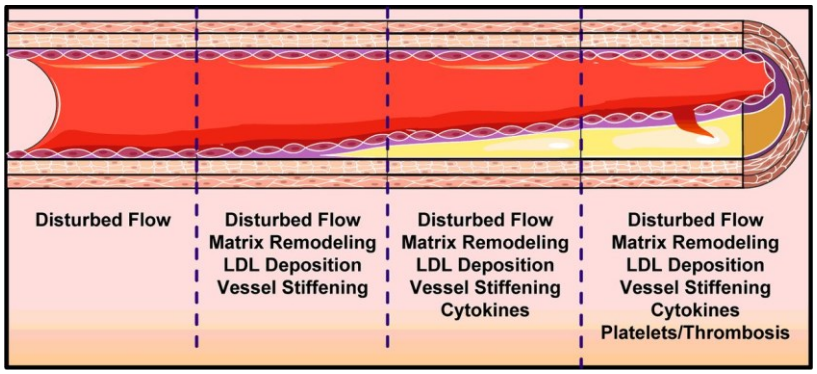

Figure 1.8 Vessel stiffening seen with atherosclerotic plaque formation reduces compliance and increases risk for cardiovascular events. Reprinted from Yurdagul et al (2016) in accordance with guidelines from the Portland Press and the Biochemical Journal.

1.5.1.1 Flow-based arterial remodeling

In normoxic conditions, arteries undergo routine remodeling in response to changes in shear stress and circumferential stretch as blood flow changes so that normal arterial wall tension is maintained [201]. In response to increased flow, a type of flow related remodeling called outward remodeling may occur dependent on the production of NO through shear stress [202]. NO is crucial to this type of arterial remodeling since it has the ability to halt the proliferation and promote the apoptosis of vascular smooth muscle cells [203]. Nitric oxide works alongside gelatinase matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9 to carry out the outward remodeling of blood vessels [204]. In instances of reduced blood flow, factors like platelet-derived growth factor and transforming growth factor-β mediate remodeling by increasing the proliferation of vascular smooth muscle cells, depositing and cross-linking more collagen, and reorganizing vessel structure with the help of MMPs [205, 206].

As discussed before (***section 1.3.1.2***), there is evidence that acute exposure to high altitude leads to increased blood flow [207]. Residents of high altitudes show even greater forearm blood flow in comparison to low altitude residents traveling to high altitudes [86], suggesting that chronic exposure to high altitude may result in changes to significant changes in arterial structure and function. Whether acute exposure to low altitude and corresponding changes to blood flow lead to arterial remodeling is not known.

1.5.1.2 Angiogenesis

Mammalian vasculature utilizes angiogenesis, an adaptive process of sprouting new capillaries from pre-existing vessels to form new capillary networks in low oxygen conditions.

[208, 209]. These conditions are most often caused by poor circulation or rapid oxygen consumption in a number of physiological or pathological processes, or due to exposure to altitudes high enough to cause hypoxia [210]. HIF1α (described in *section 1.4*) is sensitive to reduced oxygen tension capable of upregulating vascular endothelial growth factor (VEGF-A), a potent angiogenic factor important to new capillary formation in hypoxic conditions [211] [212].

VEGF-A induces the proliferation of endothelial cells and causes endothelial permeabilization and can be up regulated by other growth factors like transforming growth factor beta (TGF-B), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) to trigger capillary tube formation [209]. New capillary networks are made of endothelial cell tubes without vascular smooth muscle cells or adventitial structures and cells [209] [213]. Angiogenic growth of capillary tubes to increase capillary density serves to increase blood perfusion of hypoxic tissue and restore oxygen and nutrition supply locally [209, 214] (shown in **Figure 1.9** below).

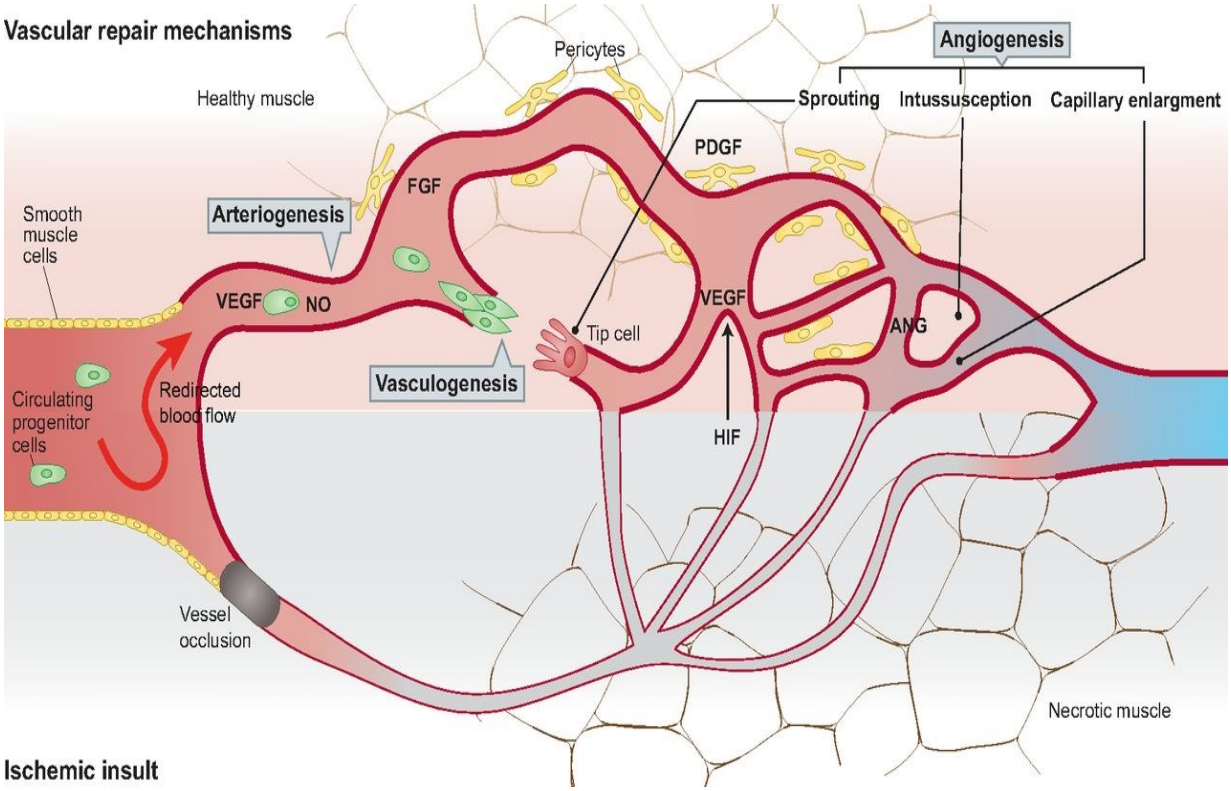

Figure 1.9 Angiogenic processes (sprouting, intussusception, and capillary enlargement) can take place after an arterial occlusion (that causes hypoxia). Hypoxic tissue expresses hypoxia-inducible factor (HIF), enabling the production of angiogenic proteins. Arteriogenesis is also involved to develop collateral vessels, so the downstream hypoxic area may achieve improved blood perfusion. VEFG = vascular endothelial growth factor; ANG = angiopoietin; NO = nitric oxide; FGF = fibroblast growth factor. Reprinted from Dragneva et al (2013) as per the Creative Commons license 3.0 of Disease Models and Mechanisms.

Heavy upregulation of VEGF-A has been shown in subjects ascending Mount Everest with levels rising significantly above sea level values at 5000 m and subsiding within 20 days of acclimatization at 6000 m [215]. Interestingly, other have deliberated if angiogenesis may contribute to the development of high altitude cerebral edema (HACE) as angiogenesis entails dissolution of the capillary basement membranes and degradation of the extracellular matrix which leads to capillary leakage [216]. Another small report of subjects (n=5) ascending the highest peak in Bolivia (6522 m) showed that individuals with the highest increase in VEGF-A expression experienced more severe acute mountain sickness in comparison to individuals whose VEGF-A levels very not increased significantly [126]. Whether the expression of angiogenic factors is changed in individuals ascending to low or moderate altitudes has not been studied systematically.

1.5.1.3 Arteriogenesis

Arteriogenesis, a process induced independently of hypoxia can also improve blood perfusion in an area. It is the growth of functional collateral arteries from pre-existing arterio-arteriolar anastomoses [209]. This process is triggered by physical forces such as altered shear stress that a collateral vessel may experience when blood flow increases. Whether arteriogenesis in involved in responses to altitude exposure is not known, but it may play a role in the cardioprotective effect of residing at low and moderate altitudes where hypoxia is insignificant. Arteriogenesis is known to occur in cases of arterial occlusion, where a large pressure difference will develop in the pre-existing arterioles connecting upstream and downstream branches. As a result, wall remodeling will occur to enlarge the collateral arterioles into arteries [217] (shown in **Figure 1.9** above).

Much of the transformation when a collateral vessel becomes a conduit artery occurs in the smooth muscle cells, which increase their tissue mass by up to 3-fold in mice and more than 20-fold in humans [218]. It has been shown that the typical processes in arteriogenesis include vascular smooth muscle cell proliferation, changes to the phenotype of these cells, and development of a neointima [219]. In coronary collateral vessel growth, two zones of growth have been identified: a highly active neointima and a less active media [218]. In these zones, matrix metalloproteinases (MMPs) MMP-2 and MMP-9 are very active, whereas TIMP-1, a tissue inhibitor of metalloproteinase is only expressed in the tunica media. In arteriogenesis, there is an accumulation of macrophages in the adventitia as a result of inflammatory processes utilizing adhesion molecules [220]. This results in production of growth factors and MMPs [221] by activated fibroblasts and macrophages leading to an enlargement of collateral vessels [222].

Some believe that arteriogenesis has the potential to restore a significant amount of blood flow to an ischemic area following arterial occlusion [219]. However, others argue that because shear stress in a collateral vessel drops drastically as the vessel enlarges, the process of arteriogenesis can be self-limiting and restore only up to 40% of the maximal conductance of the replaced artery [217, 223]. Key differences between angiogenesis and arteriogenesis are highlighted in **Table 1.4**.

Differences Between Angiogenesis and Arteriogenesis		
	Angiogenesis	**Arteriogenesis**
Definition	Formation of new capillaries through sprouting or intussusception	Growth of pre-existing collateral vessels
Source	Pre-existing capillaries	Pre-existing arterioles
Oxygen status	Hypoxia	Normoxia
Trigger	Ischemia	Shear stress
Cellular mechanism	Inflammation because of ischemic focal tissue damage	Inflammation due to increased shear stress

Table 1.4 Arteriogenesis and angiogenesis have key differences described here. Adapted from Schaper and Scholz (2003) [224].

In the case of occlusion, endothelial cells of collateral vessels in coronary vessels have been shown to develop a swollen appearance and longitudinal bulges and no long lining up with the direction of the blood flow [219]. This activated endothelium has shown increased levels of eNOS, monocyte chemoattractant protein (MCP), TGF-B, intracellular adhesion molecule 1(ICAM-1) and vascular cell adhesion protein (VCAM) [225]. Subsequent leakage of erythrocytes, plasma proteins, and platelets into the vascular wall indicate increased permeability of the endothelium, which also experiences an increased adherence of monocytes [226]. Activated endothelium in these developing collateral vessels then see an upregulation in molecules involved in the proliferation and migration of cells including MMP-2, tissue plasminogen activator (t-PA),

focal adhesion kinase (FAK) and then integrins a5B1 (fibronectin receptor) and avB3 (vitronectin receptor).

The effects altitude exposure can have on arterial structure and remodeling (including angiogenic and arteriogenic adaptations) have not been investigated in humans in a systematic way. The key to understanding the cardioprotective effect seen in humans residing at low and moderate altitudes may lie in a methodical examination of how arterial structure and remodeling, together and apart from the effects of endogenous substances, are altered with exposure to various altitudes.

1.5.2 Arterial responses to increased barometric pressure

It is well documented that cardiac hemodynamics are negatively affected in conditions of high barometric pressure, with the most notable work done in diving animals and humans. Angiograms of the harbor seal during a dive showed sudden arterial constriction in the vascular beds of muscle, skin, kidney, liver, spleen and all vascular beds except for the brain and heart [227]. There was a significant decrease of organ blood perfusion upon the dive being initiated [227]. In a recent study with the California sea lion, it was reported that systemic vascular resistance in these animals increase two fold with long duration dives as a result of vasoconstriction [228]. In humans, a typical diving response (i.e., being exposed to increased barometric pressure) is vasoconstriction of several vascular beds and reduced blood flow to capillary beds [229]. An increase in mean arterial pressure in humans during a dive has been reported by several studies [230, 231] and increased resistance to blood flow has been cited as the causative agent [229]. Although endogenous factors may play a role, vasoconstriction seen with diving illustrates the ability of increased barometric pressure to suddenly and acutely cause a reduction in diameter of mammalian vasculature.

1.5.3 Arterial responses to decreased barometric pressure

Increases in barometric pressure lead to acute arterial vasoconstriction in humans and other mammals. There is a lack of literature studying the mechanical effect of acutely reduced barometric pressure in normoxic conditions on the vasculature of humans or other mammals. Arterial structure may be contrariwise affected in instances of lowered barometric pressure (i.e., altitude above sea level). Interestingly, differences in the arterial structure of high-altitude residents (chronic exposure) and lowlanders have been identified and it is suspected residents of high altitude may have reduced systemic wall thickness and larger lumen diameter which is similar in magnitude to individuals with chronic exercise exposure [193, 232]. Although not studied in the context of altitude yet, chronic exercise induced changes in the circumferential strain placed on blood vessels are thought to stimulate structural arterial remodeling and produce an anti-atherogenic effect, resulting specifically in an overall larger lumen [233].

Taken with reports of a larger carotid diameter in lowlanders upon chronic exposure to high altitude (5000 m), it may be inferred that arterial remodeling is an adaptive response to high altitude [234]. It should be noted that most studies investigating the effects of altitude on arterial structure have focused on moderate, high and extreme altitudes where the effects of hypoxia must be considered. It is difficult to predict exactly how small increases in altitude (and reduction in barometric pressure) would affect arterial structure and remodeling without investigation in animal models and humans.

1.6 Objectives

There exists a notable amount of literature linking reductions of morbidity and mortality from various cardiovascular diseases with low and moderate altitudes of residence (up to 3000 m above sea level). However, the effects of low altitudes (normoxic conditions), both in experimental animals and humans are not well studied. This dissertation aims to explore a mechanism through which low altitudes may offer a protective cardiovascular effect for humans. Through three experimental projects, we studied how low altitude simulation (normoxic barometric pressure reduction) affects vascular structure *ex vivo*, and *in vivo* in healthy and diseased mice. The specifics of each project are as follows:

1. We studied the effects of normoxic low altitude simulation on isolated arterial segments to observe how a conduit artery may react passively and actively to reduced barometric pressure. This has never been described in scientific literature.
2. We studied the cardiac hemodynamics of healthy mice under normoxic low altitude simulation, to see if an overall hemodynamic effect could be observed.
3. We investigated whether normoxic low altitude simulation could be used as a therapy to improve cardiac perfusion in a murine model of myocardial infarction, post MI.
4. We investigated whether normoxic low altitude simulation therapy could improve blood perfusion in a systemic atherosclerotic disease such as peripheral arterial disease (PAD) in a murine model of hindlimb ischemia.

Chapter 2 Normoxic low altitude simulation alters murine vascular function *ex vivo* and hemodynamics *in vivo*

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Keywords

low altitude, moderate altitude, lowered barometric pressure, altitude hemodynamics

Abstract

Humans living at low and moderate altitudes (500 m – 3000 m) above sea level are at a lower risk for developing cardiovascular diseases. We hypothesize the lowered barometric pressure at these altitudes enhances vasodilation in the systemic vasculature through reduced external compressive forces on arteries. This could be promoting an anti-artherogenic environment and protecting altitude dwellers from cardiovascular diseases.

First, we studied the effects of normoxic low altitude simulation on the resistance arteries of healthy C57BL6 mice (13.3±1 weeks) *ex vivo* using a pressure myograph system. Inside a custom constructed hypobaric chamber where mesenteric arteries (n=14) were exposed to barometric pressures of 754 mmHg, 714 mmHg and 674 mmHg, we used step wise manipulation of perfusion pressures (4-140 mmHg) or flow rates (0-70 μL/min) to assess myogenic tone in the presence or absence of L-NAME and meclofenamate. We observed a marked increase in vessel diameters at 714 mmHg and 674 mmHg (20.9±9.3% and 28.2±8.6%, respectively) compared to 754 mmHg ($p<0.01$) in response to increased perfusion pressure. Flow-mediated vasodilation was enhanced under hypobaric conditions with and without L-NAME and Meclofenamate. Vascular resistance was reduced at 674 mmHg vs. 754 mmHg (2.14±0.60 mmHg*min/μL vs. 3.21±0.49 mmHg*min/μL, $p<0.05$). Next, C57BL6 mice underwent left-ventricular catheterization during consecutive acute exposures to 754 mmHg, 714 mmHg, and 674 mmHg to study the *in vivo* hemodynamic effects of normoxic low altitude simulation. Total systemic vascular resistance was reduced by lowered barometric pressure (10.09±0.15 mmHg*min/μL at 754 mmHg vs. 8.11±1.45 and 8.18±1.24 mmHg*min/μL at 714 mmHg and 674 mmHg, respectively; $p<0.05$). Significant increases in stroke volume and cardiac output from 754 mmHg to 714 mmHg and 674 mmHg ($p<0.05$) were observed.

New and Noteworthy

We found that normoxic low altitude simulation boosted arterial vasodilation *ex vivo* independent of nitric oxide and prostaglandin-mediated vasodilator pathways. Additionally, we showed that artificially simulating altitude improved hemodynamics *in vivo*, with improved cardiac output and reduced systemic vascular resistance. This finding may have utility for understanding the reduced incidence of cardiovascular diseases found at low and moderate altitudes above sea level and could lead to a non-invasive therapy for cardiovascular diseases.

Introduction

Cardiovascular diseases, including coronary artery disease, heart failure, and stroke, are very common and are associated with significant morbidity and mortality [235]. A significant proportion of cardiovascular diseases are related to atherosclerosis, which is in turn associated with exposure to risk factors. The INTERHEART international study of myocardial infarction (MI), which included 15,152 cases and 14,820 controls from 52 countries worldwide, linked 90% of the population attributable risk fraction for MI to exposure to modifiable risk factors, including abnormal lipids, smoking, hypertension, diabetes, obesity, psychosocial factors, low consumption of fruits and vegetables, consumption of alcohol, and low levels of physical activity [9]. While these canonical risk factors explain much of the risk for atherosclerotic cardiovascular events, other exposures may modify the risk.

Mortimer *et al*. reported a link between residence at low and moderate altitudes (1220-2135) m and lower mortality from coronary artery disease (CAD) in 1977, after evaluating mortality rates of Caucasian men in five counties in New Mexico [3]. While this initial report was criticized as confounding [23], subsequent studies have found similar associations between higher altitude of residence and lower mortality from coronary artery disease and stroke [1, 2, 20]. Conversely, one report from Yemen suggested that rates of acute coronary syndrome are higher in people living at higher altitude, however, this study also demonstrated significantly increased prevalence of other risk factors and previous cardiovascular events that may explain this result [37]. A larger study of 1198 Greek subjects identified a protective effect of living at 950 m [21] after adjustment for differences in other risk factors. The largest study of altitude and cardiovascular disease included 1.64 million German Swiss residents living between 259 and 1960 m, and included mortality data, sociodemographic information, and places of birth and residence.

The investigators demonstrated a relative risk reduction of -22% per 1000 m of altitude above sea level for MI and -12% per 1000 m for stroke [24]. A limitation of this report was the lack of adjustment for differences in risk factor prevalence. Altitude is also associated with lower all-cause mortality in the general American population [25]. Despite the limitations of prior reports, there appears to be a link between altitude of residence and protection from atherosclerotic cardiovascular disease.

Increases in barometric pressure are associated with hemodynamic changes that might affect vascular function and atherosclerosis. A study of ten healthy volunteers undergoing simulated dives at pressures between 1.6 and 3 atmospheres confirmed significant increases in ventricular afterload with increased barometric pressure [236]. A similar study reported increases in serum N-terminal pro brain natriuretic peptide after scuba diving, suggesting hemodynamic changes due to this exposure [237]. Conversely, exposure to hypobaric hypoxia was associated with improved myocardial perfusion in six male patients with coronary artery disease, supporting the theory that lower ambient barometric pressure might have beneficial effects on coronary physiology [182]. Since ambient barometric pressure varies inversely with altitude, variations in barometric pressure associated with altitude could alter vascular function in physiologically significant ways and alter susceptibility to cardiovascular disease.

To explore whether the changes in ambient barometric pressure seen with relevant altitude differences could alter arterial function, we performed a series of tests of arterial function *ex vivo* and cardiovascular function *in vivo* in a murine model. We hypothesized that normoxic low altitude simulation (by lowering barometric pressure) would reduce resistance across systemic arteries by reducing the external compressive forces on the arteries and increase the pressure difference across

the arterial wall (transmural pressure), allowing these vessels to distend and maintain a larger luminal diameter.

Methods

Ethical approval

All protocols were approved by the University of Alberta Animal Policy and Welfare Committee (UAPWC) in accordance with the Canadian Council on Animal Care (CCAC) guidelines.

Vascular Function: *Ex vivo* (Pressure Myograph System)

Sixteen-week-old male C57BL6 mice (Charles River; Vilmington, MA) were given access to standard chow and water *ad libitum* and were housed in a 12h-12h light-dark cycle. Mice were euthanized through sodium pentobarbital administered intraperitoneally, and their mesenteries were removed and immersed in freshly prepared cold physiological salt solution (PSS: 10 HEPES, 5.5 glucose, 1.56 $CaCl_2$, 4.7 KCl, 142 NaCl, 1.17 $MgSO_4$, 1.18 KH_2PO_4 (in mM), pH 7.5). Two second order resistance arteries were dissected from surrounding connective and adipose tissues and mounted in a pressure myograph system (Living Systems Instrumentation; Burlington, VT). Vessels were tied onto a glass cannula with a thin suture on either side such as the vessel was immersed in a PSS bath maintained at a temperature of 37 °C. Intravascular pressure and flow were measured and alterable using pressure and flow control systems. A peristaltic pump was used to maintain specific rates of flow across the lumen of vessels mounted in the pressure myograph

system. To mimic physiological conditions as best as possible, the vessels were oriented as such that flow would be applied in the same direction as *in vivo* blood flow. The vessels were equilibrated for 40 minutes during which the bathing PSS solution was changed multiple times and the vessels underwent stepwise increases in perfusion pressure from 60 mmHg to 80 mmHg. Ultimately, the vessels were maintained at a perfusion pressure of 60 mmHg, the approximate *in vivo* mesenteric arterial pressure of mice.

Drugs

Vessels were exposed to phenylephrine (PE) and methacholine (MCh) before the start of the experimental protocol to ensure the vessel was intact and capable of responding to pressure and flow stimuli. In some protocols (n=8), one bath of the pressure myograph was infused with inhibitors of NO synthase and prostacyclin to inhibit the action of major endogenous endothelial vasodilators. This was done in order to study the effect of altitude simulation (i.e., barometric pressure) on mesenteric arteries isolated from the vasoactive influences of nitric oxide and prostacyclins. In Ca^{2+} free protocols, $1x10^{-4}$ M papaverine was added to both baths (25 µl of $1x10^{-2}$ M in a 2.5 ml bath) to achieve maximal dilation for the blood vessels at an equilibrated intravascular pressure of 60 mmHg.

Normoxic low altitude simulation

The entire myograph system was enclosed within a custom designed 26.9 x 14.6 x 5.5 cm acrylic barometric pressure-controlled chamber (**Figure 2.1a**, McMurtry), capable of being sealed

and to simulate various low altitude conditions as necessary. A control laboratory pressure (754 mmHg) was established on the pressure sensor in relation to the barometric pressure denoted by the meteorological service of Canada at the Edmonton, Alberta station **(Figure 2.7)** [4]. Pressure conditions of 714 mmHg and 674 mmHg were assigned as 40 and 80 mmHg below barometric pressure, respectively. Conditions were maintained through a vacuum controller (Buchi V-850; New Castle, DE). These conditions were chosen to mimic near sea level conditions, 523 m above sea level, and 1001 m altitude above sea level, respectively. As altitude was not directly manipulated in these experiments, further discussion of these experiments will refer the directly manipulated barometric pressures instead. Although oxygen saturation in healthy humans is known to be maintained above 95% at 2000 m of altitude [238], normoxia inside the barometric pressure chamber was ensured with an oxygen probe at all altitude simulation conditions prior to commencement of the *in vivo* and *ex vivo* experiments.

A pressure transducer and flow regulator coupled with a peristaltic pump mechanism (Living Systems Instrumentation; Burtlington, VT) was set up so it could be manipulated from outside upon sealing the barometric pressure chamber around the myograph system. The addition of any drugs in the myograph baths required opening of the chamber (and bringing the vessels back to control barometric pressure). For the purposes of the experiment, three normoxic low altitude simulation conditions were simulated using the following barometric pressures: 754 mmHg (control barometric pressure, 714 mmHg, and 674 mmHg, followed by a return to 754 mmHg (control) at the end of the experiment.

Measurements

Vessel diameter and wall thickness were recorded with the help of a micrometer coupled with a STEMI 2000 microscope (Zeiss; North York, ON). Measurements were taken after every intervention when the myograph had been sealed inside of the barometric pressure-controlled chamber. A transparent lid allowed for micrometer measurements. Desiccants lined the inner walls of the chamber to ensure visibility of the vessels was not diminished due to increasing humidity in the chamber.

Pressure Volume Analysis: *In vivo* (Left-ventricular Catheterization)

Left ventricular catheterization procedures were completed on male C57BL6 mice (aged 4-6 months old, n=8) using a FTH-1212B-4518 1.2 F admittance catheter (Transonic/SciSence; Toronto, ON) inserted via the carotid artery into the left ventricle as previously described [239]. The surgery was performed under 2.0% isoflurane anesthesia, with the animal breathing 100% oxygen. The mouse was operated on inside of a custom constructed cylindrical 20.1 x 26.67 cm acrylic chamber (**Figure 2.5a**, McMurtry), sealable to maintain barometric pressure after catheter insertion into the left ventricle. LabChart 3 (ADInstruments; Sydney, Australia) provided real time data and derived pressure volume loops from several measured and calculated hemodynamic variables. The barometric pressure inside the chamber was recorded for 5 minutes at four altitude conditions (corresponding to 754 mmHg, 714 mmHg, 674 mmHg, and a return to 754 mmHg as per *ex vivo* protocols) and pressure-time relationships were recorded in the aorta before advancing the catheter through the aortic valve into the LV where pressure-time relationships were recorded again. The animal was euthanized through cervical dislocation following the protocol

Statistical Analysis

The functional data generated from the myograph work is largely presented as a mean and standard errors of raw lumen diameters of mesenteric vessels in response to our experimental conditions of changes in barometric pressures, perfusion pressures, and intraluminal flow. The significance of the difference in mean values of our continuous variables between groups was determined by a two-way ANOVA, with a Bonferroni post-hoc test for multiple comparisons, using the statistical software IBM SPSS Statistics 21 (Armonk, NY). The *in vivo* data is represented as means and standard errors of raw values with significance between the differences of means determined through a one-way ANOVA.

Results

Normoxic low altitude simulation increased flow mediated vasodilation in murine resistance arteries. Murine second order mesenteric arteries [n=8] mounted on a pressure myograph **[Figure 2.1a]** responded with increased lumen diameter across a range of physiologically relevant flow volumes **[Figure 2.1b]**, as expected. The same arterial segments responded with significantly greater increases in lumen diameter under conditions of an applied reduction in barometric pressure to either 714 mmHg (mimicking 523 m altitude) or 674 mmHg (mimicking 1001 m altitude), with the larger barometric pressure reduction associated with larger increases in lumen diameter across a range of flow volumes. Even at a flow volume of 0 µl/min, an increase in lumen diameter was observed once ambient barometric pressure was reduced. A very large dilation was seen with a 80 mmHg reduction in barometric pressure (674 mmHg) at 70 µl/min.

Normoxic low altitude simulation increased passive and active pressure mediated vasodilation in murine resistance arteries. Murine second order mesenteric arteries mounted on a pressure myograph in Ca^{2+} free conditions [n=7] responded with increased lumen diameter across a range of physiologically relevant intraluminal pressures **[Figure 2.2a]**, again as expected. Low altitude simulation was associated with increased lumen diameter, with the greatest change in lumen diameter observed at the lowest perfusion pressures. Similarly, murine second order mesenteric arteries mounted on a pressure myograph with a physiological concentration of Ca^{2+} [n=8] responded with increased lumen diameter across a range of physiologically relevant intraluminal pressures **[Figure 2.2b]**, as expected. Although the applied low altitude simulation

(i.e., reduction in ambient barometric pressure) was associated with dose-dependent increases in lumen diameter, as in the Ca^{2+} free experiments, the magnitude of increases in lumen diameter were somewhat smaller at a given perfusion pressure for the arteries in the presence of physiological Ca^{2+}.

Increases in murine resistance artery lumen diameter in response to low altitude simulation are not significantly attenuated by the presence of inhibitors of endothelial function. Murine resistance arteries [n=8] mounted on a pressure myograph at control barometric pressure (754 mmHg) responded with increased lumen diameter with increased perfusion pressures, but the presence of inhibitors of endothelial function, including L-NAME and meclofenamate, attenuated the maximal percentage increase in lumen diameter achieved from 163.5±21.1% to 58.6±27.1% ($p < 0.05$) **[Figure 2.3a]**. Under conditions of low altitude simulation, however, the same murine resistance arteries demonstrated similar percentage increases in lumen diameter regardless of whether L-NAME and meclofenamate were present **[Figure 2.3b, Figure 2.3c]**.

Murine resistance artery lumen diameter varied directly with dose-dependent increases in methacholine and application of normoxic low altitude simulation through acute reductions in barometric pressure. At control barometric pressure (754 mmHg; near sea level), murine resistance arteries mounted on a pressure myograph [n=12] responded with increasing lumen diameters as increasing doses of methacholine were added to the bath **[Figure 2.4]**. In the same murine resistance arteries, low altitude simulation augmented lumen diameter in a dose-dependent manner. The application of reductions of ambient barometric pressure to 714 mmHg and 674

mmHg induced increases in lumen diameter greater than any dose of methacholine alone at control barometric pressure (754 mmHg). Highly dilated vessel lumens were observed with the application of 674 mmHg and the highest doses of methacholine.

Acute simulation of normoxic low altitude is associated with increased stroke volume, increased cardiac output, and decreased total systemic vascular resistance in freely breathing mice in vivo. Representative pressure-volume relationships obtained via invasive LV catheterization through the carotid artery in a closed chest procedure at 754 mmHg **[Figure 2.5a]**, 714 mmHg **[Figure 2.5b]**, and 674 mmHg **[Figure 2.5c]** show a rightward shift of the pressure volume relationship after 5 minutes of exposure to 714 mmHg **[Figure 2.5b]**, with a further right shift and a decreased systolic blood pressure trend with exposure to 674 mmHg **[Figure 2.5c]**. The parameters showed in **[Figures 2.6a-f]** are measured and calculated values from the inserted catheter. Invasive pressure-volume hemodynamic analyses showed preserved mean arterial pressures and dp/dt max **[Figures 2.6b, f]** across the barometric pressure conditions of 754 mmHg, 714 mmHg, and 674 mmHg. Stroke volume (SV) **[2.6c]** and cardiac output (CO) **[2.6d]** were increased significantly ($n=9$, $p<0.05$) at 674 mmHg and 714 mmHg as compared to 754 mmHg. SV and CO indices returned to near baseline values when the barometric pressure was returned to 754 mmHg at the end of each protocol, pointing to the transient nature of the induced effects.

Discussion

We performed a set of experiments to evaluate how changes in barometric pressure (mimicking changes in altitude) alter the function of murine resistance arteries *ex vivo* as well as the hemodynamics of intact murine circulation. Our most significant finding is the ability of barometric pressure alone to influence vascular diameter, with or without the aid of major endogenous endothelial factors. Our work shows that inhibiting nitric oxide and prostacyclin activity does not dampen vasodilation induced by normoxic low altitude simulation. We believe this occurs due to a reduction in the external compressive forces on the arteries as barometric pressure is reduced, increasing transmural pressure and increasing vascular diameter **[Figure 2.]**. This finding in *ex vivo* arterial segments mounted on a pressure myograph system was corroborated by reduced systemic vascular resistance *in vivo* in our mouse model, in which we found significant improvements in stroke volume and cardiac output of healthy catheterized mice.

We achieved a 90% increase in vasodilation of arterial segments *ex vivo* at a physiological intraluminal perfusion pressure of 60 mmHg with an 80-mmHg reduction in barometric pressure in the presence or absence of inhibitors of major endogenous endothelial vasodilators. This vasodilation through normoxic low altitude simulation has not been reported before in *ex vivo* laboratory settings. Utilizing reductions in barometric pressure to cause passive distention of arteries could become a novel method to address pathologies that entail endothelial dysfunction and exhibit limited production and secretion of important endogenous vasodilators. Impaired bioavailability of Nitric oxide (NO) is implicated heavily manifestations of atherosclerotic conditions such as peripheral arterial disease (PAD) and thrombotic cardiovascular diseases (e.g., acute MI and stroke) [240]. As NO and other systemic vasodilators such as prostacyclin (PGI_2) and endothelium derived hyperpolarizing factor (EDHF) rely on endothelial function, a dilation

mechanism to compensate for reduced bioavailability of these factors may be of critical importance in preventing further arterial narrowing seen with atherosclerosis [241] [128].

In vivo, stroke volume and cardiac output were increased while total systemic vascular resistance was reduced in healthy mice by over 20% with barometric pressure reductions of 40 mmHg (from 754 mmHg to 714 mmHg). This was accomplished without changing heart rate or contractility. We show that reducing barometric pressure lessens the external compressive forces on systemic arteries and causes increased distention of these vessels, independently of endothelial factors (shown by our *ex vivo* experiments). The resulting decrease in systemic vascular resistance may be contributing to the observed higher stroke volume and cardiac output *in vivo*.

Limitations and strengths

A limitation of our study is the lack of measurement of the partial pressure of oxygen inside our chamber when low altitude was simulated with the arteries enclosed within our custom constructed hypobaric chamber. We cannot completely exclude that very minor reductions in the partial pressure of oxygen may contribute to arterial vasodilation in the perfused arterial segments, but we suspect this effect would be small since the reduction in pO_2 would only be around 11% (to ~124 mmHg) even at the largest reductions in barometric pressure. Such minor reductions in levels of P_{O2} generally do not constitute a hypoxic environment for mammalian tissues [55-57]. Moreover, in the case of the catheterized *in vivo* mice, these animals were breathing 100% oxygen during the experiment, excluding hypoxia as a contributing factor during these experiments. In addition, as our experiments evaluated acute responses, and we cannot exclude chronic adaptations that would attenuate or reverse the changes we observed. Significant strengths of the work include

pharmacological dissection of the observed vasodilation to exclude major endothelium-dependent components of this phenomenon, and confirmation of the changes using invasive hemodynamic measurements in an intact *in vivo* model.

Clinical implications

Our data support that small reductions in barometric pressure that are seen with low elevations above sea level are associated with measurable changes in blood vessel function (i.e. enhanced resistance artery vasodilation) and cardiovascular function in an intact murine circulation. These changes, which are associated with a lower risk for MI and stroke in humans, could potentially explain the epidemiologic cardioprotective phenomenon of low and moderate altitude dwellers. This suggests the potential of normoxic low altitude simulation to be harnessed for therapeutic benefit.

Since the clinical approach to acute ischemic disorders like MI and ischemic stroke involve improving blood flood to ischemic tissues, or reperfusion [242], a non-invasive mechanical technique of lowering ambient barometric pressure (and thus simulating low altitude) might improve blood flow to ischemic tissues in these disorders. Similarly, since the therapeutic approach for managing congestive heart failure is afterload reduction [243], lowering ambient barometric pressure to reduce vascular resistance systemically could have therapeutic benefits, at least acutely. Altitude simulation is a current and commercially viable everyday reality, occurring in passenger aircraft [244, 245], and negative pressure hospital rooms [246]. Therefore, further preclinical work evaluating hypobaric exposure, with or without supplemental oxygen, is justified.

To conclude, we show enhanced vasodilation of murine resistance arteries *ex vivo* and decreased stroke volume with improved cardiac output *in vivo* after application of normoxic low altitude simulation (using reductions in barometric pressure). These effects appear independent of sensitivity to major endogenous vasodilators and may have a potential therapeutic benefit in treating acute forms of cardiovascular disease.

Disclosures

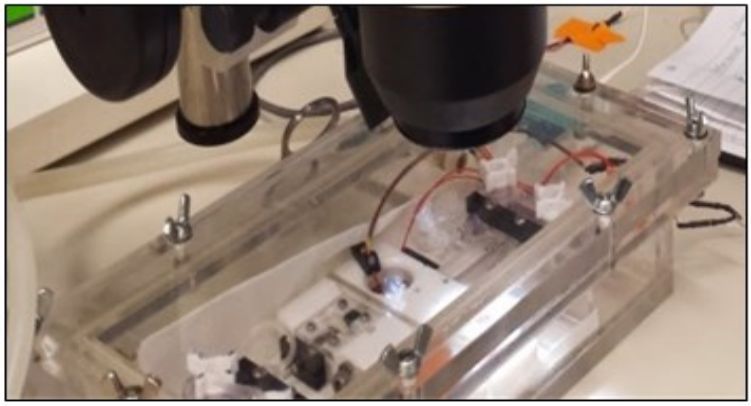
There are no competing interests declared for any of the authors.

Grants

This work is funded by the Canadian Institutes for Health Research.

Figures

[a]



[b]

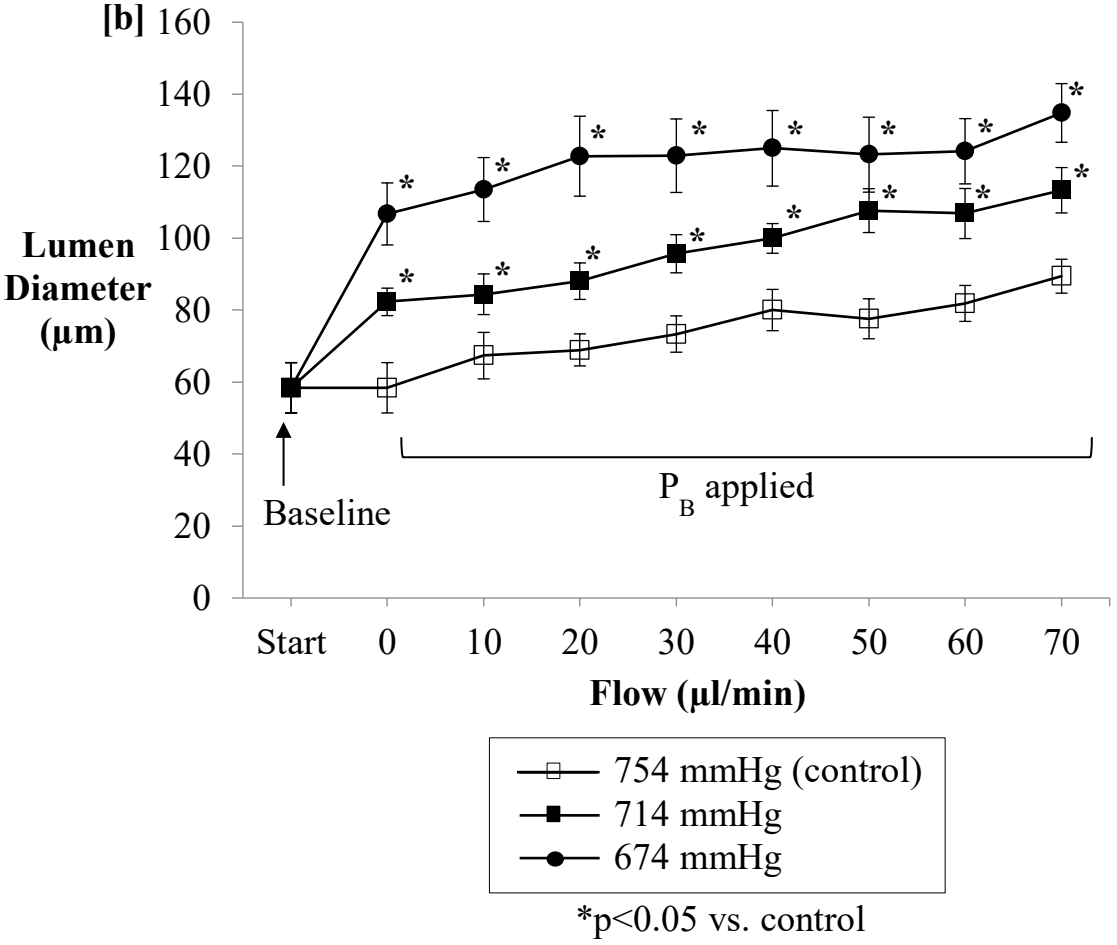
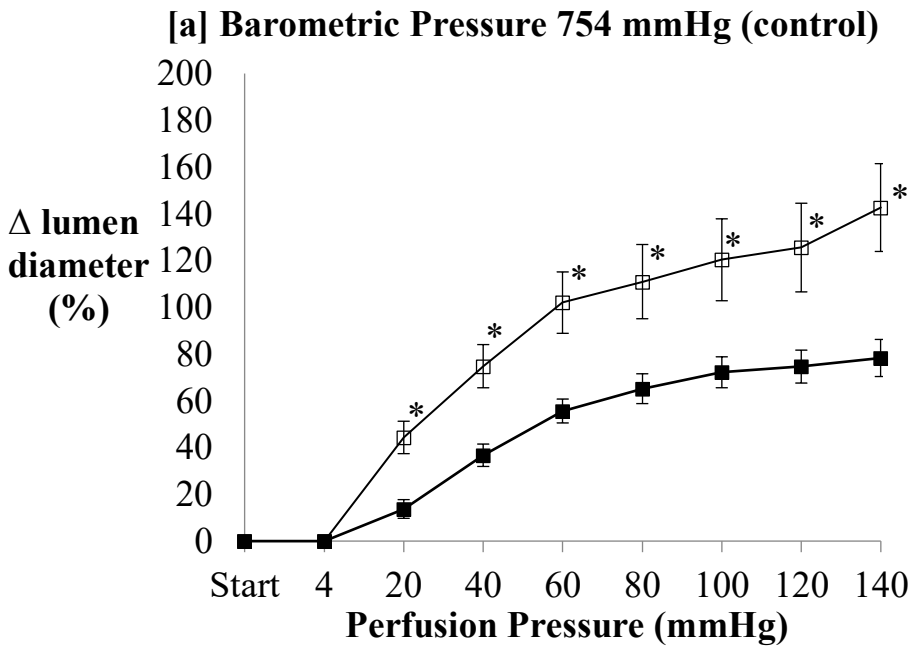
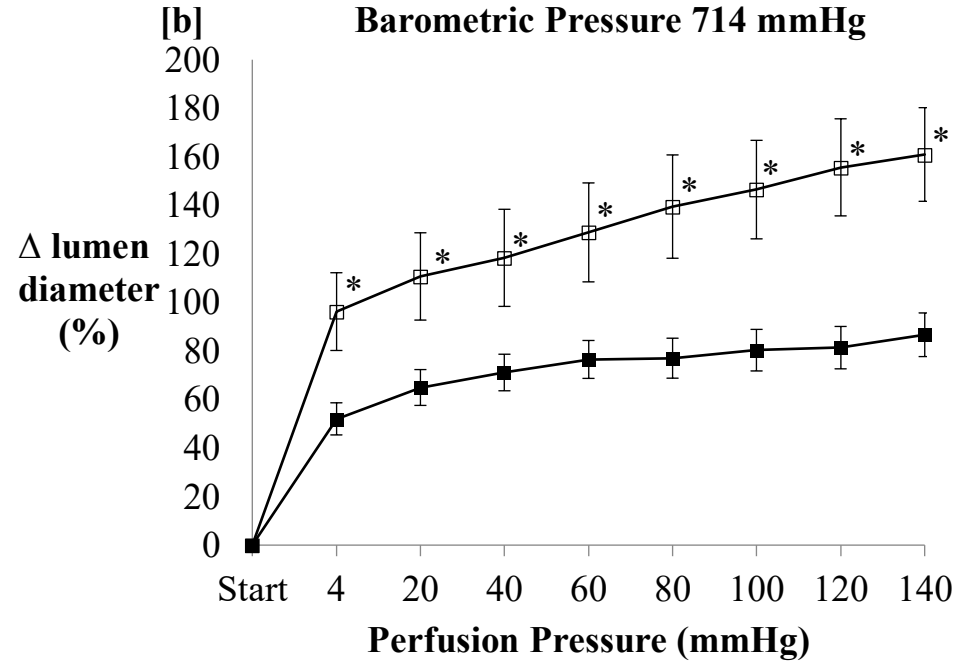

Lumen Diameter (µm)
160
140
120
100
80
60
40
20
0
Start
0
10
20
30
40
50
60
70
Flow (µl/min)
Baseline
P_B applied
754 mmHg (control)
714 mmHg
674 mmHg
*p<0.05 vs. control

Figure 2.1 Segments of murine mesenteric arteries were mounted in a pressure myograph which was in turn placed within a specially constructed chamber to allow low altitude simulation using reductions in barometric pressure **[a]**. The lumen diameter of perfused segments of murine mesenteric artery increased as the internal flow through the arterial segments increased across a range of physiologically relevant flow volumes **[b]**. Reductions of barometric pressure around the perfused arterial segments from 754 mmHg to 714 mmHg and 674 mmHg significantly increased the lumen diameter in response to increased flow volume. Values shown are means ± SE.


[a] Barometric Pressure 754 mmHg (control)
Δ lumen diameter (%)
200
180
160
140
120
100
80
60
40
20
0
Start
4
20
40
60
80
100
120
140
Perfusion Pressure (mmHg)


[b] Barometric Pressure 714 mmHg
Δ lumen diameter (%)
200
180
160
140
120
100
80
60
40
20
0
Start
4
20
40
60
80
100
120
140
Perfusion Pressure (mmHg)

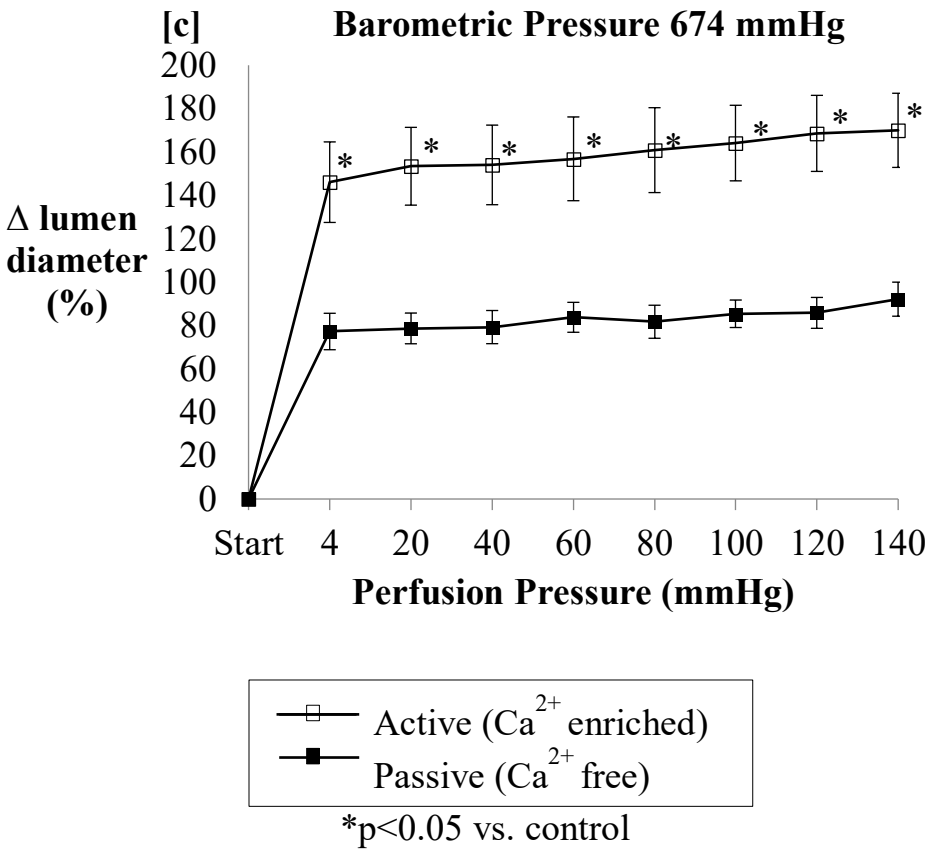
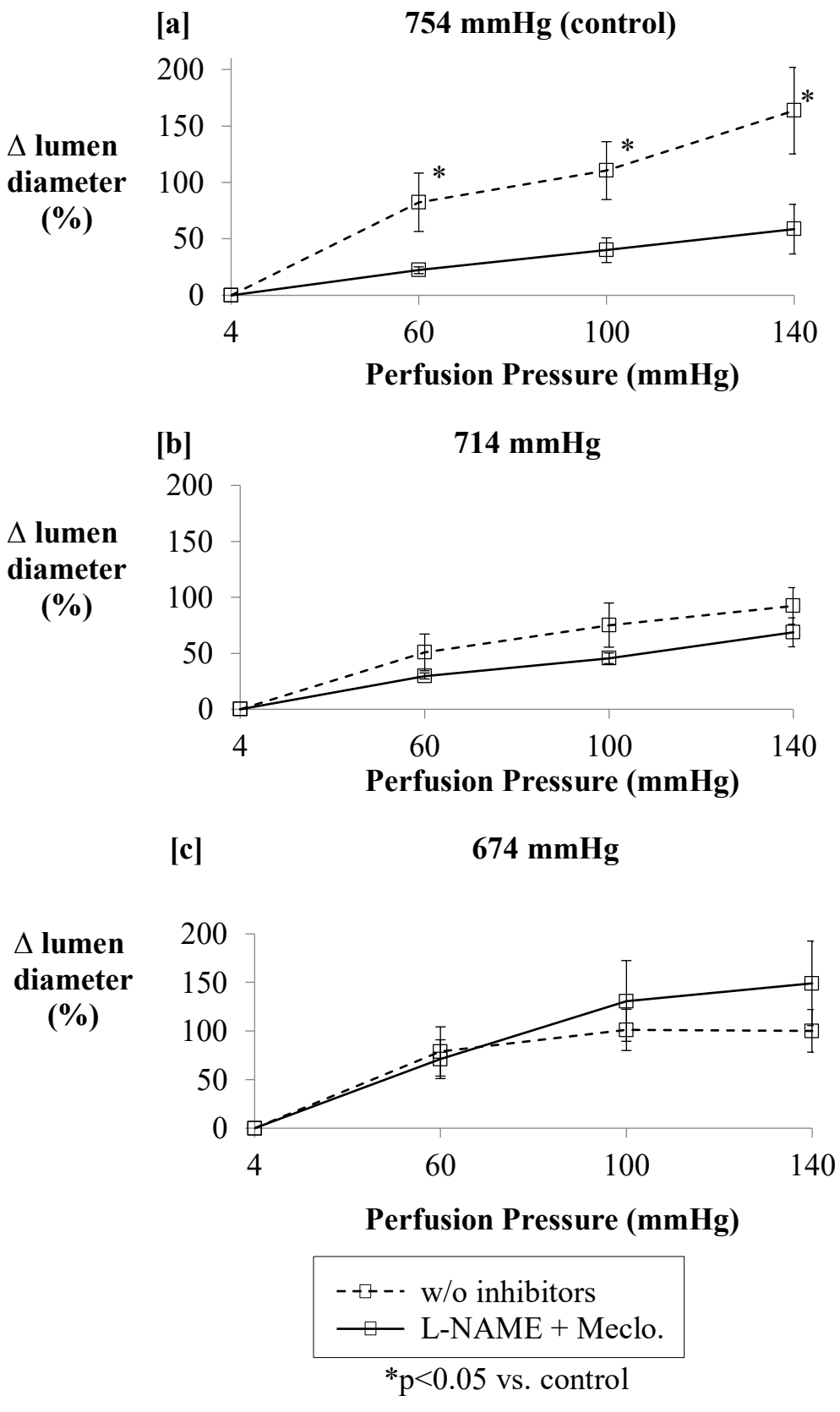


Figure 2.2 The lumen diameter of perfused segments of murine mesenteric artery increased as internal perfusion pressure increased across a range of physiologically relevant perfusion pressures for both Ca^{2+}-free **[a]** and Ca^{2+}-enriched **[b]** conditions. Normoxic altitude simulation through reductions in barometric pressure around the perfused arterial segments from 754 mmHg to 714 mmHg and 674 mmHg significantly increased the lumen diameter in response to increased internal perfusion pressure, particularly at lower perfusion pressures. Values shown are means ± SE.



*p<0.05 vs. control

Figure 2.3 In perfused segments of murine mesenteric artery at control barometric pressure, the percentage of vasodilation from baseline in response to increased perfusion pressure is higher in the absence of inhibitors of endothelial function [a]. However, when the barometric pressure surrounding the perfused arterial segment is reduced from 754 mmHg to 714 mmHg [b] and 674 mmHg [c], arterial segment percentage vasodilation is similar with or without inhibitors of endothelial function, supporting that endothelium contributes less to the vasodilation under these conditions. Values are mean percentage vasodilation ± SE.

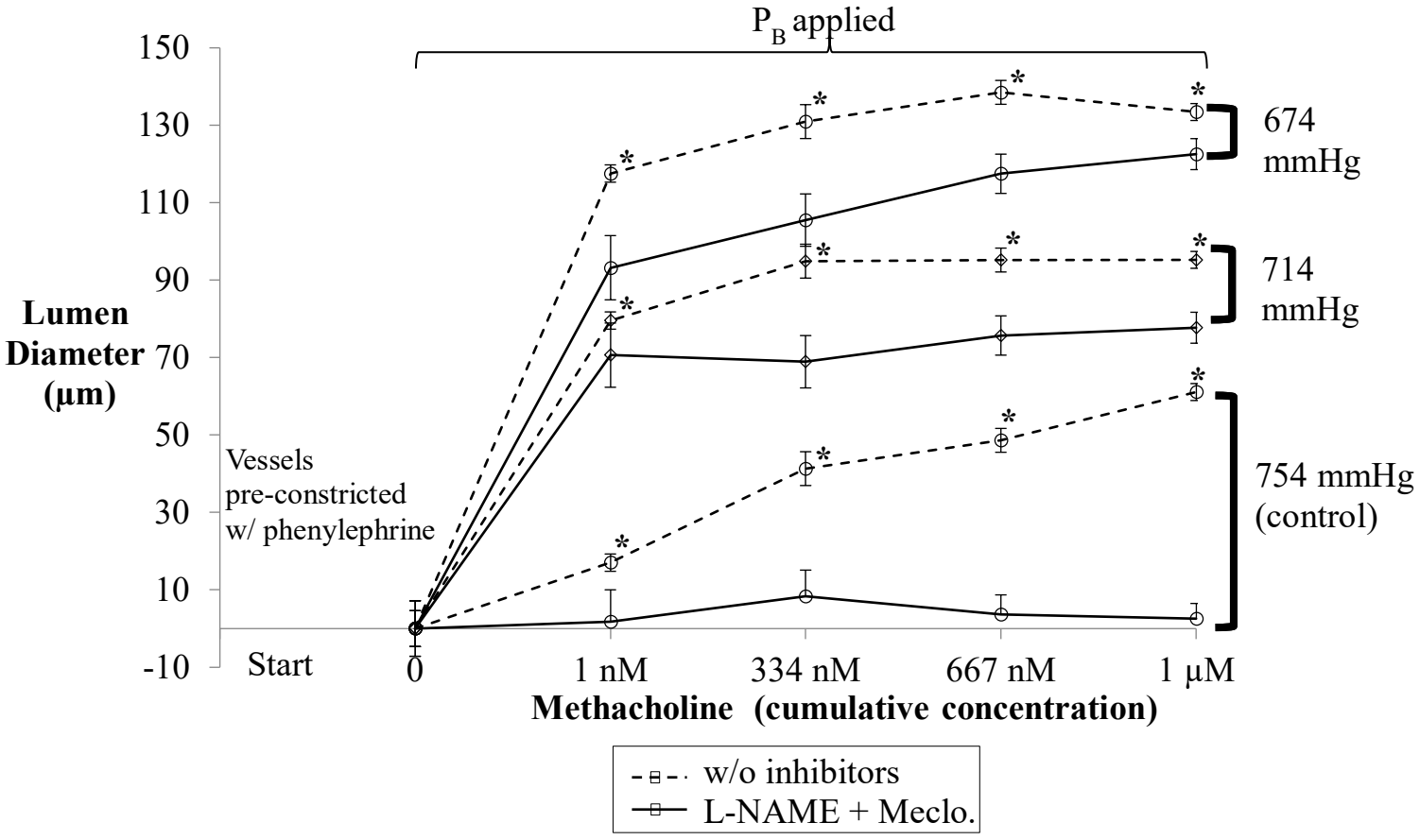


Figure 2.4 In pre-constricted perfused segments of murine mesenteric artery, increasing doses of methacholine significantly increased lumen diameter in the absence of inhibitors of endothelial function, at all conditions of low altitude simulation used (i.e., whether the barometric pressure surrounding the perfused arterial segment was 754 mmHg, 714 mmHg, or 674 mmHg). However, reducing the barometric pressure surrounding the perfused arterial segment to 714 mmHg and 674 mmHg substantially increased lumen diameter, even more than maximal doses of methacholine at 754 mmHg. Values shown are means ± SE.

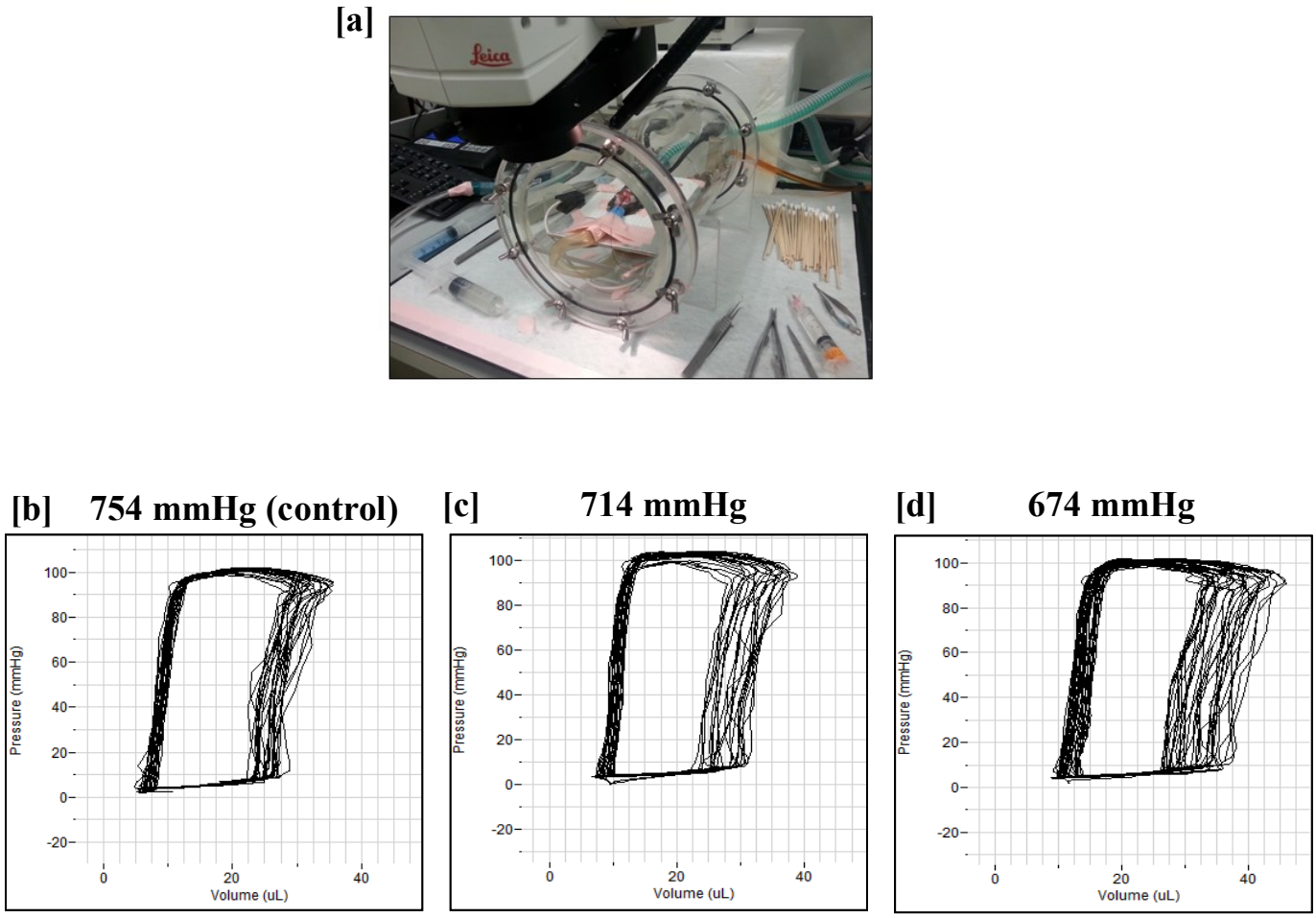


Figure 2.5 Pressure-volume loops were obtained using a conductance catheter placed in the left ventricle of anaesthetized intact mice breathing oxygen enriched air inside a hypobaric chamber **[a]**. Hemodynamics were measured at chamber pressures of 754 mmHg **[b]**, 714 mmHg **[c]**, and 674 mmHg **[d]** for 5 minutes at each condition in each mouse. Acute reductions in barometric pressure around the mice were associated with visible increases in stroke volume **[b-d]**. The pressure volume loops shown are a representative example, taken from one mouse over the period of approximately 5 seconds.

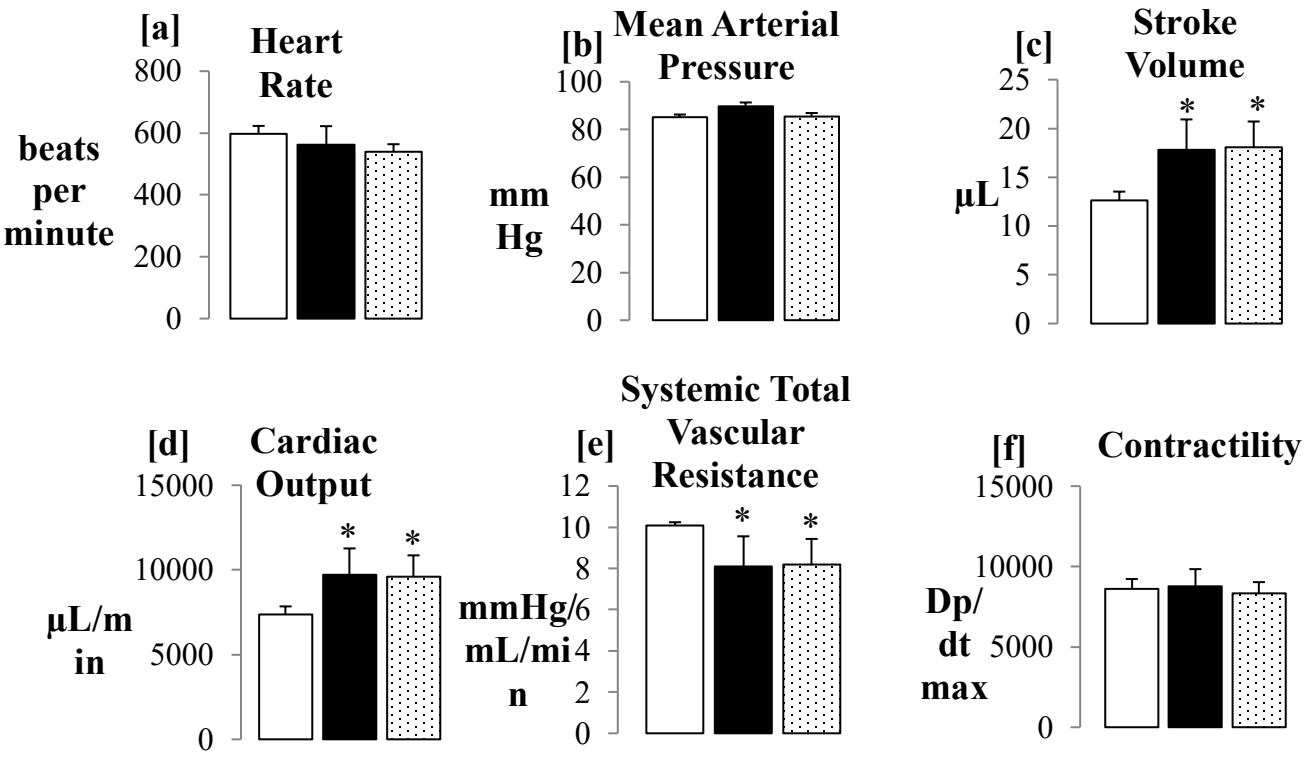


Figure 2.6 Hemodynamic parameters were obtained through invasive left-ventricular catheterization *in vivo* with normoxic low altitude simulation (using barometric pressures of 754 mmHg, 714 mmHg, and 674 mmHg). There were no significant differences in heart rate [a] or mean arterial pressure [b]. There were statistically significant increases in stroke volume [c] and cardiac output [d]. Calculated total systemic vascular resistance was also reduced with normoxic low altitude simulation [e]. Left ventricular contractility, as measured by dP/dt, was not changed by normoxic low altitude simulation [f]. Values shown are means ± SE.

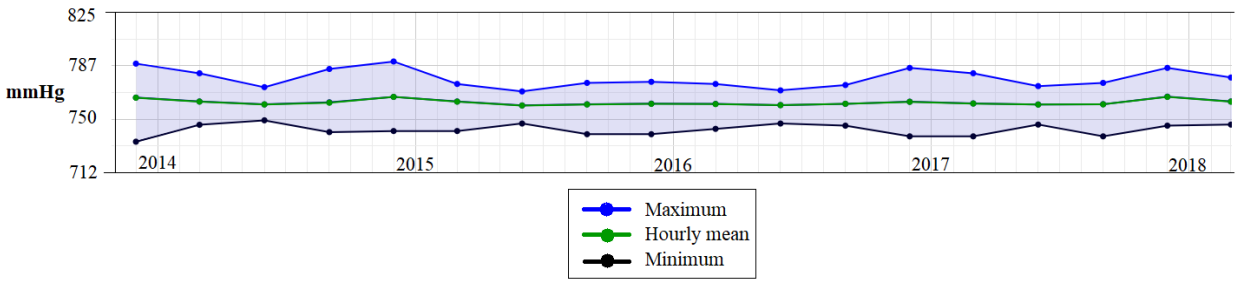


Figure 2.7 5-year quarterly barometric pressure data values (published by the Edmonton, Canada weather station) show that fluctuations between 727 mmHg and 788 mmHg are seen dependent on the season, whereas mean values lie near 760 mmHg. Adapted from Environment and Climate Change Canada (2019) [4].

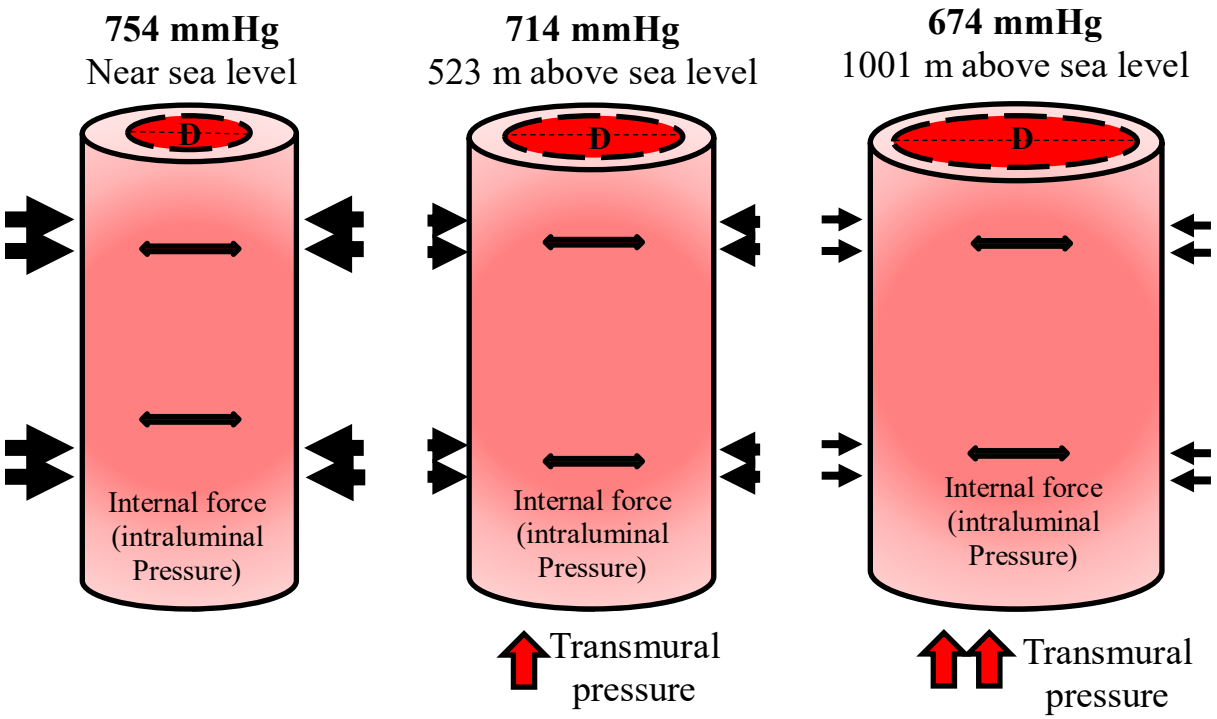


Figure 2.8 Reduction in the external compressive forces on the arteries as barometric pressure is reduced (with increased altitude) increases transmural pressure and vascular diameter (D) passively. Black arrows (➡) indicate the relative influence of barometric pressure in different altitude conditions, whereas red double-headed arrows (⟷) illustrate the internal force (intraluminal pressure, which we assume to be constant for the purposes of our experiments).

Chapter 3 Normoxic Low Altitude Simulation Improves Cardiac Function and Reduces Infarct Size in LAD-ligated Mice

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Keywords

low altitude simulation, lowered air pressure, altitude, hemodynamics, myocardial infarction, echocardiography

Abstract

Residence at low and moderate altitudes above sea level is associated with lower risk for myocardial infarction (MI) and better survival post-MI. We have previously shown that normoxic low altitude simulation achieved by small reductions in barometric pressure enhances arterial vasodilation *ex vivo* and reduces afterload *in vivo*. We hypothesize that reduced systemic vascular resistance (i.e., afterload reduction) through therapeutic normoxic low altitude simulation after MI would improve cardiac function.

We performed left-anterior descending artery (LAD) ligation on three-month old C57BL6 mice. Control group mice (n=12) recovered at 754 mmHg for one week. Treatment group mice (n=13) were placed in a hypobaric pressure chamber to recover from surgery for 3-hours daily at 714 mmHg in order to simulate an elevation of 523 m above sea level for 1 week. Echocardiographic evaluation of left ventricular (LV) function was performed for on Day 1 and Day 8. Post-treatment, there was a 14.2±5.3% improvement in ejection fraction for treatment group mice ($p<0.01$ versus Day 1), and no change for control mice. Cardiac output and stroke volume increased by 11.48±3.9 mL/min and 24.33±8.3 μL respectively in treatment group mice ($p<0.01$ versus Day 1), while control mice showed no significant improvement. Infarct size was significantly reduced in treatment group mice. We conclude that normoxic low altitude simulation through reduced barometric pressure improves myocardial function and reduces infarct size in mice.

Introduction

The dangers of altitude are thought to lie primarily with hypoxia due to linear reductions in the partial pressure of oxygen as altitude increases. However, there are consistent epidemiological reports linking low and moderate altitudes of residence and lower mortality from coronary artery disease (CAD) and stroke [1-3, 20]. The mechanism is not understood, but these findings persist even after adjustment for modifiable patient risk factors such as smoking, hypertension, diabetes, abnormal lipids, alcohol consumption, and low levels of physical activity [9].

Many studies of hemodynamics have hinted that a vascular response to altitude may be organ and vascular bed specific, with a reported decrease in blood flow of brachial, common femoral, superficial femoral, and deep femoral arteries with no change in the diameter of the carotid arteries of eleven healthy subjects ascending to 5330 m [247]. Weil *et al.* theorized a vasodilation in the cerebral and coronary circulation at altitude but at the expense of vasoconstriction of the peripheral circulation [248] in 1969. Two years later, the Weil team went to on to report a significant decrease in the forearm venous compliance of subjects measured during 10 days of exposure to 4300 m [207]. As instances of acute mountain sickness, high altitude cerebral edema, and symptoms of insufficient cerebral oxygenation such as reductions in reaction time, altered night vision and headaches are uncommon at altitudes of up to 3000 m [249], it may be possible that hemodynamics differ significantly between altitudes over 3000 m and lower altitudes.

Shultz et al, in 2014, reported that oxygen saturation of the blood is at 91% at an altitude of 3440 m and is accompanied with slightly lowered central blood pressure when going from an

altitude of 400m to 3440 m [250]. In 2003, Wyss et al showed improved myocardial blood flow up to 4500 m in 10 healthy adults during an ascent to altitude measured by positron emission tomography during exercise, adenosine administration, and exercise [65]. These studies hint at the potential of altitude to safely increase blood flow in various areas of the body without inducing altitude related illnesses associated with hypoxia.

We have previously demonstrated significant increases in arterial diameter *ex vivo* with artificial application of normoxic low altitudes (523 m – 1001 m) through small reductions in barometric pressure. Additionally, we have shown significant increases in cardiac output, stroke volume, and decreases in systemic vascular resistance measured through left ventricular catheterization of healthy mice at 714 mmHg (mimicking an altitude of ~1000 m). We hypothesized that normoxic low altitude simulation could improve left ventricular function by reducing systemic vascular resistance (i.e., afterload) and improving blood flow to the ischemic coronary area to reduce infarct size.

Methods

Ethical approval

All experimental protocols were approved by the Animal Policy and Welfare Committee (UAPWC) at the University of Alberta in accordance with the Canadian Council on Animal Care (CCAC) guidelines.

LAD-ligation surgery

Sixteen-week-old C57BL6 male mice (Charles River; Vilmington, MA) were given access to standard chow and water ad-libitum and were housed on a 12h-12h light-dark cycle. Mice (n=17)

were anaesthetized through intubation with 2.5% isofluorane on a heated surgical platform, where a 3 mm incision was used to perform left- thoracotomy between the second and third ribs. The left anterior descending artery was located, and a 7-0 polyethylene suture was tied around it without damaging the artery. The suture was tightened as to completely and permanently restrict blood flow through the left anterior descending artery. 5-0 polyethylene sutures were used to carefully close the chest, layer by layer as previously described [251].

The animals were split into groups of two, a control group and an experimental group. Immediately following the surgery, the control group animals were allowed to regain consciousness and recover at control barometric pressure with access to standard chow and water ad-libitum. Animals in the experimental group were allowed to regain consciousness and immediately placed in an altitude simulation chamber with a barometric pressure of 714 mmHg for a period of 3 hours. After the normoxic low altitude treatment, these animals were allowed to continue recovery at 754 mmHg (near sea level conditions) with access to standard chow and water ad-libitum on a 12h-12h light-dark cycle. The experimental group of animals was administered low altitude simulation at a 714 mmHg of barometric pressure daily for the next 7 days.

Echocardiography

24-hours following the LAD ligation surgery, echocardiographic assessment using the VEVO 2100 ultrasound system (Visualsonics, Toronto, Canada) was performed on all animals to verify that a major myocardial infarction had occurred. Animals were anesthetized with 1.5% isoflurane in oxygen through the course of imaging, done with a high-frequency 40 MHz linear array transducer. The animals were placed on a heating pad to maintain 37C body temperature and were constantly monitored through ECG limb electrodes. We aimed to achieve heart rates of 400

beats per minute or above for the images acquired. The left ventricular chamber of all animals was imaged in parasternal long-axis and short axis views. Left ventricular M-mode imaging was conducted through tracing with the transducer at a sweep speed of 700 Hz at the papillary muscle level. These M-mode images were carefully taken at the papillary muscle level of the left ventricle in short axis orientation to ensure imaging of the correct section of the ventricle.

On day 8 following LAD-ligation surgery, echocardiography was performed on all animals was performed once again to observe any changes in cardiac function that had occurred over the experimental period. Echocardiographic images were analyzed for standard parameters using the LV tracing method of M-mode images at the level of papillary muscles, corroborated by the Simpson's method.

Normoxic low altitude simulation

For normoxic low altitude simulation, animals were placed inside of a custom constructed cylindrical 20.1 x 26.67 cm chamber [**Figure 3.1**, Custom Built – McMurtry], sealable to maintain a lowered barometric pressure. A control pressure was established on the pressure sensor in relation to the barometric pressure denoted by the meteorological service of Canada (Edmonton, Alberta station). A pressure of 714 mmHg (mimicking 523 m of altitude), approximately 40 mmHg below the barometric pressure of the laboratory was maintained through a vacuum controller (Buchi V-850; New Castle, DE) for a period of 3 hours daily with the experimental animals enclosed. This pressure was chosen to reflect a condition of low altitude, without the effects of hypoxia on the animals. Further discussion of these experiments will refer the directly manipulated corresponding barometric pressures instead of altitude, which was simulated through reductions in barometric pressure.

Histology and Infarct size

To measure infarct size, hearts were excised from both groups of mice at Day 8 and flash frozen in Tissue-Plus O.C.T. compound (Fischer Scientific, Waltham, MA, USA) using liquid nitrogen. The hearts were sliced at the papillary level using a cryostat and stained with a standard protocol of Masson's Trichrome Stain which stains cardiomyocytes red [252]. Infarct size was calculated as a percentage of the necrotic area versus area at risk.

Western Blot Analysis

Western blotting was performed using a HIF1α antibody (CAT #3716, Cell Signaling, Danvers, MA, USA) with standard technique using 25 μg of protein per sample obtained from peri-infarct cardiac tissue. We normalized expression to Actin to correct for loading differences. The films were developed and quantified using densitometry technique in the public domain Image J program (National Institutes of Health, Bethesda, MD, USA). The ImageJ "Gel Analysis" function was used, with background correction using a "rolling ball" method with a radius of 4 times the width of the band. The output was a value for each band proportional to the Integrated Density Value (IDV) of that band.

Lectin Perfusion

All imaging was performed with an EVOS FL LED fluorescence microscope (Invitrogen, Waltham, MA, USA). 5 mg of lectin fluorescein ricinus communis agglutinin I (Vector Laboratories Inc, Burlingame, CA, USA) was injected via a central venous cannula for 5 minutes prior to sacrifice, cardiac tissue isolation and flash freezing. Tissues were sliced 20 μm thick and fixed with 4% paraformaldehyde and imaged. For semi-quantification, peri-infarct zone fields

were evaluated and regions of interest were semi-quantified in arbitrary fluorescence units using Image J (National Institutes of Health, Bethesda, MD, USA). For quantification, the background was excluded from measured green florescence units with background correction using a "rolling ball" method with a radius of 50.0 pixels. The program was used to retrieve the RGB (red, blue, green) profile of the selected area, providing the intensity of each color in the region. Lectin perfusion was imaged in the green channel, hence the green intensity signal in the ROI (region of interest) was recorded for comparison of lectin perfusion between the control samples and treatment tissue samples.

Statistical analyses

All data are presented as mean ± SEM. SPSS Statistics 21 (IBM, Armonk, NY, USA) was used to conduct all statistical analysis. Effects of the low altitude simulation condition were evaluated using the unpaired t-tests for comparison between non-treatment and treatment groups where $p<0.05$ was considered significant.

Data availability

All data generated or analyzed during this study are included in the published article.

Results

Echocardiographic data showed significant improvement in left ventricular function in animals treated with normoxic low altitude simulation treatment after LAD ligation surgery. After LAD ligation surgery animals were assigned to 7 days of treatment in the custom-built altitude

chamber **[Figure 3.1]** or no treatment. Echocardiographic M-mode data obtained on Day 1 of the study post LAD ligation showed a clear akinesis of the anterior wall of the left ventricle in both groups of animals **[Figure 3.2 a, b]**. Repetition of the echocardiogram on these animals after 7 days, the control animals that were left to recover at near sea level conditions (754 mmHg) showed no improvement in the function of their anterior left ventricular wall **[Figure 3.3a]**. However, animals treated with 714 mmHg corresponding to an altitude of 523 m above sea level for a period of 7 days after the LAD ligation surgery showed a very noticeable movement in their anterior LV wall **[Figure 3.3b]**.

Through echocardiographic imaging of the animals in parasternal long axis mode and short axis mode at the apical, papillary, and base of the left ventricle, we were able to quantify standard cardiac function parameters **[Figure 3.4]** using the Simpson's method for accuracy and reproducibility. We found no significant differences in heart rate **[Figure 3.4a]** of the control vs. treatment animals at Day 1 or Day 7 after the LAD ligation surgery. Both control and treatment group animals started with similar fractional shortening measurements at Day 1. However, at Day 7, the animals that had been treated with normoxic low altitude simulation at 714 mmHg showed an improvement in their fractional shortening of 9.27±3.2 % **[Figure 3.4b]**. Similarly, control and treatment group animals started at similarly diminished levels of ejection fraction Day 1 after the LAD ligation. At Day 7, however, treatment animals showed a 14.06±5.1% increase in their ejection fraction values compared to animals who did not receive normoxic low altitude simulation treatment **[Figure 3.4c]**.

Following the same trend, stroke volume and cardiac output measurements in animals that received normoxic low altitude simulation at 714 mmHg vs. the control animals that recovered at near sea level conditions (754 mmHg) for a period of 7 days were significantly improved. Increases

in stroke volume of 12.71±5.2 µL **[Figure 3.4d]** and an increase of 8.58±2.38 µL/min **[Figure 3.4e]** were observed in treatment animals vs. control animals.

Histological staining showed a significantly reduced infarct size in animals that received normoxic low altitude simulation (714 mmHg) after LAD ligation surgery. At Day 7 after the LAD ligation surgery, the hearts of select animals [n=4 per group] were excised and stained with Masson's Trichrome stain to reveal a significantly reduced infarct size in animals that received the normoxic low altitude simulation treatment after the myocardial infarction vs. animals that did not receive treatment **[Figure 3.5].** In comparison to animals that did not have a myocardial infarction **[Figure 3.5a]**, animals that were left to recover from the LAD ligation surgery at control conditions (754 mmHg) **[Figure 3.5b]** had a much larger size of myocardial infarction scar tissue than animals who were exposed to 714 mmHg **[Figure 3.5c]** after the LAD ligation surgery. A reduction of 23.6±6.4% in infarct size was observed between the group treated with normoxic low altitude simulation vs. the group that received no treatment when normalized to a "no infarct" image **[Figure 3.5d]**.

Western blot analysis shows reduced HIF-1α expression in peri-infarct tissue of animals who received normoxic low altitude simulation after LAD ligation surgery. Western blot analysis of peri-infarct tissue excised from select animals after Day 7 of the study showed a significantly reduced expression of HIF1α in animals that received treatment for 7 days after LAD ligation surgery **[Figure 3.6a, b].** This trend was reversed for HIF-OH expression, which was significantly higher in animals that were treated with normoxic low altitude simulation at 714 mmHg for 7 days

after the LAD ligation surgery **[Figure 3.6a, c].** All bands were normalized to actin expression to correct for loading differences in the gel.

Lectin perfusion imaging showed no significant differences in peri-infarct tissue areas between treatment animals and control animals. After a period of 7 days post LAD ligation surgery, lectin was perfused into select animals before excision of hearts for lectin perfusion analysis. The analysis showed no significant differences in the lectin perfusion (as measured by green signal) in the heart tissue of animals that recovered at near sea level conditions**[Figure 3.7a, b, c]** vs. treatment animals that received 7 days of normoxic low altitude treatment at 714 mmHg **[Figure 3.7 d, e, f].** The lectin ischemic reserve, an index of lectin perfusion, was indifferent between the two groups of animals when the green signal intensity means of both groups were normalized to an image showing the lectin perfusion in the cardiac tissue in animal with no myocardial infarction **[Figure 3.7e].**

Discussion

We conducted a set of experiments to determine whether positive changes in cardiac function may be brought on by using normoxic low altitude simulation as an acute course of treatment after the induction of a severe myocardial infarction in mice. The most significant finding of our current study is improved fractional shortening, ejection fraction, stroke volume, and cardiac output with 7 days of normoxic low altitude treatment in mice with severe MI. Furthermore, our work shows that cardiac function improvement after MI due to barometric pressure treatment differs from the HIF1α mediated response to MI observed in untreated mice [253].

We achieved significant kinetic improvement of the left anterior wall of the left ventricle through a moderate reduction of 40 mmHg in barometric pressure, from 754 mmHg to 714 mmHg for 3 hours a day over a week **[Figure 3.3b]**. This was evident through M- mode echocardiograms showing a lack of anterior LV wall movement in animals that did not receive lowered barometric pressure treatment **[Figure 3.2b]**. As non-kinetic areas of the anterior LV wall at the level of the papillary muscles are characteristic of a severe myocardial infarction, we then utilized histology to check for any differences in the infarct size of the animals. We confirmed that the excised hearts of treatment mice exhibited a size of infarction roughly 11% smaller than untreated mice **[Figure 3.5]**.

HIF1α activation in peri-infarcted area is an established downstream effect of ischemic tissue due to myocardial infarction. HIF1α is activated in hypoxic conditions to allow ischemic tissues to better deal with lowered oxygenation and has been implicated heavily in the induction of angiogenesis, limiting infarct size, and improving myocardial function after acute coronary

occlusion in mice [254]. In our study, we show a reduced expression of HIF1α **[Figure 3.6]** after myocardial infarction in the peri-infarct tissue of animals treated with normoxic low altitude simulation after LAD ligation surgery compared to the control group animals. As HIF1α up-regulation is increased linearly with level of hypoxia [255], a reduction in penumbra HIF-1α expression after low altitude treatment points to a less hypoxic ischemic zone. This could mean that low altitude simulation could lead to significant improvements in oxygenation of the peri-infarct zone, improving levels of oxygen in the area without the need of HIF-1α downstream effects such as neovascularization signalled by VEGF. The possibility of reducing infarct size has previously been achieved through mechanical preconditioning of the myocardium [256], however, reductions in infarct size using a normoxic low altitude simulation treatment have never been shown before. Lectin staining between the control and intervention group revealed no significant differences in the microvascular network **[Figure 3.7]**, implying that the benefit we observe may be due to mechanically improved tissue perfusion alone as opposed to neovascularization.

We conducted this study to obtain insight into the beneficial effect of low altitude simulation through reduced barometric pressure as a "damage control" and therapeutic factor in instances of ischemic cardiac injury. Our earlier proof-of-principle *ex vivo* work has shown a reliable and significant dilation of compliant blood vessels leading to a significantly reduced systemic vascular resistance *in vivo* under low altitude simulation conditions. We believe that this reduced vascular resistance is critical in increased blood perfusion to the penumbra of the ischemic zone following myocardial infarction, resulting in a smaller infarction. Improvement of blood flow to the area at risk through collateral vessels immediately following infarction, even in the instance of complete occlusion of the left anterior descending artery, may be a first and important step to reducing infarct size through barometric pressure reduction. In humans, the size of infarction

following primary angioplasty treatment for myocardial infarction is an established predictor of 1-year all-cause mortality [257], so targeting the ischemic penumbra for salvage may have important clinical benefits.

Limitations and strengths

A potential limitation of the current study is the lack of measurement of the partial pressure of oxygen inside of our custom constructed hypobaric chamber when animals were being treated with altitude simulation. As the chamber is small and had multiple animals inside for treatment at a time, we cannot completely exclude the possibility of rising carbon dioxide and diminishing oxygen levels for short periods of time before the vacuum pump air inlet/outlet would flush out air as a means of maintaining pressure. However, we expect that these fluctuations were minor and generally would not be considered to constitute a hypoxic [55, 56] or hypercapnic [57] environment.

Another limitation to our study is that we do not measure any effects of chronic exposure to low altitude simulation or acclimatization to this altitude. Our current study aimed to explore the effect of acutely applied low altitude simulation using reductions in barometric pressure as a therapeutic tool, so we evaluated acute responses and cannot be sure of any chronic effects of low altitude exposure that may diminish or reverse the changes we observed. It is possible that the effects of longer-term exposures to simulated low altitude could reduce the therapeutic benefit of seen with reductions in barometric pressure. Some of these effects could be symptoms of mild altitude sickness, breathing difficulties, and anxiety or stress behaviours in animal models.

A strength of the work is clear cut demonstration of the therapeutic benefit using a clinically relevant model of myocardial infarction, the LAD ligation model, as measured by

clinically relevant techniques, including echocardiography. Moreover, since altitude chambers are commercially available, our findings could be translated into humans in a straightforward way.

Clinical implications

Our data shows that moderate reductions in barometric pressure that are experienced at common low altitudes of residence can be therapeutically harnessed to reduce infarct size and improve cardiac function in mice models of MI. This may be able to explain the epidemiological studies showing that individuals living at low and moderate altitudes above have lower risk of MI and stroke. We may be able to apply normoxic low altitude simulation as a therapeutic approach to acute ischemic disorders in humans, as a non-invasive complement to pharmacological and mechanical inventions that aim to re-perfuse ischemic tissues and reduced afterload.

Fortunately, simulating altitude is a very accessible means of potential treatment, as altitude simulation is an everyday reality in passenger aircraft [244, 245], and negative pressure hospital rooms [246]. Altitude simulation therapy is a unique, highly practical and non-invasive therapy to treat a plethora of ischemic disorders such as myocardial infarction, stroke, or to treat exacerbations of heart failure. In conclusion, the results from our study demonstrate the potential of normoxic low altitude simulation after a myocardial infarction to reduce infarct size and improve left ventricular function. This work may have potential for clinical translations to humans with myocardial infarction.

Acknowledgements

We thank the Cardiovascular Research Group at the University of Alberta for usage of the VEVO 2100 echocardiography system and EVOS FL system.

Sources of Funding

This study was supported by grants from the Canadian Institute of Health Research and the Heart of Stroke Foundation of Canada.

Highlights

- Normoxic low altitude simulation of 523 m above sea level reduced infarct size in MI mice.
- Normoxic low altitude simulation improves fractional shortening, ejection fraction, stroke volume, and cardiac output in post-MI mice.
- Normoxic low altitude simulation improves cardiac function in MI mice without increased activation of HIF-1α.

Figures

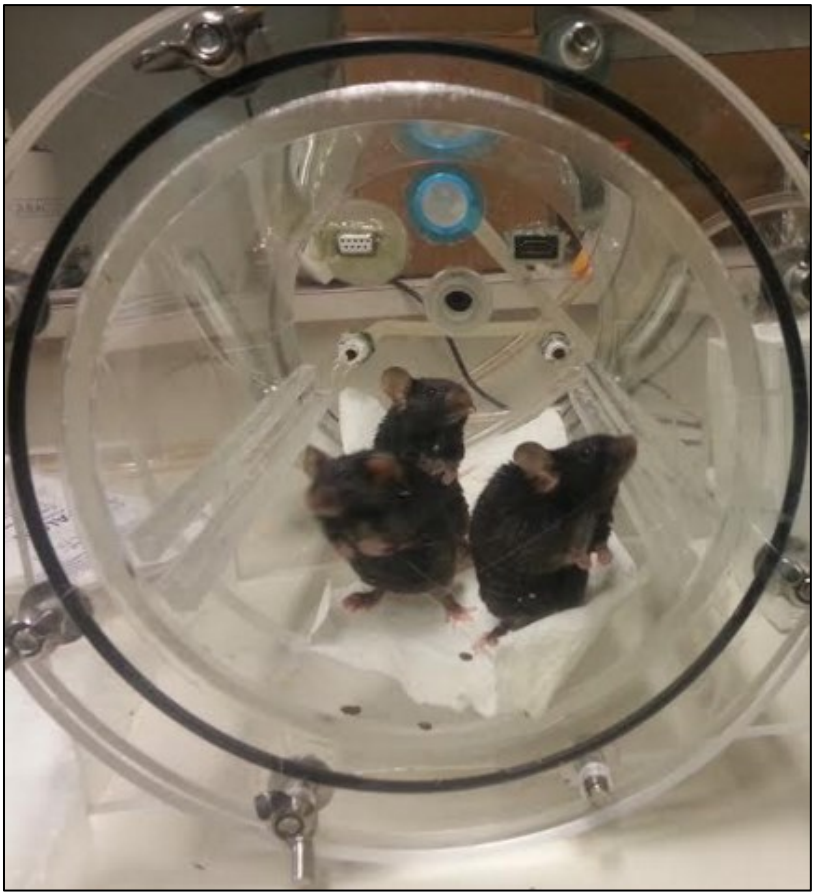

Figure 3.1 Experimental animals were placed within a specially constructed chamber to administer normoxic low altitude simulation through small reductions in barometric pressure. The animals were exposed to 40 mmHg barometric pressure reductions for a period of 3 hours daily for 1 week post the LAD ligation surgery to study the therapeutic potential of normoxic low altitude simulation on cardiac function after myocardial infarction.

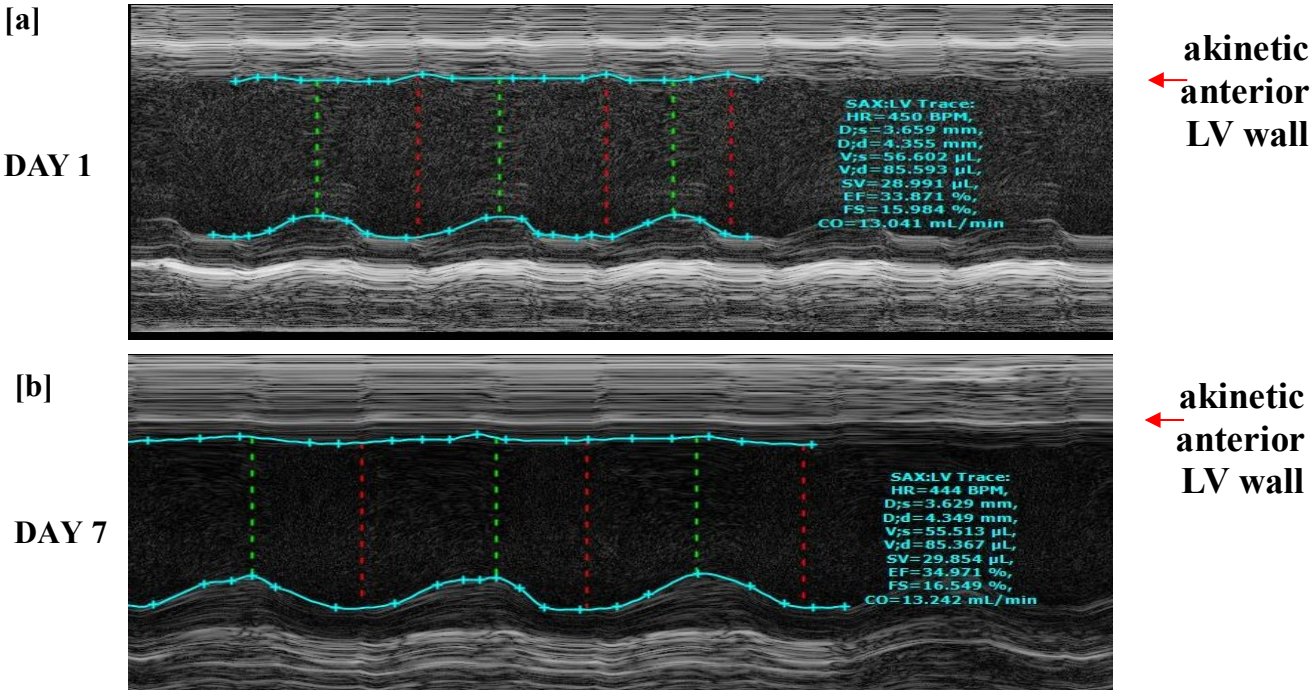

Figure 3.2 M-mode images taken from animals that underwent LAD ligation and were not treated with normoxic low altitude simulation. The images shown are 24 hours after the LAD ligation **[a]** and 7 days after the LAD ligation surgery **[b]** – both images depict reduced left ventricular function, with a lack of motion in the anterior left ventricular wall at Day 1 and Day 7.

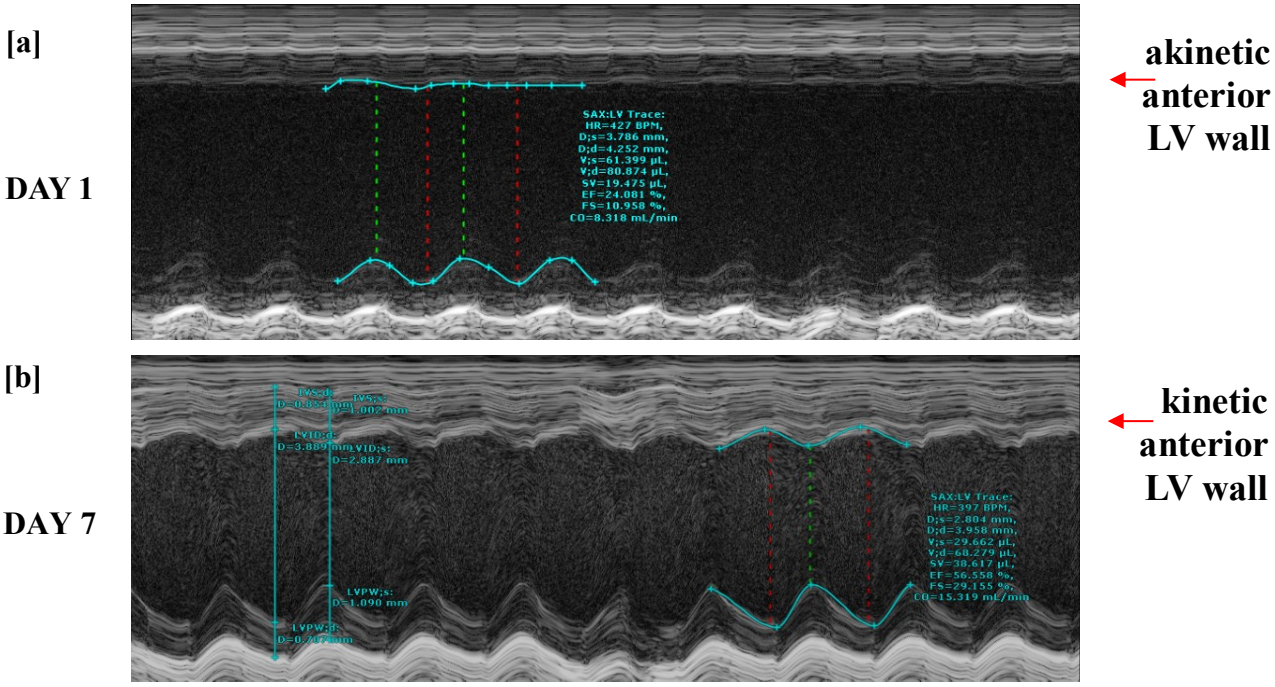

Figure 3.3 M-mode images taken from animals that underwent LAD ligation (n=13 per group) and were treated with normoxic low altitude simulation in the custom constructed hypobaric chamber at 714 mmHg 3 hours daily for 7 days post LAD ligation surgery. The images shown are 24 hours after the LAD ligation **[a]** and 7 days after the LAD ligation surgery **[b]**. There is marked akinesis of the left anterior left ventricular wall at Day 1. At Day 7, this akinesis is replaced with dramatically improved movement of the anterior LV wall.

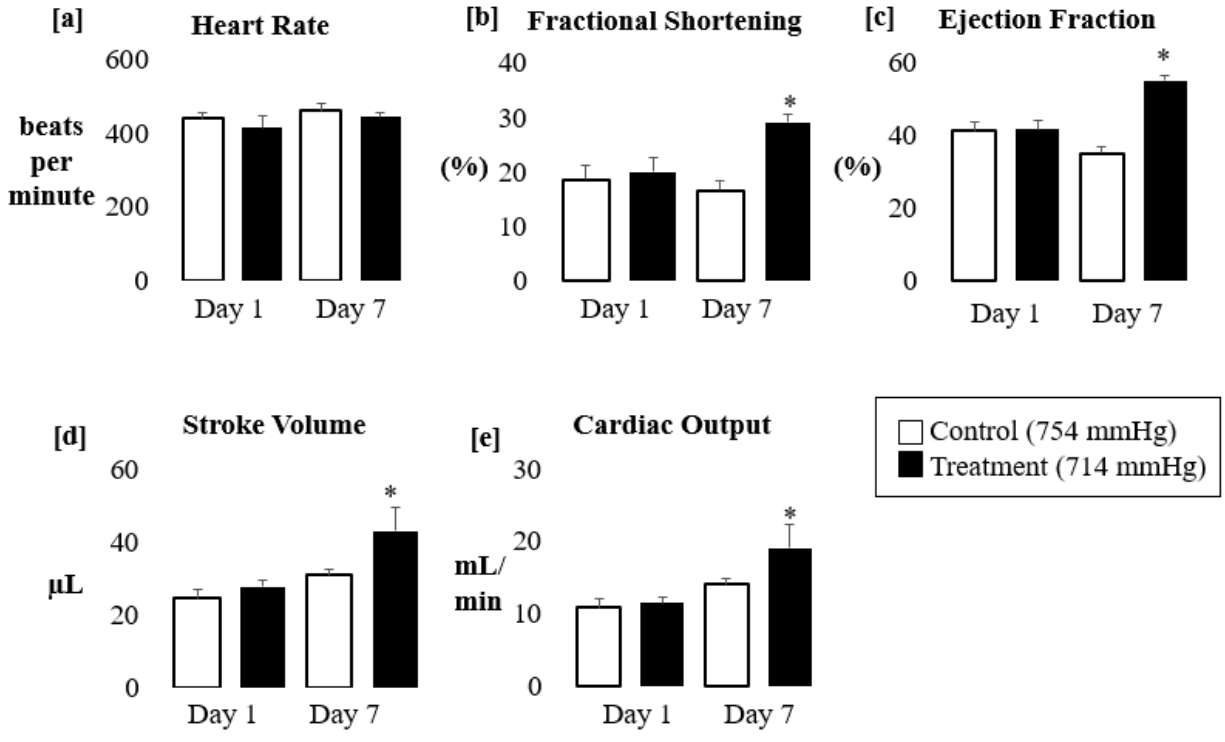

Figure 3.4 Hemodynamic parameters were obtained through echocardiography for experimental and untreated animals at Day 1 and Day 7 after LAD ligation surgery. There were no significant differences in heart rate **[a]** in both set of animals from Day 1 to Day 7. There were statistically significant increases in fractional shortening **[b]**, ejection fraction **[c]** stroke volume **[d]** and cardiac output **[e]** in animals that received 3 hours normoxic low altitude simulation (at 714 mmHg) for 7 days in comparison to control animals. Improvements in left ventricular function as shown by these parameters were absent from animals that did not receive normoxic low altitude simulation treatment.

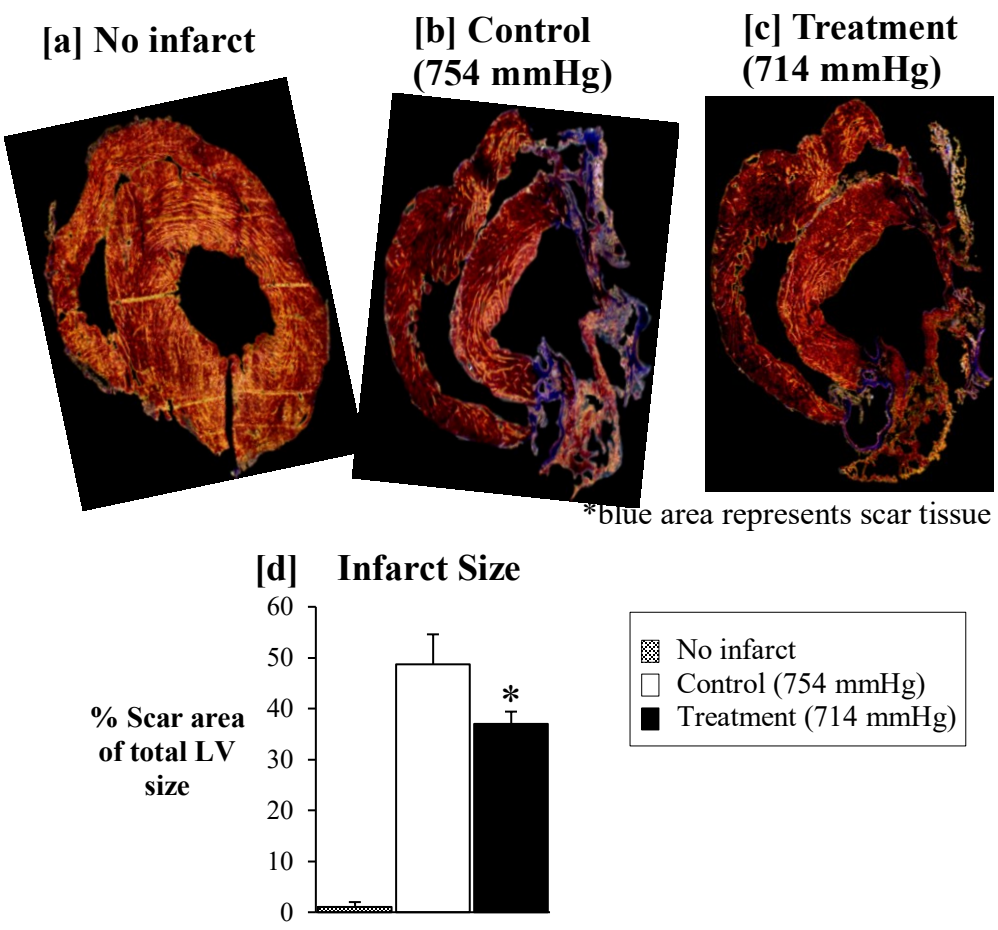

Figure 3.5 Excised heart stained with Masson's Trichrome **[a-c]** (n=4) show a significantly higher infarct size in control animals vs. animals that received normoxic low altitude simulation treatment **[d]**. Values shown are means ± SE.

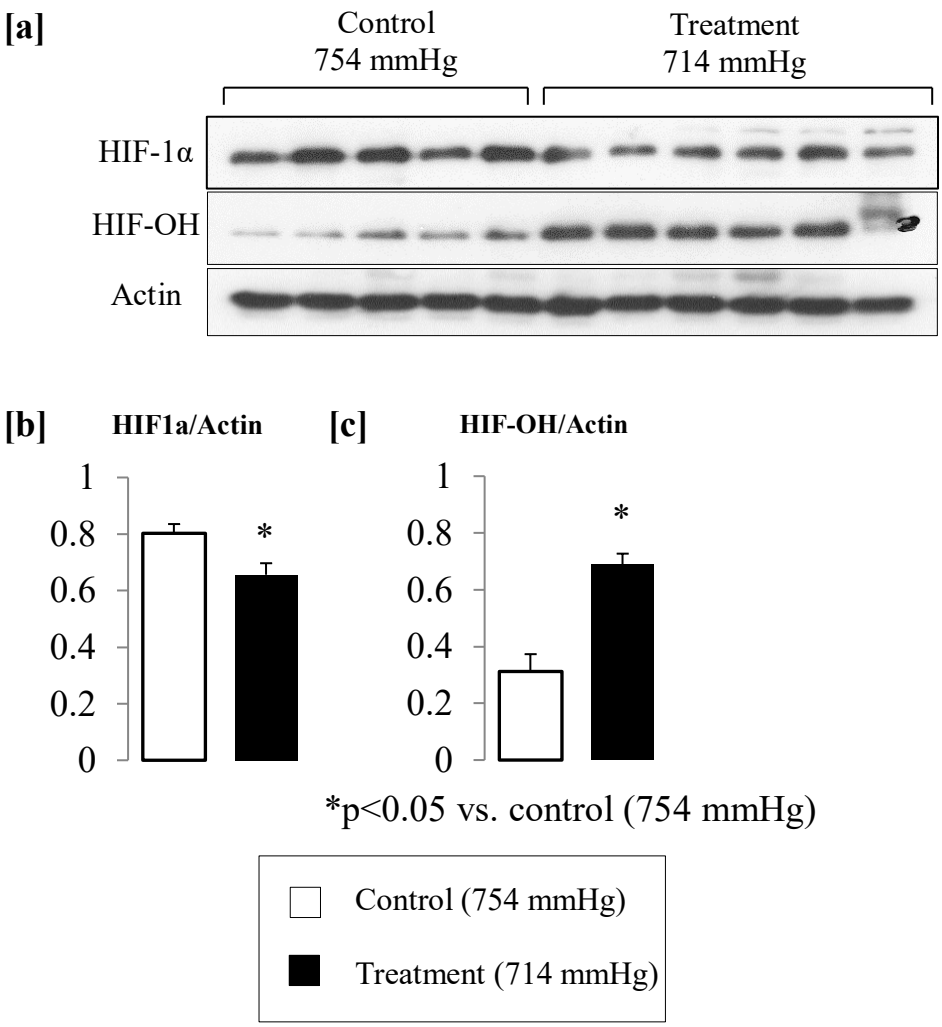

Figure 3.6 Western blot staining shows decreased HIF-1α expression in peri-infarct tissue obtained from treatment animals vs. control animals [a,b]. HIF-OH shows an opposite trend, with significantly more degraded HIF-1α in treatment animals vs. control animals [c]. The values were normalized to actin to control for loading differences and analyzed with ImageJ Software.

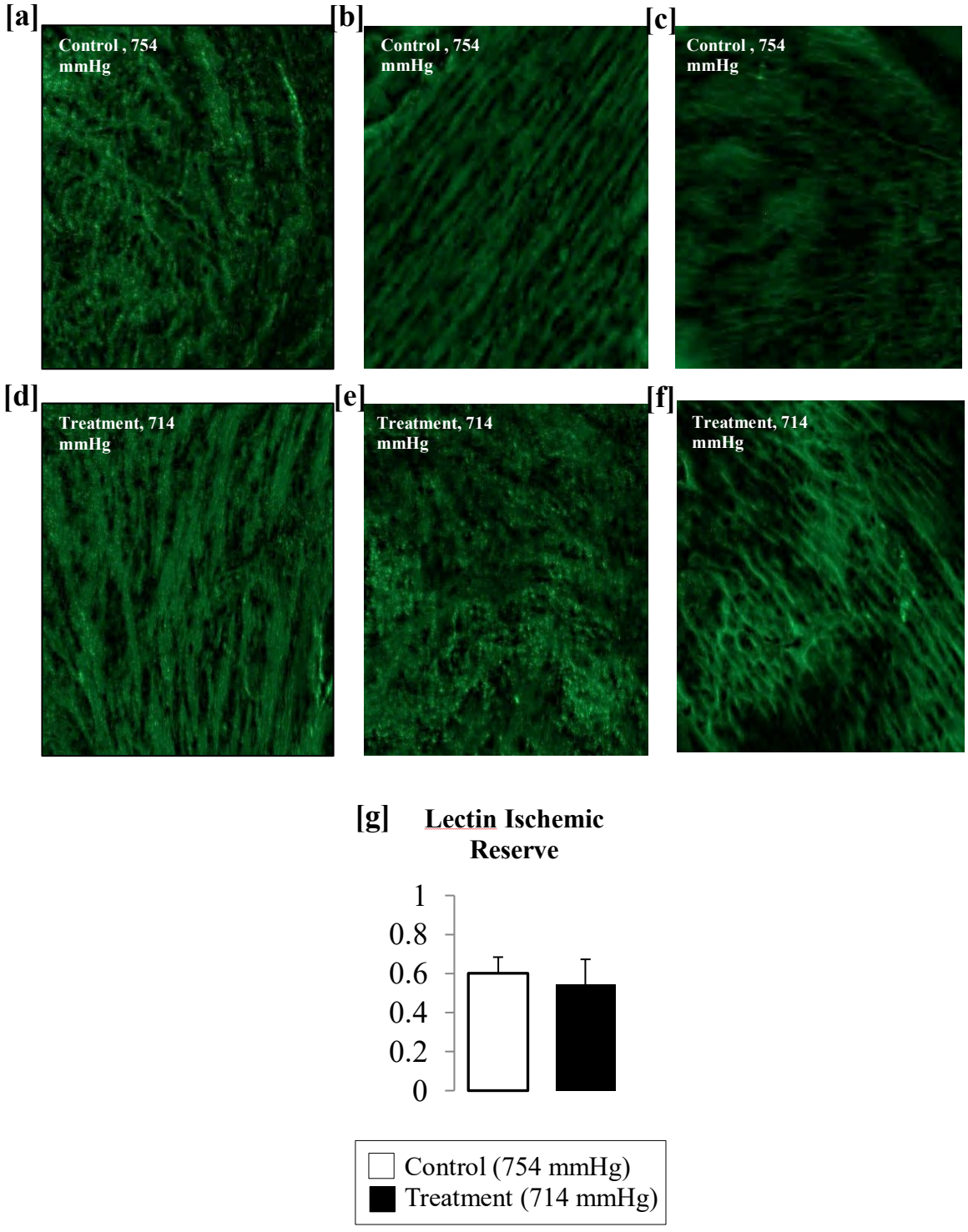

Figure 3.7 Lectin perfusion staining shows no significant difference in perfusion in peri-infarct tissue obtained from treatment animals **[a-c]** vs. control animals **[d-f]**.

Chapter 4 Normoxic low altitude simulation (at 714 mmHg) improves limb blood perfusion in mice with hindlimb ischemia

Running head: Low altitude simulation improves hindlimb ischemia

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Keywords

low altitude simulation, lowered barometric pressure, altitude hemodynamics, peripheral arterial disease

Abstract

Humans have fewer cardiovascular events and improved outcomes after cardiovascular events when living at low and moderate altitudes (<3000 m) above sea level. We have previously shown that low altitude simulation using reductions in barometric pressure enhances vasodilation *ex vivo* in arterial segments and reduces systemic vascular resistance *in vivo* and can also improve left ventricular function after a myocardial infarction. We hypothesize that low altitude simulation could also improve hindlimb ischemia, a model of peripheral artery disease in humans.

We performed femoral artery ligation to generate hindlimb ischemia in three-month old C57BL6 mice. Control group mice (n=10) recovered at 754 mmHg (control) for 14 days. Treatment group mice (n=15) were placed in a low altitude simulation chamber (at 714 mmHg) to recover from surgery for 3-hours daily for 14 days. Hindlimb perfusion imaging using a laser doppler line scanner was performed for all mice prior to the surgery, and then on days 1, 3, 7 and 14 post-surgery. At two weeks, ischemic reserve was significantly higher in the treatment group mice (0.50±0.13 vs. 0.20±0.06; p=0.01). Treatment group mice had higher functional scores and were able to walk better at two weeks. Approximately 3 times less HIF1α was found via Western blotting and a small but statistically significant improvement of lectin perfusion in calf tissue of treatment mice was observed.

We conclude that low altitude simulation improves blood perfusion in murine hindlimb ischemia. This approach may have therapeutic implications for humans with peripheral artery disease.

New and noteworthy

We have previously found that low altitude simulation improved hemodynamics *in vivo*, with higher cardiac output and reduced systemic vascular resistance, and could be used in mice to improve cardiac blood perfusion after myocardial infarction. We now apply this model of improving blood flow in a mouse model of hindlimb ischemia. This finding could lead to a non-invasive treatment to supplement current therapies for peripheral arterial disease.

Introduction

Atherosclerosis contributes to a myriad of cardiovascular diseases, including peripheral arterial disease (PAD), which is caused by atherosclerotic plaques in the leg arteries leading to arterial stenosis or occlusion [258]. The resulting ischemia can be very severe, causing pain on rest and tissue loss through ulceration and gangrene [259]. PAD is also associated with stroke, myocardial infarction (MI), vascular dementia, renovascular disease, and mesenteric disease [259, 260]. The Global Burden of disease study states that PAD was responsible for over 40,000 deaths in 2013 alone and patients with severe symptomatic large vessel PAD had a 25% chance of death from cardiovascular causes within a year [261]. The 1-year mortality of patients presenting with severe, chronic PAD can be up to 45% [262]. However, a large percentage of PAD cases are not symptomatic, and many countries do not screen for PAD [263]. Data from the NHANES study of 2174 persons in the United States showed that more than 95% of patients with PAD presented with one of more cardiovascular risk factors [264] which include diabetes, hyperlipidemia, hypertension, metabolic syndrome, older age, and smoking status among others [265]. While these risk factors explain much of the risk for atherosclerosis leading to PAD, other exposures may modify the risk.

Several studies have reported associations between a higher altitude of residence and lower mortality from coronary artery disease, myocardial infarction, and stroke [1, 2, 20, 21]. A notable study of individuals living between 259 and 1960 m showed a risk reduction of -22% per 1000 m of altitude above sea level for MI and -12% per 1000 m for stroke [24]. Interestingly, others have reported negative consequences of altitude on cardiovascular health in humans with a history of previous cardiovascular events [37, 112]. There is some evidence that altitude exposure may alter vascular function. A 2001 study suggested that higher altitude residents exhibited a superior ability to increase blood flow velocity as a response to muscular ischemia, compared to lowland dwellers

being conditioned to higher altitude. However, this study did not provide a sea level baseline for lowland dwellers, making it difficult to infer changes to the vascular function of the lowlanders reliably [266]. Another study presented that altitude exposure in lowlanders caused persistent impairment in vascular function, potentially mediated by oxidative stress and sympathoexcitation [267]. However, these studies were conducted at altitudes above 5000 m, a higher altitude than previous studies showing cardiovascular benefit from altitude. Although it seems that high altitude can have detrimental effects to vascular function, low and moderate altitudes above sea level are strongly linked with lower all-cause mortality in the general US population [25]. Despite the limitations of prior reports, there appears to be a strong correlation between low and moderate altitude of residence and protection from atherosclerotic cardiovascular disease. In our previous work with mouse models of MI, we showed that low altitude simulation treatment could be used to improve left ventricular function after MI [268] by increasing blood perfusion to the ischemic area, leading us to believe altitude simulation may also be beneficial in therapeutic use for PAD.

Currently, PAD is managed with risk factor reduction and exercise is commonly prescribed to improve mildly symptomatic presentations of the disease [269]. For patients presenting with severe symptoms or tissue loss, revascularization is required through surgery or angioplasty. However, it is often difficult to achieve revascularization and amputation may become necessary, leading to disability and extensive costs to the health care system and the economy [270]. Additionally, there are no effective medications for PAD [271], and therefore additional treatments to directly improve limb perfusion are needed. Based on our prior work showing improvement in left ventricular function post-MI in mice on a regimen of daily low altitude exposure in an altitude simulation chamber [268, 272], we hypothesized low altitude simulation would improve ischemic limb perfusion in a murine model of hindlimb ischemia (HLI).

Methods

Ethical approval

All protocols used in this study were approved by the University of Alberta Animal Policy and Welfare Committee (UAPWC) in accordance with the Canadian Council on Animal Care (CCAC) guidelines.

Surgical induction of hindlimb ischemia

3-month-old male C57BL6 mice (Charles River; Wilmington, MA) were given access to standard chow and water and were housed on a 12h-12h light-dark cycle. Mice (n=25) were intubated and anesthetized using 2.5% isofluorane on a heated surgical platform. A 3 mm incision was made in the left inguinal area and the common femoral and superficial femoral arteries were identified tracing along the left thigh. Two 5.0 polypropylene sutures were placed around the left superficial femoral artery tightly, taking care not to injure the adjacent vein or nerve. The suture was tightened to completely and permanently restrict blood flow through the femoral artery. The wound was closed using 5.0 prolene interrupted suture.

The animals were split into two groups, a control group (n=10) and a treatment group (n=15). Immediately after the surgery was completed, animals in the experimental group were placed in an altitude simulation chamber with a barometric pressure of 714 mmHg for a period of 3 hours to simulate low altitude before continuing recovery at room barometric pressure. We have previously shown small reductions in barometric pressure to have an acute and immediate effect on vascular function *ex vivo* and cardiovascular hemodynamics *in vivo* and we chose treatment

with 714 mmHg at 3 hours daily in light of our previous work [268, 272, 273]. Control group animals recovered at room barometric pressure (754 mmHg) following the surgery. The experimental group of animals were administered altitude simulation treatment (at 714 mmHg) daily for two weeks (14 days), for 3 hours per day. All animals were monitored twice daily to ensure the animals recovered as expected post-operatively. As per recommendation of the Veterinarian with the University of Alberta Animal Care and Use Committee, Buprenorphine was used as an analgesic. After 14 days, the animals were sacrificed using cervical dislocation and calf tissue was harvested.

Low altitude simulation

For normoxic low altitude simulation treatment, animals were placed in a specially constructed cylindrical 20.1 x 26.67 cm chamber **[Supplemental Figure S4.1**; **https://doi.org/10.6084/m9.figshare.7859972.v2]** that could be sealed to maintain a given barometric pressure. A baseline pressure was noted with a pressure sensor relative to the barometric pressure denoted by the meteorological service of Canada (Edmonton, Alberta). With the experimental animals enclosed in the chamber, low altitude simulation corresponding to a pressure of 714 mmHg (approximately 40 mmHg below the barometric pressure of the laboratory room) was maintained through a vacuum controller (Buchi V-850; New Castle, DE). This pressure was chosen carefully to simulate low altitude without inducing the effects of hypoxia in the mice.

Laser doppler imaging and analysis

Imaging to discern changes in blood perfusion were captured using a laser doppler line scanner, MoorLDLS2-IR (Moore Instruments; Delaware USA). Animals were anesthetized using 2.5% isoflurane and placed on a heated matt (37 C) for 5 minutes. The laser doppler line scanner was set to high acquire high resolution images and calibrated at 14 cm from the subject. A depilatory cream was used to remove the fur from both lower limbs thoroughly. Laser doppler images for the lower body (both limbs) were acquired immediately prior to and 5 minutes after the completion of the femoral ligation procedure. Laser doppler images were also acquired on Days 3, 7, and 14 after the surgery. Care was taken to remove any hair regrowth before each imaging session. It was ensured that ambient light in the room was consistent throughout all imaging sessions. Images were analyzed using the software accompanying the MOOR LDLS laser doppler scanner. For each image acquired, a flux image was generated showing a gradient of color from blue to red, the latter denoting a higher degree of blood perfusion. A region of interest was selected in each limb (control vs. experimental) and flux values were generated. These values were standardized to the leg that did not receive surgical intervention.

Functional scoring

We assigned Tarlov scores [274, 275] [as shown in **Table 4.1**] to evaluate any functional deficits in the control and treatment groups of mice after femoral artery ligation surgery was performed. Tarlov scoring was completed on days 7 & 14 following the femoral artery ligation surgery.

Tarlov Score	Function
0	No movement
1	Barely perceptible movement, no weight bearing
2	Frequent and vigorous movement, no weight bearing
3	Supports weight, may take 1 or 2 steps
4	Walks with only mild deficit
5	Normal but slow walking
6	Full and fast walking

Table 4.1 Tarlov scores used to assess walking ability after femoral artery ligation surgery. Adapted from Brenes et al (2013) *[275]* .

Lectin perfusion imaging and analysis

We used an EVOS FL LED fluorescence microscope (Invitrogen, Waltham, MA, USA) to complete the imaging. Mice were injected with 5 mg of lectin fluorescein ricinus communis agglutinin I (Vector Laboratories Inc, Burlingame, CA, USA) using a central venous cannula for 5 minutes prior to sacrifice, calf tissue isolation, and flash freezing. We sliced the tissues at 20 µm thick and fixed them with 4% paraformaldehyde before imaging. We used the Image J (National Institutes of Health, MA, USA) software to semi-quantify regions of interest were in arbitrary

fluorescence units. For quantification, we excluded the background from measured green fluorescence units with background correction using a "rolling ball" method with a radius of 50.0 pixels. Image J was used to retrieve the RGB (red, blue, green) profile of the selected area, providing the intensity of each color in the region. Lectin signal was imaged in the green channel, and the green intensity signal in the ROI (region of interest) was recorded for comparison of lectin signal between the control samples and treatment tissue samples.

Western blot analysis

We performed Western blotting with standard technique using 25 μg of protein per sample obtained from calf tissue of the ischemic limbs of select mice from the control (n=5) and treatment groups (n=8). We used a HIF1α antibody (cat. no#3716S) from Cell Signaling, Technology Inc of Beverly, MA, USA at a suggested 1:1000 dilution. We normalized the protein expression to actin to correct for loading differences before developing and quantifying the films using the densitometry technique in the Image J program (National Institutes of Health, MA, USA). The ImageJ "Gel Analysis" function was utilized to generate numerical values for each band that could then be quantified.

Statistical analysis

All data are presented as mean ± SEM. We used a one-way ANOVA to compare the ischemic reserve values generated from repeated laser doppler imaging of the control and treatment group of mice. Multiple comparisons were made using the Tukey's post-hoc test. The unpaired t-test was used for comparison between control and treatment groups where appropriate and $p<0.05$

was considered significant. We used the IBM SPSS Statistics 21 (Armonk, NY) software for statistical analysis.

Results

Low altitude simulation treatment significantly improved blood flow and function of the ischemic limb after femoral artery ligation. After femoral ligation surgery animals were assigned to either 14 days of normoxic low altitude treatment in the hypobaric pressure chamber or no treatment. Laser doppler images obtained immediately after the surgery showed a clear reduction in blood flow (blue in colour) versus the unaffected limb (red in color) **[Figure 4.1b, 1g]**. Upon repetition of the laser doppler imaging after 3 days, 7 days, and 14 days, the control animals that were left to recover at room barometric pressure of 754 mmHg showed minor improvement in perfusion of the ischemic hindlimb **[Figure 4.1c-e]**. However, animals treated with low altitude simulation at 714 mmHg for a period of 14 days after the femoral ligation surgery showed a very clear and significant improvement in blood perfusion to the surgically affected limb **[Figure 4.1h-j]**. Ischemic reserve values generated by normalizing the blood flow of the surgically generated ischemic limb vs. the non-ischemic limb showed statistically significant improvements the blood perfusion in the ischemic hindlimbs of the treatment group animals vs. the control group animals at Day 14 (0.5±0.3 vs. 0.2±0.1; p=0.01) **[Figure 4.2]**. Tarlov scoring showed significant ambulatory improvement in the mice that received normoxic low altitude simulation for two weeks in comparison to control mice (3.19±0.14 vs. 1.89±0.18; p=0.001) **[Figure 4.3]**.

Normoxic low altitude simulation treatment was associated with higher lectin perfusion of calf tissue in the ischemic limbs of treatment mice. To evaluate whether greater blood perfusion

with normoxic low altitude treatment was associated with enhanced neovascularization, we evaluated microvascular density of blood vessels using fluorescence microscopy of lectin-perfused ischemic hindlimb tissues. 14 days after femoral ligation surgery, lectin was injected into select animals before the excision of calf muscle in the surgical limb for lectin perfusion analysis. The analysis showed small but significant differences in the lectin signal (as measured by green signal) in the calf tissue of animals that did not receive low altitude simulation **[Figure 4.4a, b, c]** vs. treatment animals that received 14 days of normoxic low altitude simulation treatment at 714 mmHg **[Figure 4 d, e, f]**. The lectin ischemic reserve, an index of small blood vessel density (tissue vascularity), was improved in the treatment group mice vs. the control group (7.61±0.10 vs. 7.20±0.17; p=0.03) **[Figure 4.4g]**.

Normoxic low altitude simulation treatment reduced HIF-1α expression in the calf tissue of mice after femoral ligation surgery. To evaluate whether observed changes in blood perfusion could be related to differences in oxygen tension, we measured the expression of HIF-1α which is increased under hypoxic or ischemic conditions. Western blot analysis of calf tissue excised from select animals after Day 14 of the study showed a significantly reduced expression of HIF-1α in animals that received normoxic low altitude simulation treatment (at 714 mmHg) for 14 days after femoral artery ligation surgery in comparison to control animals (0.13±0.03 vs. 0.37±0.13; p=0.03) **[Figure 4.5a, b]**. All bands were normalized to actin expression to correct for loading differences in the gel.

Discussion

We performed a set of experiments to evaluate how low altitude simulation could influence lower limb blood perfusion in a murine model of PAD. Our most significant finding is the ability of normoxic low altitude simulation to significantly improve blood flow perfusion in mouse models of HLI over a short period of 14 days. A decreased expression of HIF1α in the calf tissue of treatment mice after exposure to low altitude shows that the benefit of the treatment is not due to a downstream effect of hypoxia. We have previously shown a similar therapeutic benefit in a mouse model of MI [268] and believe a mechanical mechanism where reductions in barometric pressure reduce the external compressive forces on arteries, thereby improving vessel dilation and blood flow to ischemic areas is likely **[Figure 4.6]**.

We achieved a 2-fold increase in blood perfusion of ischemic hindlimbs of treatment group mice vs. control mice by acutely simulating low altitude. This finding was accompanied by a significant functional improvement in the treatment group mice, who were able to walk and use their affected hindlimb visibly better than their control group counterparts. Enhanced perfusion and function achieved by reductions in barometric pressure has not been reported before in laboratory settings. Therapies that aim to increase angiogenesis and vasculogenesis under ischemic conditions have been considered a promising path to finding new and novel therapies for PAD. Physical agents have been used to improve hindlimb ischemia in a comparable manner. Acute thermal therapy (heat) through use of a far-infrared dry sauna at 41°C followed by 34°C has been used to increase the ischemic limb/normal side blood perfusion ratio in hindlimb ischemia models and an upregulation of eNOS in treatment animals has been cited as the reason behind the noted improvement [276]. Nitric oxide is a major endogenous vasodilator [277] and could potentially improve blood perfusion in a model of HLI through vasodilation of the vasculature. By achieving

mechanical distension of peripheral vasculature by small reductions in barometric pressure as would be seen with a slight increase in altitude, we can lessen the effects of HLI.

Low energy shockwave treatment has also been used to induce angiogenesis through VEGF receptor 2 simulation therapy to provide relief to ischemic muscles in models of HLI [278]. Laser doppler imaging of these animals showed a significant improvement in the lower limb blood perfusion of the treatment mice. To increase gastrocnemius muscle angiogenesis in HLI mice, a 2010 study used non-invasive electroporation with fibroblast growth factor-2 (FGF-2) delivery to the ischemic issue post femoral artery ligation. This study found a large improvement in the blood perfusion of the ischemic muscle [279].

We report a small but statistically significant increase in lectin density in the calf muscle tissue of animals that receive low altitude treatment. Increased lectin density is a hallmark of new blood vessel formation in the area of interest [280]. Angiogenesis is a notable downstream effect of hypoxia, regulated by upregulation of HIF-1α in ischemic tissues [281]. We report a reduction in the expression of HIF-1α in the treatment group animals after low altitude simulation. We believe that a mechanical effect of reduction in barometric pressure is reduction of external compressive forces on the systemic vasculature. This may have the effect of improving blood flow through collateral vessels and simulate arteriogenesis and lead to a functional improvement as we demonstrated. Whether arteriogenesis alone, or together with angiogenesis improves blood flow with low altitude simulation is unclear and warrants further pre-clinical work.

Limitations and strengths

A limitation to our study is the lack of measurements of oxygen partial pressure inside our chamber while the animals received treatment. Lowering barometric pressure can reduce the partial pressure of oxygen, however, we suspect this effect would be small at our chosen pressure (714 mmHg) as it mimics a low altitude of 523 m and altitudes below 2000 m are not associated with PO2 changes large to cause significant hypoxia in mammalian tissues [54-56, 238].

Additionally, laser doppler imaging struggles with a few limitations worth noting. It has been well documented that laser doppler imaging scans and generated color coded numerical values of blood perfusion can be influenced rather heavily by small changes in ambient lighting, animal body temperature, inappropriately removed fur from the skin being imaged, distance of the subject from the scanner, and changes in levels of inhaled anesthesia like isoflurane [282]. To make sure that our measurements were as accurate as possible, careful consideration was used to keep these variables as consistent as possible across measurements taken at different time points. In addition, as our experiments evaluated acute responses, and we cannot predict whether chronic adaptations to low altitude could attenuate or reverse the changes we observe in our study. Significant strengths of our work include identifying a blood perfusion benefit from an easy to implement low altitude simulation procedure.

Clinical implications

Our data support that small reductions in barometric pressure that are seen with low elevations can be used to significantly improve blood perfusion in cases of lower limb ischemia. Improvement in blood perfusion of the peripheral vasculature is associated with a lower risk for

MI and stroke in humans and this could be reason behind the epidemiologic phenomenon that people living at higher elevations have a lower risk for cardiovascular diseases. The main therapeutic approach to ischemic disorders including PAD involves improving blood flow to ischemic tissues through exercise or other more rigorous methods. A non-invasive mechanical technique of reducing barometric pressure to achieve better blood perfusion may become a valuable therapeutic tool for treating conditions like PAD. Furthermore, altitude simulation is a fairly cost effective and easy-to-implement means of treatment, occurring in passenger aircraft [244, 245], and negative pressure hospital rooms [246] every day.

To conclude, we show that we can enhance lower limb perfusion and improve limb function in mice with hindlimb ischemia with an acute application of low altitude simulation mimicking 523 m. These effects appear independent of HIF1α activity as may be seen with hypoxia and may have a potential therapeutic benefit in treating the symptoms of peripheral arterial disease.

Acknowledgements

We would like to thank Dr. Paul Jurasz and his laboratory staff for allowing us use of their laser doppler line scanner and providing us input on optimizing our hindlimb ischemia work and imaging.

Disclosures

There are no competing interests declared for any of the authors.

Grants

This work is funded by the Canadian Institutes for Health Research Canadian Institutes of Health Research (Vascular Metabolism and Angiogenesis, MOP 106515).

Figures

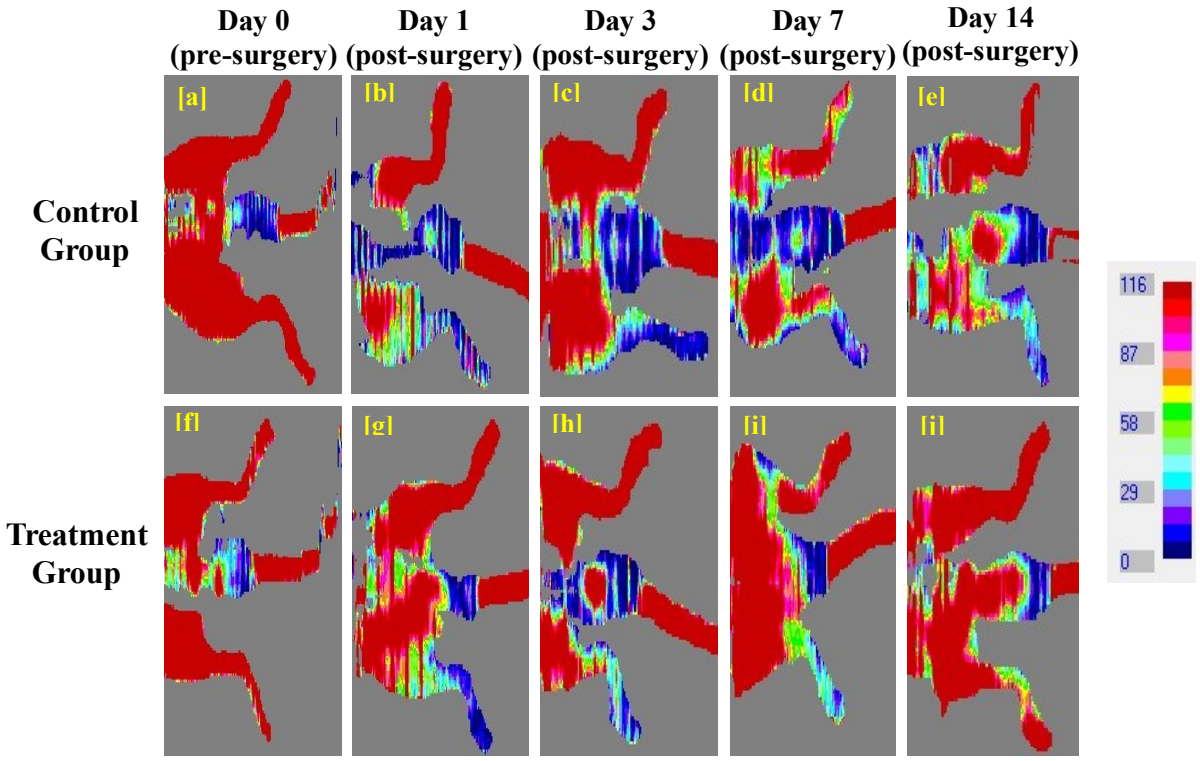


Figure 4.1 Laser doppler images reveal that treatment group mice showed a significantly greater improvement in blood perfusion in the intervention limb over the course of 14 days compared to the control group mice **[e, j]**. Low altitude simulation related to reduction in barometric pressure from 754 mmHg to 714 mmHg significantly augments the blood flow in the ischemic hindlimbs

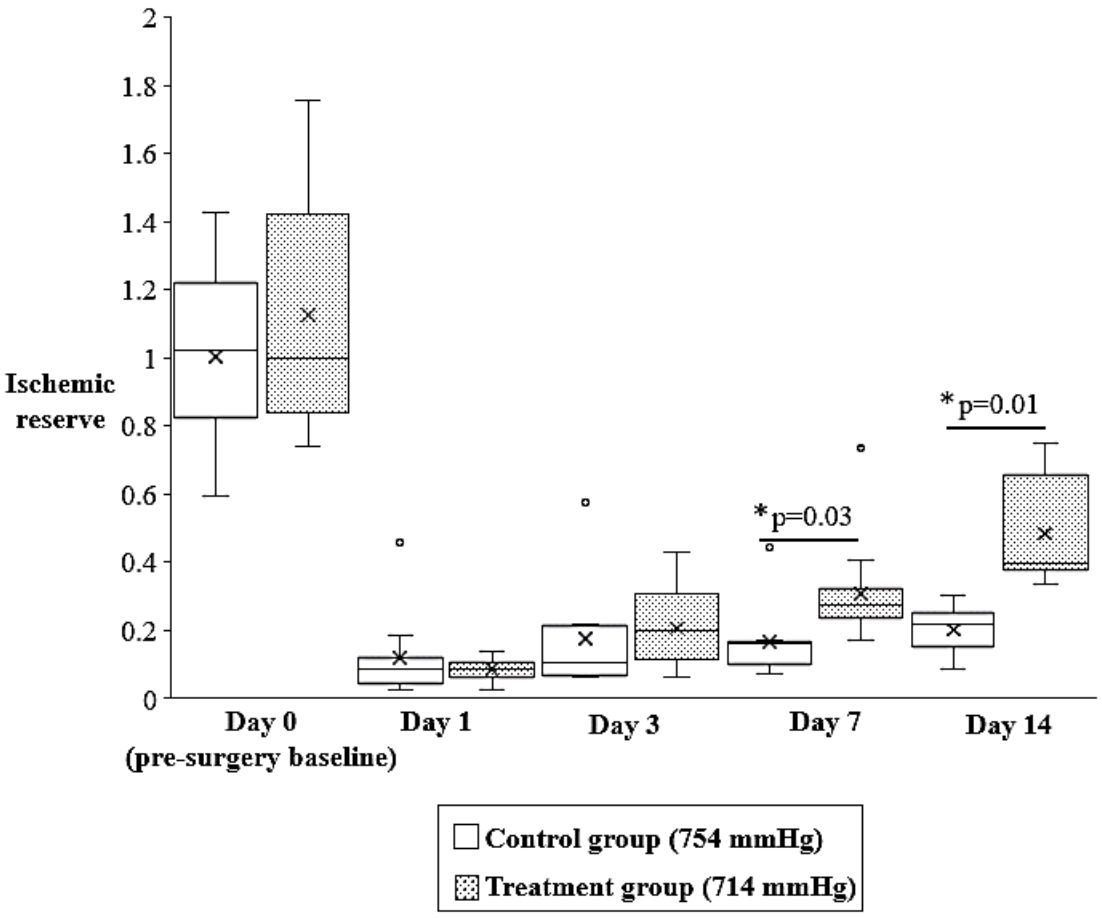


Figure 4.2 Quantitative analysis of the laser doppler image data shows improved blood perfusion in the ischemic limb of animals that received 14 days of low altitude simulation at 714 mmHg treatment vs. animals that did not. Significant differences in blood perfusion between the two groups are observed at Day 7 after the surgery and Day 14 after the surgery. Values shown are means ± SE.

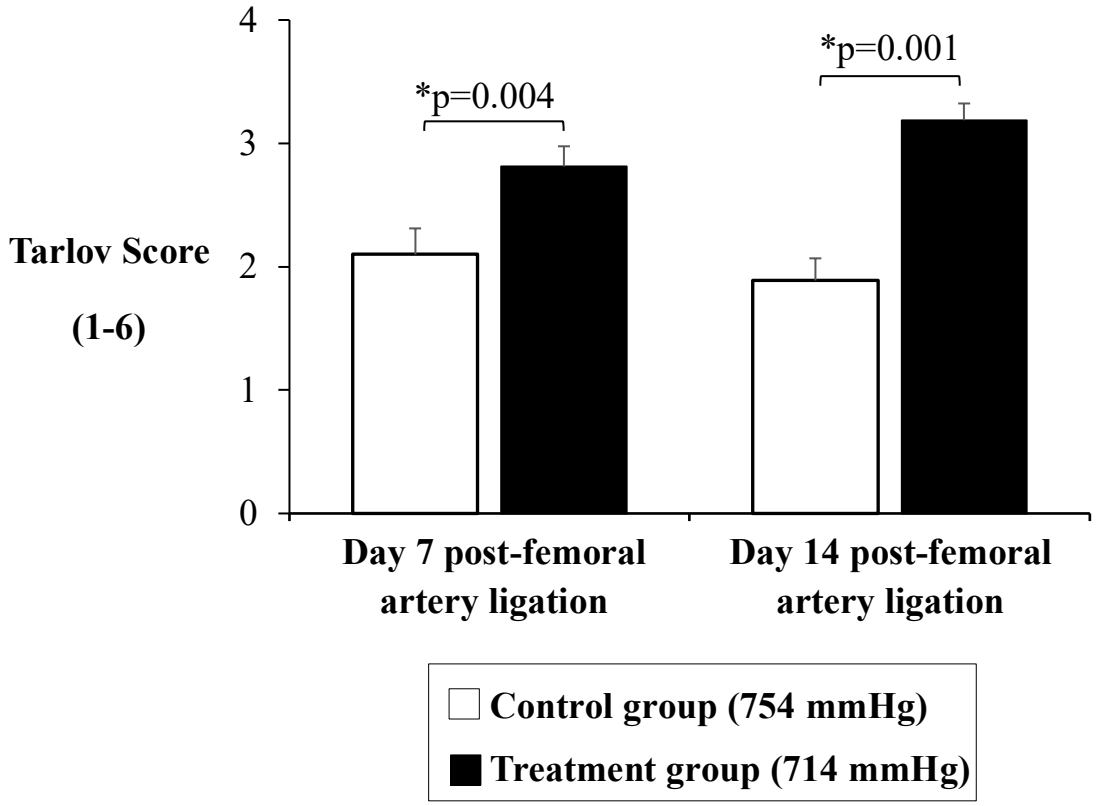
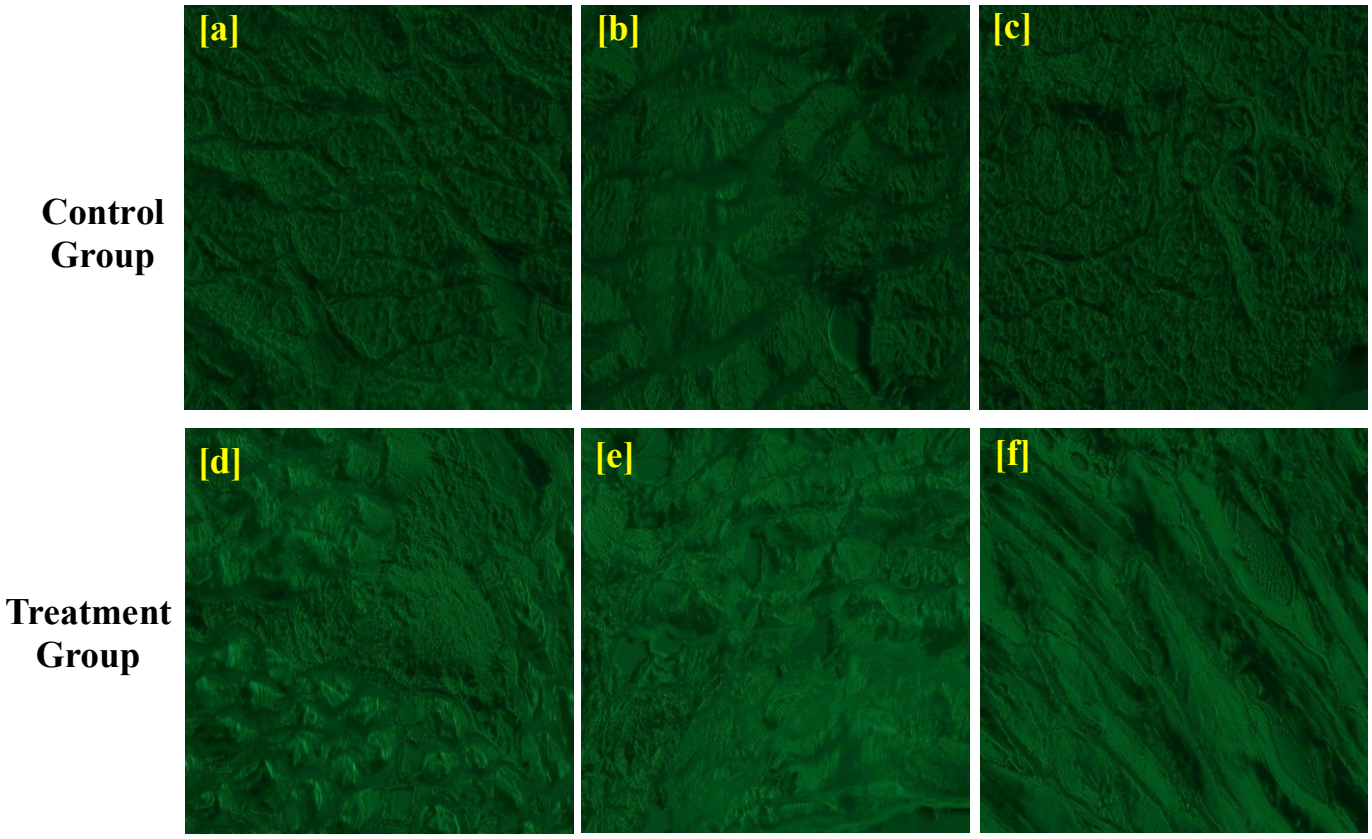


Figure 4.3 Tarlov scoring showed improved limb function and ability to walk in treatment mice at two weeks vs. control mice (3.19±0.14 vs. 1.89±0.18; p=0.001). Values shown are means ± SE.



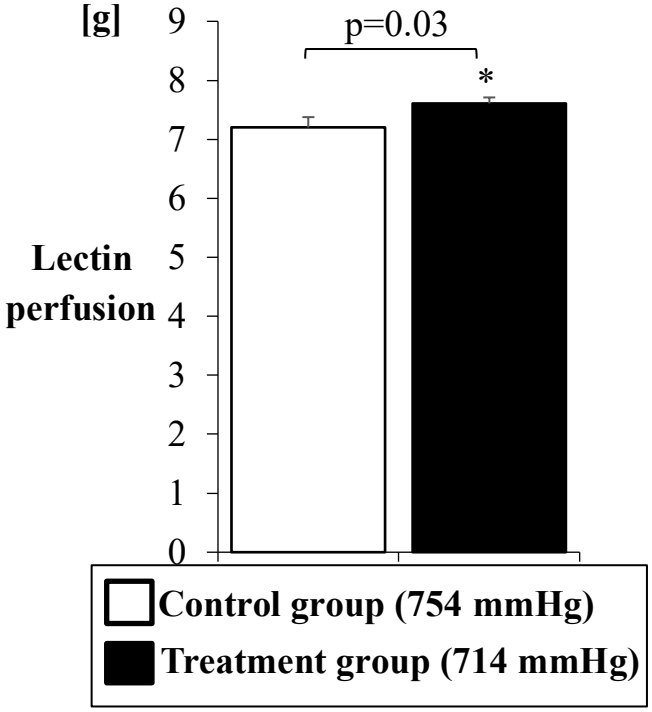


Figure 4.4 Lectin perfusion staining shows a small but statistically significant improvement of lectin signal in calf tissue extracted from the ischemic limb of normoxic low altitude simulation in treatment animals **[a-c]** vs. control animals **[d-f]**. The ischemic reserve is quantified and represented in **[g]**. Values shown are means ± SE.

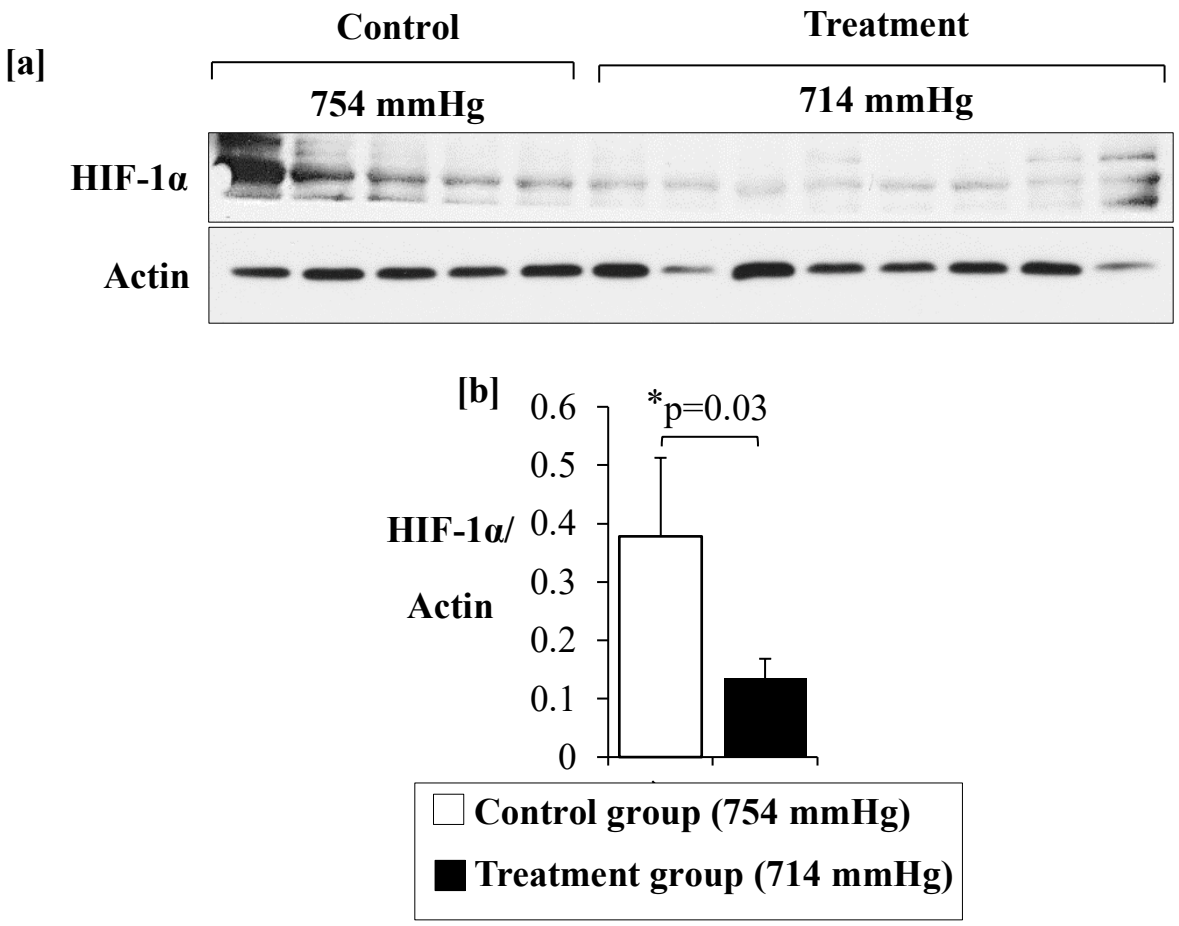


Figure 4.5 Western blotting showed a decrease in HIF-1α expression in calf tissue obtained from treatment animals vs. control animals [a,b]. The values were normalized to actin to control for lo loading differences and analyzed with ImageJ Software. Values shown are means ± SEM.

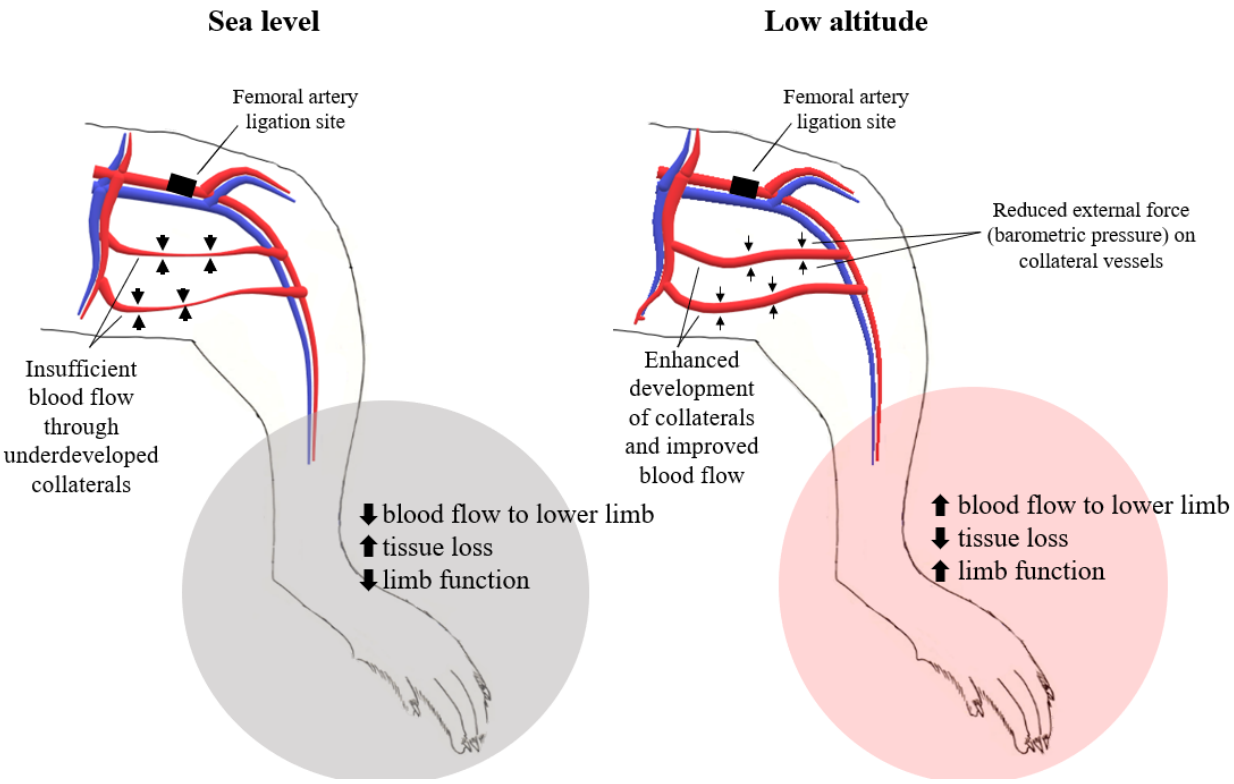


Figure 4.6 Reduced barometric pressure at low altitudes may improve blood perfusion to the lower limb after femoral artery ligation by enhancing the development of collateral vessels, reducing tissue loss and improving function in the affected limb.

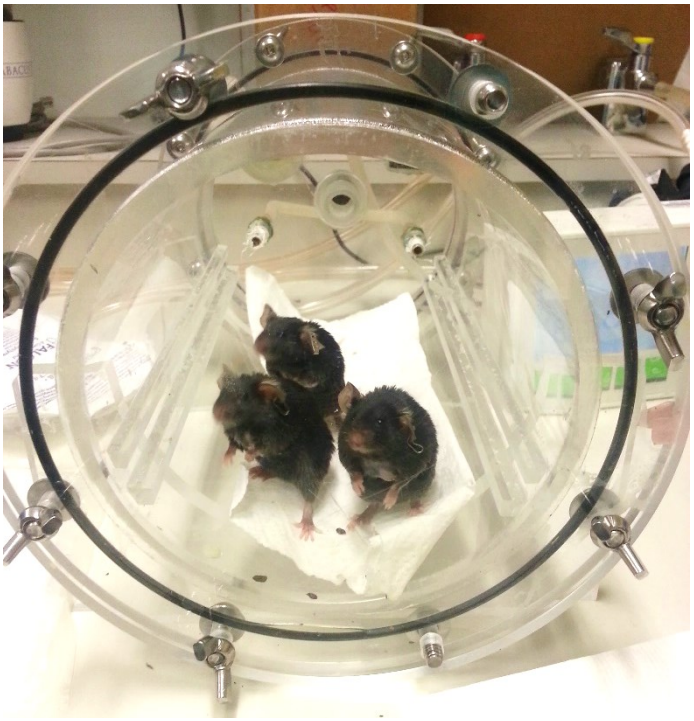


Figure S4.1 A custom made altitude simulation chamber (McMurtry) was used to treat mice with 714 mmHg.

Chapter 5 General discussion and conclusion

5.1 Discussion

To explore why residents of low and moderate altitudes that are not associated with hypoxia have less mortality from MI and other CVDs, we conducted experiments with mouse models mimicking ischemic disorders (specifically acute MI and PAD) in humans. We found that blood flow to the ischemic areas affected by both these illnesses could be improved using normoxic low altitude simulation by way of small reductions in barometric pressure. These disease models were an extension of our work with healthy mice exposed to normoxic acute low altitude simulation showing a significant improvement in cardiac output, stroke volume and reductions in systemic vascular resistance. This made for a strong conclusion to our primary *ex vivo* study (highlighted in Chapter 2) showing that reduced barometric pressure causes sudden mechanical distention of isolated resistance arteries, and this effect is independent of nitric oxide and prostacyclin.

5.1.1 Exploring the effect of normoxic low altitude simulation on isolated arterial segments *ex vivo* and healthy intact mice *in vivo*

In the study outlined in Chapter 2 of this dissertation, we performed a set of experiments to evaluate the effect of normoxic low altitude simulation (using small reductions in barometric pressure) on isolated arterial segments. Our most significant finding was the ability of reduced barometric pressure alone to increase vascular diameter, with or without the aid of major endogenous vasodilators. Our work shows that inhibiting the activity of major endothelial

vasodilators does not diminish the vascular distention induced by a small reduction in barometric pressure. This *ex vivo* response to altitude simulation has never been reported in isolated arteries before. Moreover, since we simulated low altitude under normoxic conditions, our findings challenge the previous concept that systemic arterial dilation occurs at altitude primarily as a result of hypoxia [77]. Although factors such as cold exposure or increased levels of physical activity leading to increased sympathetic activity at altitude have been explored as potential contributors to systemic vasodilation seen with altitude, the contribution of hypobaric pressure has not been previously considered [283, 284]. We are the first to show that reductions in barometric pressure alone can influence vascular diameter in normoxic conditions and hypoxia is not necessary for arterial responses to altitude.

We observed a 90% increase in the distention of arterial segments *ex vivo* at an intraluminal pressure of 60 mmHg when applying 80 mmHg of reduction in barometric pressure (mimicking 1001 m above sea level) in the presence or absence of inhibitors of nitric oxide and PGI_2. We utilized small reductions in barometric pressure to achieve arterial vasodilation, whereas, others have previously reported similar increases in arterial diameter using agonists like methacholine, bradykinin, and hydralazine [285-288]. We believe it is important that an application of normoxic low altitude simulation can dilate blood vessel diameter similarly to vasoactive agents that may be used clinically to treat cardiovascular diseases. Furthermore, since the response we observed is endothelium-independent, our finding may have important therapeutic application in situations entailing endothelial dysfunction.

To complement our *ex vivo* findings, we showed that normoxic low altitude simulation reduces systemic vascular resistance *in vivo* in healthy catheterized mice breathing 100% oxygen. We showed that stroke volume and cardiac output increased while total systemic vascular

resistance decreased by over 20% with a small reduction in barometric pressure (714 mmHg). Considering our *ex vivo* findings, we believe that reductions in barometric pressure dilate the systemic vasculature and reduce SVR. These reductions in systemic vascular resistance lead to reduced afterload and improved CO and SV without a significant change in HR. The use of pharmacologic agents to reduce impedence to LV ejection through systemic arteriolar dilation have long been used therapeutically in cardiac diseases [289-291]. As our findings show that small reductions in barometric pressure can non-invasively reduce afterload, low altitude simulation may come to have important applications in clinical use in CVDs.

5.1.2 Exploring the therapeutic benefits of low altitude simulation in murine models of human ischemic disorders

5.1.2.1 Using low altitude simulation to treat acute myocardial infarction in mice

In the study outlined in Chapter 3 of this dissertation, we showed that normoxic low altitude simulation can improve myocardial function (fractional shortening, ejection fraction, stroke volume, and cardiac output) and reduce infarct size significantly after a severe MI in mice. With echocardiograms, we show a markedly increased movement of the anterior LV wall in mice that received normoxic low altitude treatment in comparison to control mice. Although infarct size reduction and cardiac functional improvement has been achieved before in humans with administration of pharmacologic agents such as β_1 adrenergic blockers [292, 293], a non-invasive mechanical therapy using barometric pressure has never been explored before. In mice, HIF1α has previously been reported to be a major coordinator of responses to acute coronary occlusion in mice by inducing angiogenesis, limiting infarct size, and improving myocardial function[254].

Our work contradicts this notion and shows that improved cardiac function after MI due to normoxic low altitude simulation treatment can occur without HIF1α mediated responses [253]. As we show reduced HIF1α expression in the penumbra of mice that received normoxic low altitude treatment, the mechanism behind the beneficial effect of our treatment could be improvement blood perfusion to the infarcted area and generation of a more normoxic penumbra environment by mechanisms that are not HIF1α mediated.

Increased lectin perfusion is a hallmark of increased capillary density in an area of interest [280]. Interestingly, lectin perfusion showed no difference in the capillary density between the control and intervention group. This implies that the improvement in cardiac function that we have observed in normoxic low altitude simulation treated mice after 7 days of treatment is not due to neovascularization, but perhaps increased collateral development. We believe that reductions in barometric pressure may facilitate the enlargement of collateral vessels to improve blood flow to the ischemic area and improve cardiac function after MI.

5.1.2.2 Using low altitude simulation to treat hindlimb ischemia in mice

In the study outlined in Chapter 4 of this dissertation, we studied the effect of normoxic low altitude simulation on mice with induced hindlimb ischemia as a model of PAD in humans. We showed a two-fold increase in blood perfusion of the ischemic hindlimbs of treatment group mice vs. control mice by acutely simulating normoxic low altitude (using a small reduction in barometric pressure). Although unusual methods such as heat, low energy shockwaves, and electroporation with fibroblast growth factor have previously been used to encourage improvement of blood perfusion, the potential of improving blood perfusion through normoxic low altitude simulation has never been explored in animal models before.

New blood vessel formation through angiogenesis is a notable downstream effect of hypoxia, heavily regulated by upregulation of HIF1α in ischemic tissues [281]. However, we found a reduction in the expression of HIF1α in the treatment group animals after normoxic low altitude simulation but showed a small increase in lectin perfusion in the calf muscle tissue of animals that receive normoxic low altitude treatment. Lectin perfusion is a common measure of angiogenesis used heavily in hindlimb ischemia models [294]. As we show reduced expression of HIF1α in the treatment group, we predict that other angiogenic factors could be causing new capillary formation.

Fibroblast growth factor (FGF-2) is an angiogenic factor and has been shown to reduce infarct size in overexpression mouse models, and improve blood perfusion in hindlimb ischemia through angiogenesis [295, 296]. In light of our *ex vivo* work, we expect that the mechanical effect of reductions in barometric pressure may also be at play, distending non-occluded vasculature (including collaterals) to improve blood supply to the ischemic zone. We consider that a mechanism behind the success of our therapy could be low altitude simulation mediated enhancement of collateral vessel formation to improved blood flow to the ischemic area, ultimately improving limb function. Whether new capillary formation (angiogenesis) supplements collateral vessels to improve blood flow in normoxic low altitude conditions is yet to be explored.

Although our study is the first to explore mechanisms through which normoxic low altitude simulation could improve blood flow in a rodent model of hindlimb ischemia, low altitude simulation has been used recently to improve foot blood flow in human PAD patients. A Norwegian study team led by Sundby and colleagues recently reported on the effects of normoxic low altitude simulation (referred to as intermittent negative pressure treatment) using -40 mmHg below room atmospheric pressure on lower leg and foot blood flow in PAD patients. They reported reduced arterial blood flow velocity, skin blood flow, and skin temperature in the feet of healthy

volunteers when negative pressure (normoxic low altitude simulation) was applied constantly in healthy volunteers, but increased blood flow when applied intermittently [297]. Intermittent negative pressure application immediately and robustly increased peak blood flow velocity by 46% [(95% CI 36-57, $p>0.001$) above baseline in the feet of PAD patients [298].

Sundby et al. argued that constant negative pressure causes venous distention and reduces local blood flow through the venoarteriolar reflex [298]. As their study design differed considerably from our animal study in subjects, study length, and endpoints, it is difficult to interpret how normoxic low altitude simulation (or blood pressure) could be beneficial when applied in a pulsatile versus a constant manner at this time. Further pre-clinical research exploring real-time changes in blood flow of healthy and hindlimb ischemia mice may help to resolve these complexities before more clinical studies with humans are conducted. Although the mechanistic background behind normoxic low altitude simulation requires further study, low altitude simulation appears to be an attractive therapeutic avenue for human patients of PAD.

5.3 Strengths and Limitations

5.3.1 Strengths of this work

A major strength of this work includes pharmacological dissection of the altitude-related changes in lumen diameter to show that reduced barometric pressure can exert effects on the mammalian vasculature without endothelial contribution. Additionally, our *in vivo* experiments with healthy mice breathing 100% oxygen show clearly that hypoxia is not necessary for altitude mediated changes on the cardiovascular system. Perhaps most importantly, we show that HIF1α (i.e., hypoxia) mediated mechanisms are not responsible for improved outcomes seen with

normoxic low altitude simulation in our disease models and that barometric pressure can significantly influence disease outcomes in mouse models of MI and PAD. Finally, clear demonstration of the utility of normoxic low altitude simulation as a therapy in clinically relevant models of MI and PAD using clinically relevant techniques, including echocardiography and laser doppler imaging is a forte of this work.

5.3.2 Limitations of this work

A limitation of our study is the lack of measurement of the partial pressure of oxygen inside our chamber during our experiments, due to technical difficulties of obtaining oxygen readings with animals enclosed. We measured oxygen levels inside the chamber at our barometric pressure conditions of 754 mmHg and 714 mmHg prior to the *in vivo* protocols and always found oxygen levels to remain above 19.6%. Such minor reductions in barometric pressure do not generally reduce oxygen levels to an extent that would constitute a hypoxic environment for mammalian tissues [55-57], however, it is difficult to ascertain any fluctuations in oxygen levels without direct measurement in the chamber at all times. In our protocol with healthy mice *in vivo*, animals were breathing 100% oxygen while enclosed in the altitude simulation chamber, excluding hypoxia as a contributing factor during these experiments. Additionally, control group animals in the LAD ligation experiments and hindlimb ischemia experiments were not exposed to the same handling as the treatment group animals. Control animals were left to recover in their cages, not exposed to the daily handling and exposure to the barometric pressure chamber as the other animals had. Another limitation of our study was the technical inability to measure heart rate and blood pressure of the MI and PAD mice when enclosed in the chamber under normoxic low altitude simulation conditions. Finally, a major limitation to our work lies in the acute nature of our experiments. We

did not investigate the effects of longer-term altitude simulation *ex vivo* or *in vivo* and cannot predict if chronic adaptations (acclimatization) would attenuate or reverse the effects we found.

5.4 Major contributions

This thesis has been founded on the epidemiological connection between low altitude and reduced morbidity and mortality from cardiovascular diseases and we have used novel methods to investigate potential mechanisms behind this phenomenon. To dissect the physiological effect of exposure to low altitude, we have studied the effects of normoxic low altitude simulation (through reduced barometric pressure) on the isolated artery in a laboratory environment. We then studied the effects of reductions in barometric pressure on live, healthy mice and found that minor reductions in barometric pressure are associated with significant improvements in stroke volume, cardiac output, and significant reduction in systemic vascular resistance in mice. Finally, we generated murine models of acute myocardial infarction and peripheral arterial disease to study whether a reduction in barometric pressure (under normoxic conditions) can be used in a therapeutic capacity. We present a unique therapeutic application of normoxic low altitude simulation in mouse models of MI and PAD, which has not been previously done in a laboratory environment. We believe that further pre-clinical work may justify the use of clinical trials studying whether small reductions in barometric pressure may be applied as part of existing current therapies for various cardiovascular diseases.

5.5 Conclusions

Our data show that normoxic low altitude simulation can improve ventricular function and reduce infarct size after MI and improve blood flow to peripheral tissue in hindlimb ischemia. Dissecting the physiological effects of low altitude on *ex vivo* arterial segments and intact healthy mice, we found that low altitude simulation increases transmural pressure and distends the vasculature, decreasing vascular resistance overall. This may provide a mechanism behind epidemiological studies showing that individuals living at higher altitudes have lower risk of MI and stroke and is highlighted in **Figure 5.1** below.

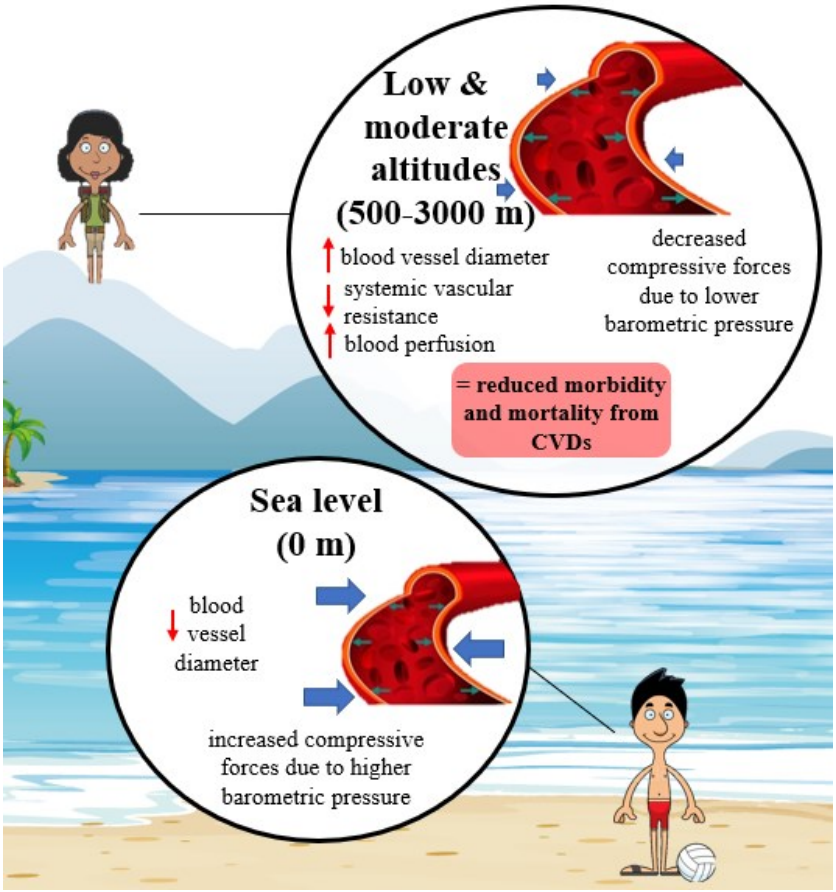


Figure 5.1 Epidemiological studies have shown reduced morbidity and mortality from many cardiovascular diseases in individuals residing at altitude. Through our experimental work with *ex vivo* arterial segments and intact mice, we propose that reduced barometric pressure corresponding to low altitudes can cause mechanical distention of the mammalian arterial tree, decreasing systemic vascular resistance and creating a cardioprotective environment. This schematic was created by Anmol Shahid (2019) using royalty free graphic art.

We show that normoxic low altitude simulation reduces systemic vascular resistance and increase cardiac output and may increase perfusion to ischemic tissue without concomitant neovascularization. Since the therapeutic approach to human ischemic disorders involve improving blood flood, or reperfusion [242], a non-invasive mechanical technique like normoxic low altitude simulation might be useful as a therapeutic tool to improve blood flow to ischemic tissues. Altitude simulation is a current and commercially viable everyday reality, occurring in passenger aircraft [244, 245], and negative pressure hospital rooms [246]. Therefore, further preclinical work evaluating normoxic low altitude simulation, with or without supplemental oxygen, may be justified. Chronic exposure experiments with animal models could provide additional insight into the cardioprotective benefit of low altitudes. Future directions could entail more investigations of normoxic low altitude simulation in other cardiovascular pathologies, including hypertension and stroke.

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