The Influence of Carbon Monoxide on Vascular Reactivity

by

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Abstract

Background: Carbon monoxide alters cardiovascular function through its actions as a signaling molecule and by limiting oxygen delivery in the blood, favouring pro-dilatory and anti-constrictor effects within the vasculature. The impacts of mild carbon monoxide exposure on vascular responses to exercise and sympathetic vasoconstriction have not been previously assessed in humans. We hypothesized that mild increases in circulating carbon monoxide would increase the magnitude of vasodilation in response to exercise, and inhibit α_1 adrenergic vasoconstriction.

Methods: Cardiovascular function was assessed via measurement of heart rate (ECG), mean arterial pressure (finger plethysmography), brachial artery diameter (B-mode ultrasound), forearm blood flow (FBF) (duplex ultrasound) and vascular conductance (FVC) (calculated from mean arterial pressure and FBF) in 19 young healthy volunteers (10 females) before and after carbon monoxide inhalation, and compared against a second control day without carbon monoxide exposure in a randomized control trial design. Measures were collected during resting baseline, rhythmic handgrip exercise (15% of maximum voluntary contraction) to assess the vascular response to exercise, and during continued exercise following a venous bolus infusion of the α_1 adrenergic agonist phenylephrine to assess sympathomimetic vasoconstriction. The magnitude of vasoconstriction during the initial peak constrictor response and between 90- and 120-seconds post-infusion were quantified as indices of α_1 adrenergic sensitivity. Forearm vascular data were obtained in both the exercising and resting arms to investigate the effects of carbon monoxide in both working and inactive tissue.

Results: Handgrip exercise elicited mild increases in heart rate ($P<0.05$), and substantial increases in FBF and FVC in the exercising arm ($P<0.05$). Phenylephrine infusion elicited decreases in heart rate ($P<0.05$), increases in mean arterial pressure ($P<0.05$), and reductions in FBF and FVC in both the exercising and resting arms ($P<0.05$). Carbon monoxide inhalation increased circulating carboxyhemoglobin saturation

to 5.6 ± 1.2%. No differences in cardiovascular responses were detected with carbon monoxide exposure, either in relative (percent change) or absolute measures.

Conclusions and Significance: Our data indicate that mild carbon monoxide does not alter functional vascular reactivity to exercise nor to α_1 adrenergic stimulation. These findings conflict with previous works demonstrating greater vasodilation with higher levels of exposure and attenuated vasoconstriction in isolated models, suggesting that alternate compensatory mechanisms abolish these effects at the present level of carbon monoxide exposure in humans. These findings improve our understanding of vascular function during moderate perturbations in vasoactive signaling, and support that mild carbon monoxide exposure does not constitute a significant threat to vascular regulation and oxygen delivery during physical activity.

Preface

This thesis is an original work by Scott F. Thrall. The research conducted for this project received ethics approval from the University of Alberta – Research Ethics Board under the project name: The Impact of Carbon Monoxide and Altitude on Vascular Function (Pro00096251**)**. Clinical trial approval was obtained from HealthCanada (HC6-24-c241154), and is registered on Clinicaltrials.gov (identifier: NCT04928183).

The research conducted for this thesis is from a subset of data from a larger study investigating various aspects of vascular regulation with carbon monoxide, co-led by Nicholas Cheung and myself.

Dr. Craig Steinback and I contributed to the conception of the experimental design, and I am responsible for the acquisition, analysis, and interpretation of the data, and the writing of the manuscript. Through data acquisition, Nicholas Cheung contributed with protocol coordination and brachial ultrasound, and Dr. Craig Steinback, Dr. Michael Tymko, Emily Vanden Berg, and Andrew Steele contributed assisted with phlebotomy and pharmacological infusions.

I would like to thank Dr. Craig Steinback for his mentorship and steadfast support throughout my degree. Thank you to Dr. Michael Stickland and Dr. Christopher Hearon Jr. for their support on my supervisory committee. Thank you to all of my fellow graduate students, university faculty, family, and local waitstaff who supported and encouraged my development throughout my degree; it takes a village.

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(G; H) and vascular conductance (I; J) from steady-state handgrip exercise to late phenylephrine responses

List of Abbreviations and Symbols

AC	Adenylyl cyclase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BKca	Large conductance calcium-activated potassium channel
BV	Blood volume
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanine monophosphate
CO	Carbon monoxide
EDH	Endothelium-derived hyperpolarization
EDHF	Endothelium-derived hyperpolarizing factor
FBF	Forearm blood flow
FVC	Forearm vascular conductance
HO	Heme oxygenase
IKca	Intermediate conductance calcium-activated potassium channel
IP_3	Inositol triphosphate
IV	Intravenous
K^+	Potassium
MVC	Maximum voluntary contraction
NE	Norepinephrine
NO	Nitric oxide
NPY	Neuropeptide Y
PaO_2	Partial pressure of arterial oxygen
PG	Prostaglandin

PKA	Protein kinase A
PKG	Protein kinase G
PLC	Phospholipase C
PO2	Partial pressure of oxygen
sGC	Soluble guanylyl cyclase
SKca	Small conductance calcium-activated potassium channel
SNA	Sympathetic nerve activity

List of Equations

Equation 1. Ohm's Law: $I = \frac{\Delta V}{R}$

Equation 2. Volumetric blood flow: $Q = \frac{\Delta P}{R}$

Equation 3. Poiseuille's Law: $R = \frac{8 \times L \times \eta}{\pi \times r\text{^}4}$

Equation 4. Nadler formulae: $Males: BV = (0.3669 \times H\text{^}3) + (0.03219 \times W) + 0.6041$

$$Females: BV = (0.3561 \times H\text{^}3) + (0.03308 \times W) + 0.1833$$

Equation 5. Forearm blood flow: $FBF = MBV \times \pi \times radius^2 \times 60s$

Equation 6. Forearm vascular conductance: $FVC = \frac{FBF}{MAP}$

Chapter 1: Introduction

1.1 Background

Rapid and effective delivery of oxygen to tissue is crucial to support optimal organ function. Matching of convective oxygen delivery via the bloodstream to tissue is regulated by changes in arterial vascular tone. During periods of heightened metabolic activity, such as exercise, signals promoting the relaxation of vascular smooth muscle induce vasodilation, increasing blood flow and oxygen delivery to tissue while simultaneously enhancing the removal of metabolic by-products (Laughlin & Korzick, 2001).

In response to stressors, the sympathetic nervous system is activated, sending efferent signals to target tissues throughout the body. Sympathetic discharge promotes vasoconstriction, serving to restrain blood flow and increase arterial blood pressure (Shoemaker *et al.*, 2015). Sympathetic vasoconstriction and local vasodilation mechanisms work at odds to one another, and the resultant vascular tone is the product of integration between the various signaling mechanisms at play. In particular, local vasodilatory signaling in working muscle tissue acts to inhibit sympathetic vasoconstriction, proportionally to the intensity of exercise, in a phenomenon referred to as functional sympatholysis (Remensnyder *et al.*, 1962). The mechanisms involved in sympatholysis are as yet unclear.

Commonly regarded as an environmental toxin, carbon monoxide has been found to participate in an array of signaling cascades involved in the control of blood flow. Direct actions of carbon monoxide predominantly induce vasodilation (Ndisang *et al.*, 2004). Additionally, carbon monoxide's capacity to displace oxygen from hemoglobin in erythrocytes and reduce the oxygen content of the blood necessitates further local vasodilatory responses to maintain adequate oxygen delivery to tissue. These effects are exaggerated during exercise, evoking augmented blood flow responses to the working muscle to maintain oxygenation with moderate to severe carbon monoxide exposure (González-Alonso *et al.*, 2001)

Investigations of the vascular effects of carbon monoxide have demonstrated evidence of interactions between its vasodilatory actions and opposing α_1 adrenergic vasoconstriction (mimicking the

primary actions of the sympathetic nervous system) in isolated animal models, whereby carbon monoxide signaling inhibits α_1 adrenergic vasoconstriction (Wang *et al.*, 1997*a*; Sammut *et al.*, 1998; Caudill *et al.*, 1998; Kaide *et al.*, 2004; Koçer *et al.*, 2018). Moreover, carbon monoxide has been shown to inhibit the activity of key sympatholytic signals, yet blood flow to the working muscle is maintained (González-Alonso *et al.*, 2002; Kirby *et al.*, 2008); the mechanisms underpinning this process are unclear, but may implicate a compensatory sympatholytic capacity of carbon monoxide. Despite recent progress in the characterization of both carbon monoxide-mediated vasodilation and α_1 adrenergic sensitivity, the underlying mechanisms for this interaction are unclear, and whether this effect is reproducible in humans is unknown. The aim of this study was to investigate the effects of mild carbon monoxide exposure on (1) vascular responses to exercise and (2) vascular reactivity to α_1 adrenergic stimulation.

1.2 Research Hypotheses

We hypothesized that moderate carbon monoxide exposure, akin to levels experienced with environmental exposure, would: (1) increase vasodilation and blood flow to exercising muscle as a compensatory response to the reduced oxygen content within the blood, and (2) impair α_1 adrenergic vasoconstriction in resting and exercising muscle.

1.3 Significance

Carbon monoxide is produced endogenously as a signaling molecule in health and disease (Ndisang *et al.*, 2004), and environmental exposure to pollution may result in elevated levels in the body. Characterizing the influence of carbon monoxide on vascular reactivity may improve our understanding of the cardiovascular consequences of disease states involving dysregulation of endogenous carbon monoxide production (Barbagallo *et al.*, 2012), as well as for individuals chronically exposed to carbon monoxide such as firefighters and smokers. Additionally, determining the functional impact *in vivo* of the

known vascular effects of carbon monoxide on sympatholysis from isolated animal models may shed light on the complex and integrative mechanisms involved in this enigmatic phenomenon.

Chapter 2: Literature Review

2.1 Introduction to vascular function

2.1.1 Anatomy and function of the arterial circulation

The cardiovascular system as a whole is fundamentally comprised of the heart as a pump, the arterial circulation for distribution of blood to tissue throughout the body, and the venous circulation for collection and return of blood back to the heart. During systole, blood ejected from the left ventricle of the heart enters the arterial circulation under high pressure, flowing along its pressure gradient. Ohm's Law (**Equation 1)** states that the magnitude of a flowing electric current (I) is proportional to the voltage (ΔV) (*i.e.,* the driving force), divided by the resistance of the system (R).

Equation 1. Ohm's Law:

$$I = \frac{\Delta V}{R}$$

Analogously, volumetric blood flow (Q) through a vascular bed is proportional to the driving force, namely the arterio-venous pressure gradient (ΔP), divided by the resistance of the vascular network (R) (**Equation 2)**.

Equation 2. Volumetric blood flow:

$$Q = \frac{\Delta P}{R}$$

In this instance, resistance is in turn determined according to a derivation of Poiseuille's Law (**Equation 3)**, which states that the resistance (R) to flow within a cylindrical pipe is a proportional product of the length of the pipe (L), the viscosity of the fluid (η), and the inverse of the internal radius (r) to the fourth power.

Equation 3. Poiseuille's Law:

$$R = \frac{8 \times L \times \eta}{\pi \times r\text{^}4}$$

As the viscosity of blood and the length of arterial networks cannot be readily adjusted on a moment-to-moment basis, precise regulation and distribution of blood flow throughout the systemic circulation is primarily accomplished via adjustments in arterial intralumenal cross-sectional area, with vasodilation reducing resistance to facilitate flow, and vasoconstriction increasing resistance to limit flow. Blood flow to a given vascular bed may be augmented to ensure adequate delivery of oxygen and metabolic substrates and for the clearance of metabolic byproducts and waste from tissue during periods of heightened metabolic activity, or restricted to limit overperfusion to maintain local and systemic arterial pressure.

Arteries are comprised of a central circular layer of smooth muscle, with an internal lining of a single-cell layer of epithelial cells known as the endothelium, and an external layer of collagenous connective tissue. Vascular tone is mediated by changes in the contractile state of the vascular smooth muscle. Contraction of the vascular smooth muscle is dependent on the concentration of intracellular calcium. Opening of voltage- and receptor-operated ion channels in the cell membrane results in an influx of calcium, which further compounds with opening of calcium-activated calcium channels in the sarcoplasmic reticulum to release intracellular stores, ultimately activating myosin to induce contraction concentrically and constrict the artery (Clifford & Hellsten, 2004). Conversely, inhibition of these calcium channels reduces intracellular calcium, inhibiting contraction and reducing tension to relax the artery, permitting dilation (Clifford & Hellsten, 2004). The endothelium assists in the regulation of vascular tone by integrating chemical and mechanical stimuli and transmitting vasoactive signaling molecules to the vascular smooth muscle.

2.1.2 Exercise hyperemia

The variable metabolic requirements of tissue necessitate rapid and tight regulation of vascular tone to ensure oxygen delivery is maintained. During exercise, the oxygen demand of skeletal muscle

increases up to ~30-fold, and is matched by proportional increases in blood flow (Andersen & Saltin, 1985). To date, no individual signaling cascade has been identified as singularly requisite for vasodilation during exercise; rather, a host of mechanisms have been detailed, with unique activation pathways and layers of redundancy between them (Laughlin & Korzick, 2001). These signals arise from (1) the skeletal muscle itself as a product of elevated metabolic activity, (2) the endothelium in response to chemical and mechanical stimulation, and (3) systemic humoral factors (Laughlin & Korzick, 2001). Most prominently among these signals are nitric oxide (NO), prostaglandins (PGs; *e.g.,* prostacyclin), and endothelium-derived hyperpolarizing factors (EDHF) (Clifford & Hellsten, 2004). Activation of soluble guanylyl cyclase (sGC) by NO stimulates the conversion of GDP to cGMP, activating protein kinase G (PKG) which promotes vasodilation directly through inhibiting myosin activation, increased calcium sequestration to the sarcoplasmic reticulum, and increased K^+ influx via the activation of large-conductance calcium-activated K^+ channels (BKca) to hyperpolarize the cell (Burgoyne & Eaton, 2010). Secondly, activation of adenylyl cyclase by PGs catalyzes the conversion of ATP to cAMP to then activate protein kinase A (PKA), exerting similar effects to PKG on contractility by opening hyperpolarizing K^+ channels and promoting the inactivation of myosin (Burgoyne & Eaton, 2010). Thirdly, EDHF-mediated hyperpolarization of the vascular smooth muscle is achieved through the actions of non-PKG/PKA cascades, emphasizing the activation of various K^+ channels in the endothelium and vascular smooth muscle (Luksha *et al.*, 2009). Recent evidence suggests that notable players in this process are small (SKca) and intermediate calcium-activated K^+ channels (IKca) inducing hyperpolarization of the endothelium itself, conducting across to the vascular smooth muscle directly via myoendothelial gap junctions, termed endothelium-derived hyperpolarization (EDH) (Garland & Dora, 2017). The basic signaling cascades involved in vasodilation are illustrated in **Figure 1**.

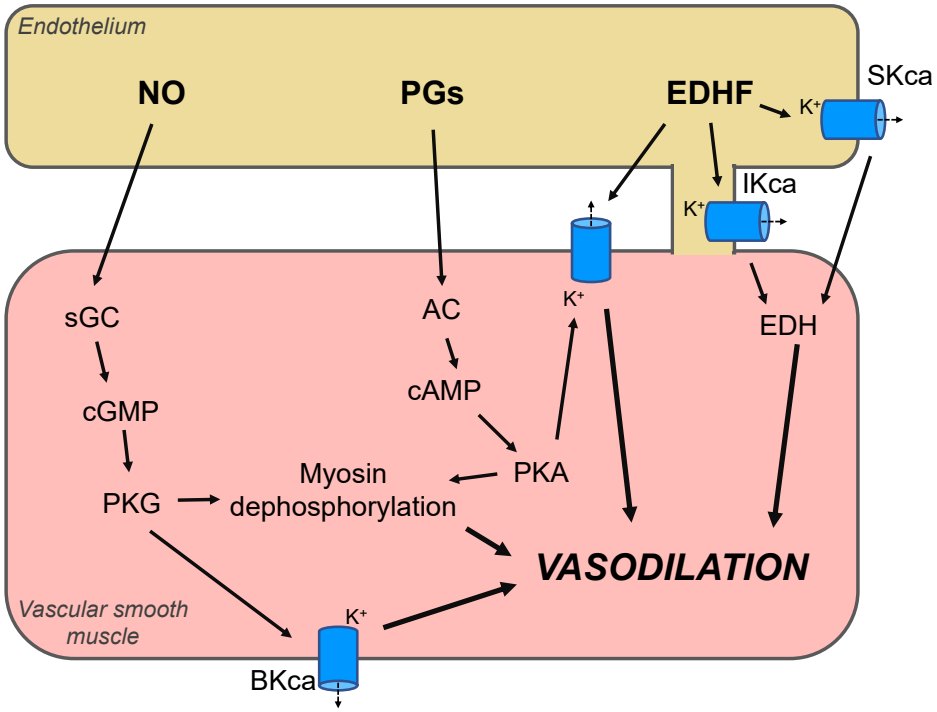

Figure 1. Vasodilation signaling cascades. Nitric oxide (NO), primarily of endothelial origin, activates soluble guanylyl cyclase (sGC) which catalyzes the formation of cGMP to activate protein kinase G (PKG). PKG stimulates vasodilation by increasing the permeability of large-conductance calcium activated K^+ channels (BKca) and by facilitating the dephosphorylation and inactivation of myosin. Prostaglandins (PGs) released by the endothelium activate adenylyl cyclase to promote the formation of cAMP and activation of protein kinase A (PKA). Vasodilation is mediated by PKA through activation of various K^+ channels as well as through the dephosphorylation of myosin. Endothelium-derived hyperpolarizing factors (EDHF), incompletely characterized as-yet, activate K^+ channels in both the endothelium and vascular smooth muscle to hyperpolarize the cell and inhibit contraction. Specifically, activation of small (SKca) and intermediate calcium-activated K^+ channels (IKca) in the endothelium and along myoendothelial projections connecting the endothelium and vascular smooth muscle facilitate K^+ efflux from the endothelium, with the resultant endothelium-derived hyperpolarization (EDH) conducting into the vascular smooth muscle via gap junctions in the myoendothelial projections. Numerous K^+ channels are involved in vasodilation; BKca, IKca and SKca channels are indicated for emphasis.

2.2 Neural control of vasculature

2.2.1 Autonomic innervation of the vasculature

The autonomic nervous system plays a critical role in the maintenance of systemic blood pressure and blood flow distribution, both tonically at rest and in response to acute and chronic stressors. Sympathetic nerve fiber bundles ubiquitously innervate the systemic vasculature, and efferent sympathetic nervous system activity (SNA) from the central nervous system results in the release of norepinephrine (NE), ATP and neuropeptide Y from sympathetic nerve terminals upon the vascular smooth muscle. These three sympathetic neurotransmitters act in concert to affect rapid and sustained contraction of blood vessels, each contributing a distinct component of the response. The brunt of the vasoconstrictor response is mediated by binding of NE to α_1 and α_2 adrenoreceptors, resulting in a large influx of calcium that propagates along the vascular smooth muscle (Wier *et al.*, 2009). This effect is complemented by binding of ATP to purinergic receptors affecting a rapid, transient release of calcium, ostensibly to "prime" the vasculature for adrenergic constriction (Wier *et al.*, 2009). Binding of neuropeptide Y results in a slower, sustained contraction, effectively maintaining the initial response to NE (Wier *et al.*, 2009). Vasoconstrictor signaling is limited by prejunctional autoreceptors for all three neurotransmitters expressed on the sympathetic nerve terminals, all with the function of inhibiting further neurotransmitter release in negative feedback loops (Shepherd & Vanhoutte, 1985). In addition to the vasoconstrictor effects of α adrenoreceptors, β_2 adrenoreceptors expressed within the vascular smooth muscle mediate vasodilation via AC-mediated pathways (Queen & Ferro, 2006).

Relative receptor density varies by tissue, with α_1 receptors expressed to a greater extent in larger arteries (*i.e.,* feeder arteries, 1st order and 2nd order arterioles), *vs.* α_2 receptors becoming the dominant subtype in the smaller branching arterioles (Ohyanagi *et al.*, 1991). Activation of α_1 adrenoreceptors increases intracellular calcium via production of IP3, whereas α_2 adrenoreceptors act to inhibit adenylyl cyclase activity, reducing intracellular cAMP and PKA (Taylor & Cassagnol, 2021). While both receptor subtypes work in concert to affect vasoconstriction upon activation with NE, post-junctional α_1

adrenoreceptors are the primary mediators of sympathetic vasoconstriction (Zang *et al.*, 2006). Conversely, β_2 adrenoreceptors expressed in the endothelium and vascular smooth muscle affect vasodilation via activation of adenylyl cyclase (Queen & Ferro, 2006). In vascular smooth muscle, PKA acts to directly inhibit myosin activation, whereas in the endothelium, PKA facilitates vasodilation via activation of nitric oxide synthase (Queen & Ferro, 2006). The basic signaling cascades of α_1, α_2 and β_2 adrenoreceptor activation are depicted in **Figure 2**.

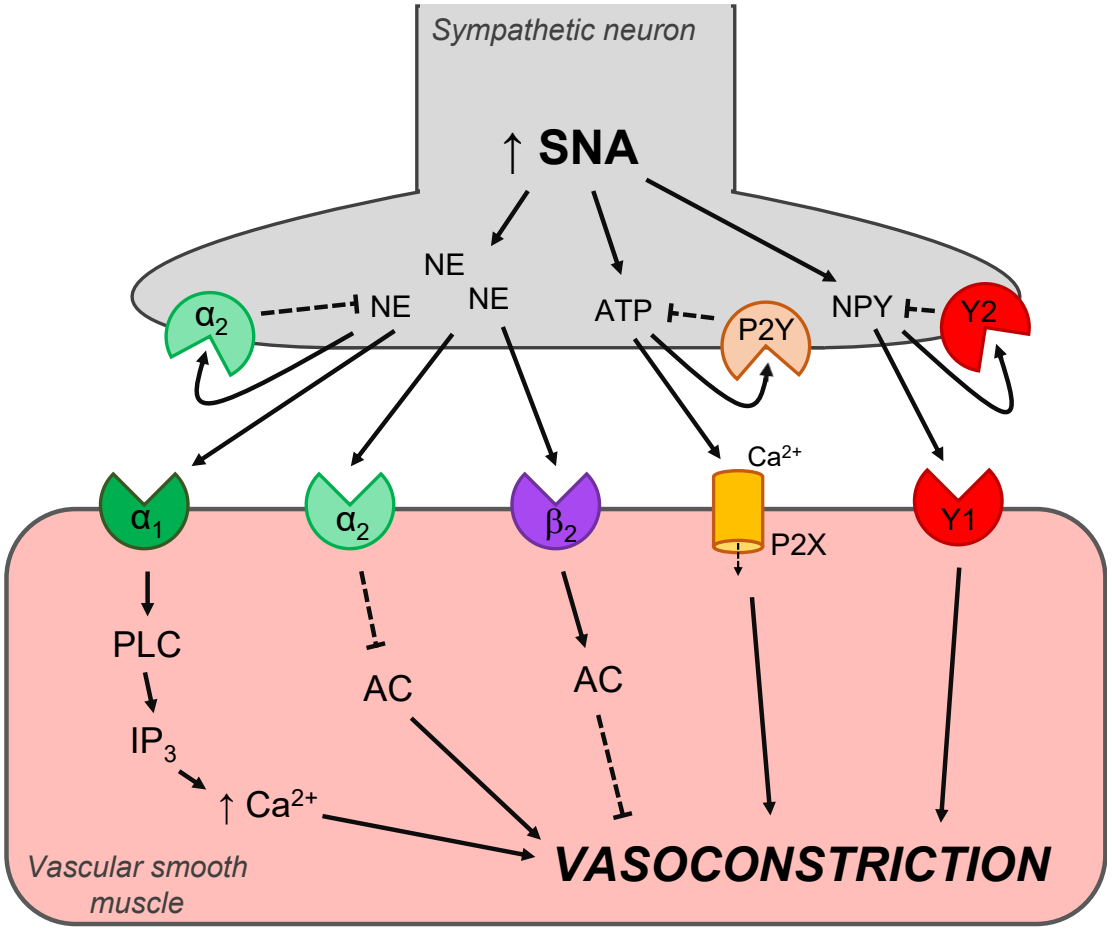

Figure 2. Vasoconstriction signaling cascades. Efferent sympathetic nerve activity (SNA) stimulates the co-release of norepinephrine (NE), ATP and neuropeptide Y (NPY). Downstream effects of NE binding differ by receptor subtype. Activation of α_1 adrenoreceptors stimulates phospholipase C (PLC) and the subsequent production of inositol triphosphate (IP_3), which activates calcium channels and increases intracellular calcium (Ca^{2+}) concentration to induce contraction. Activated α_2 adrenoreceptors inhibit adenylyl cyclase (AC) and its associated vasodilatory cascades, producing vasoconstriction. Conversely, activation of β_2 adrenoreceptors activates AC to inhibit vasoconstriction. Activation of ATP-sensitive P2X receptors and NPY-sensitive Y1 receptors produce transient and sustained vasoconstrictor effects respectively to bolster α-adrenoreceptor-mediated vasoconstriction. Binding of each sympathetic neurotransmitter (*i.e.*, NE, ATP, & NPY) to their respective pre-junctional autoreceptors (*i.e.*, α_2, P2Y, & Y2) inhibits their continued release. Hatched lines indicate inhibitory processes.

2.2.2 Neurovascular transduction

Neural control of vascular tone is the product of sympathetic outflow and corresponding degree of neurotransmitter release, weighed against the downstream transduction of the received signal (Shoemaker *et al.*, 2015). The moniker of neurovascular transduction encompasses a myriad of effects occurring downstream of sympathetic nerve fiber action potentials to adjust the resultant vasoconstrictor influence, including the release and reuptake of neurotransmitters, and the prevalence and sensitivity of the awaiting receptors (Tymko *et al.*, 2021). Intra-individual neurovascular transduction is modified under certain environmental conditions (*e.g.*, hypoxia), pathologies (*e.g.*, hypertension), and as a product of healthy ageing (Schlaich *et al.*, 2004; Seals & Dinenno, 2004; Berthelsen *et al.*, 2020).

Among the most pertinent shifts in neurovascular transduction is the inhibition of sympathetic vasoconstriction within exercising muscle tissue, known as functional sympatholysis (Remensnyder *et al.*, 1962). During exercise, oxygen demand in the working muscle increases dramatically, and is met with proportional increases in local vasodilation and blood flow. However, mass vasodilation risks depressurization of the arterial circulation, and must therefore be matched with vasoconstriction elsewhere in the systemic vasculature to maintain pressure; a function fulfilled adequately by concomitant sympathetically mediated vasoconstriction during exercise. Afferent fibers stimulated via through blood gas perturbations (*i.e.*, hypoxia & hypercapnia; chemoreflex), accumulation of metabolites (metaboreflex), mechanical factors (mechanoreflex), and central factors (central command) elicit increases in sympathetic outflow during exercise (Shoemaker *et al.*, 2015). To reconcile the conflicting signals of exercise-mediated vasodilation against neurally-mediated vasoconstriction, the working muscle exhibits a unique ability to inhibit sympathetic neurovascular transduction proportionally to the intensity of exercise (Buckwalter & Clifford, 1999).

The mechanisms responsible for functional sympatholysis are elusive, but are believed to be mediated through particular vasodilatory signaling cascades interfering with sympathetic vasoconstrictor pathways. A-adrenergic signaling takes center stage as the primary mechanism both of sympathetic

restraint of blood flow at rest as well as during exercise (Buckwalter & Clifford, 1999; Rosenmeier *et al.*, 2003*a*; Fairfax *et al.*, 2013), although it does not hold this position exclusively. Sympatholysis of neurally-released ATP and neuropeptide Y is poorly understood on account of their relatively minor role compared to adrenergic signaling, although recent evidence suggests that these mechanisms may also contribute a modest role in sympathetic restraint (Hansen *et al.*, 2020). Within α-adrenergic sympatholysis, the respective roles of α_1 *vs.* α_2 receptors are somewhat contentious, with some reports demonstrating greater sensitivity of α_2 receptors to exercise-mediated inhibition (Buckwalter *et al.*, 2001), and others indicating the roles are more equally weighted (Rosenmeier *et al.*, 2003*a*). Despite this, recent investigations have predominantly emphasized the role of α_1 receptors for several reasons. As the dominant receptor subtype in vascular control at rest, identifying mechanisms responsible for changes in α_1 sensitivity may have greater clinical relevance, and while sympatholysis in both receptor subtypes has been shown to decline with age, this decline is greater in α_1 receptors, supporting them as a more clinically relevant target for investigation (Dinenno *et al.*, 2005; Kruse *et al.*, 2018; Hansen *et al.*, 2020). Additionally, pre-junctional α_2 receptors acting to inhibit endogenous NE release may obfuscate post-junctional vasoconstrictor effects during infusion of α_2 agonists, such as clonidine and NE. The remainder of this review will focus on the role of α_1 adrenergic mechanisms of sympatholysis.

As a principal mediator of exercise vasodilation, NO has received considerable scrutiny for its putative role in sympatholysis, with mixed results. Inhibition of NO has been found to impair sympatholysis in response to increased efferent SNA (Thomas & Victor, 1998; Chavoshan *et al.*, 2002; Jendzjowsky & DeLorey, 2013), while others found that it was not obligatory for inhibition of α-adrenergic constriction (Rosenmeier *et al.*, 2003*b*; Dinenno & Joyner, 2003; Hearon *et al.*, 2017). Conversely, combined inhibition of NO and PGs impair α-adrenergic sympatholysis (Dinenno & Joyner, 2004), but are not together wholly responsible for this process (Hearon *et al.*, 2016). The endothelium plays a prominent role, markedly improving sympatholysis when stimulated via acetylcholine or ATP infusion (Hearon *et al.*, 2016). This effect appears to be dependent on interactions within the endothelium,

as vasodilators that act via endothelium-independent mechanisms such as sodium nitroprusside (an NO donor) and adenosine had no effect on adrenergic constriction (Rosenmeier *et al.*, 2004; Kirby *et al.*, 2008; Hearon *et al.*, 2016; Terwoord *et al.*, 2021). The sympatholytic effects of acetylcholine persisted even following inhibition of NO and PG synthesis, which the authors suggest to be a product of EDH, transmitted directly to the vascular smooth muscle via gap junctions (Hearon *et al.*, 2016). These findings suggest that sympatholysis in healthy young adults is mediated, in descending order of contribution, by EDH, NO, and PGs.

A tempting upstream candidate for the co-activation of endothelial hyperpolarization, NO and PG release is intravascular ATP, released from erythrocytes. Exogenous ATP administration demonstrates an impressive capacity to attenuate α_1 adrenergic vasoconstriction (Kirby *et al.*, 2008). Decrements in ATP release documented with healthy ageing are matched by decrements in sympatholysis, which are reversed with low-level ATP infusion (Kirby *et al.*, 2012; Hearon *et al.*, 2020). Moreover, the efficacy of sympatholysis is strongly associated with physical activity. Exercise training mitigates age- and hypertension-related decrements in sympatholysis and is associated with increased purinergic receptor expression and sensitivity, and conversely, physical inactivity impairs ATP-mediated sympatholysis (Mortensen *et al.*, 2012*a*, 2012*b*, 2014). While this does not conclusively indicate intravascular ATP as obligatory for sympatholysis, it does demonstrate its significant contribution to the phenomenon, with particular emphasis on the role of hyperpolarizing factors.

2.3 Introduction to carbon monoxide

2.3.1 Sources and kinetics of carbon monoxide

Commonly regarded as an environmental toxin, carbon monoxide (CO) is widely known for its lethality with acute exposure. Less appreciated is the role of this gaseous molecule as a physiological signaling molecule, classified as a gasotransmitter and sharing many functions with the similarly classed NO and hydrogen sulfide (Cebová *et al.*, 2016). Catabolism of heme via the enzyme heme-oxygenase

endogenously produces CO concurrently with biliverdin and ferrous iron, and can also be produced through oxidation of organic molecules (Tenhunen *et al.*, 1969; Rodgers *et al.*, 1994). Endogenous CO production occurs primarily via the former pathway, with expression and regulation of heme oxygenase observed ubiquitously throughout the body as a source of CO. Two primary isoforms of heme oxygenase responsible for the generation of CO have been identified: HO-1, an inducible isoform, upregulated by various stressors, including hypoxia (Ewing *et al.*, 1994), and HO-2, expressed constitutively in select tissues (Zakhary *et al.*, 1996; Wu & Wang, 2005).

The functional significance of heme oxygenase expression, activity and resultant CO signaling is obfuscated by its similarities with NO, and the interwoven relationship of each gasotransmitter on the production, release and activity of the other. Nitric oxide synthase and NO release have been reported to be inhibited by CO in some studies (Thorup *et al.*, 1999; Ishikawa *et al.*, 2005), and augmented in others (Yang *et al.*, 2016; Choi & Kim, 2021). Conversely, NO has been found to inhibit heme oxygenase activity (Rodriguez *et al.*, 2004). Furthermore, the two molecules can alter the sensitivity of the other in their downstream signaling cascades (Ingi *et al.*, 1996; Wu *et al.*, 2002). The relationship of CO and NO on functional outcomes is clearly interdependent, and while the matter merits further scrutiny, a consistent paradigm of CO/NO interactions and activities has yet to be established.

Elimination of CO occurs via diffusion into the blood stream, binding to hemoglobin to form carboxyhemoglobin (COHb) as an intermediary, and diffusing through the alveolar membrane to be exhaled via the pulmonary system (Rodgers *et al.*, 1994). Conversely, inhaled CO readily diffuses into the pulmonary circulation, resulting in increases in COHb as a proportional product of the inhaled concentration of CO and the diffusing capacity of the lungs. Quantification of dose-response curves of inhaled CO to circulating COHb has been utilized for the assessment of pulmonary diffusing capacity as a clinical marker of pulmonary function in health and disease (Hegewald, 2009; Nambu *et al.*, 2015), as well as for the determination of systemic blood volume (Burge & Skinner, 1995; Schmidt & Prommer, 2005). As an alternative to inhalation of gaseous CO, CO can be administered in biological systems via

the use of carbon monoxide-releasing molecules (CORM), stable in solution and capable of releasing CO under biological conditions (Cebová *et al.*, 2016). These molecules may be administered intravenously, evoking dose-dependent increases in COHb and following similar mechanisms of diffusion and excretion to inhaled CO.

CO binding to hemoglobin to form COHb is analogous to the formation of oxyhemoglobin via oxygen binding, although the binding affinity of carbon monoxide is approximately 200 times that of oxygen (Sladen, 1981). As the affinity of hemoglobin for substrates increases with the degree of saturation of its available binding sites, the partial saturation of hemoglobin with carbon monoxide results in marked increases in hemoglobin affinity for oxygen as well, causing a leftward shift of the oxygen-hemoglobin dissociation curve (**Figure 3**) (Okada *et al.*, 1976; Sladen, 1981). Thus, CO may limit oxygen delivery to tissue by two mechanisms: (1) partial, yet highly stable saturation of hemoglobin by CO increases its affinity for oxygen, limiting oxygen extraction by tissue by binding to hemoglobin, and (2) occupation of available binding sites for oxygen during pulmonary gas exchange, reducing oxygen saturation of the blood proportionally to the level of CO present (Sladen, 1981).

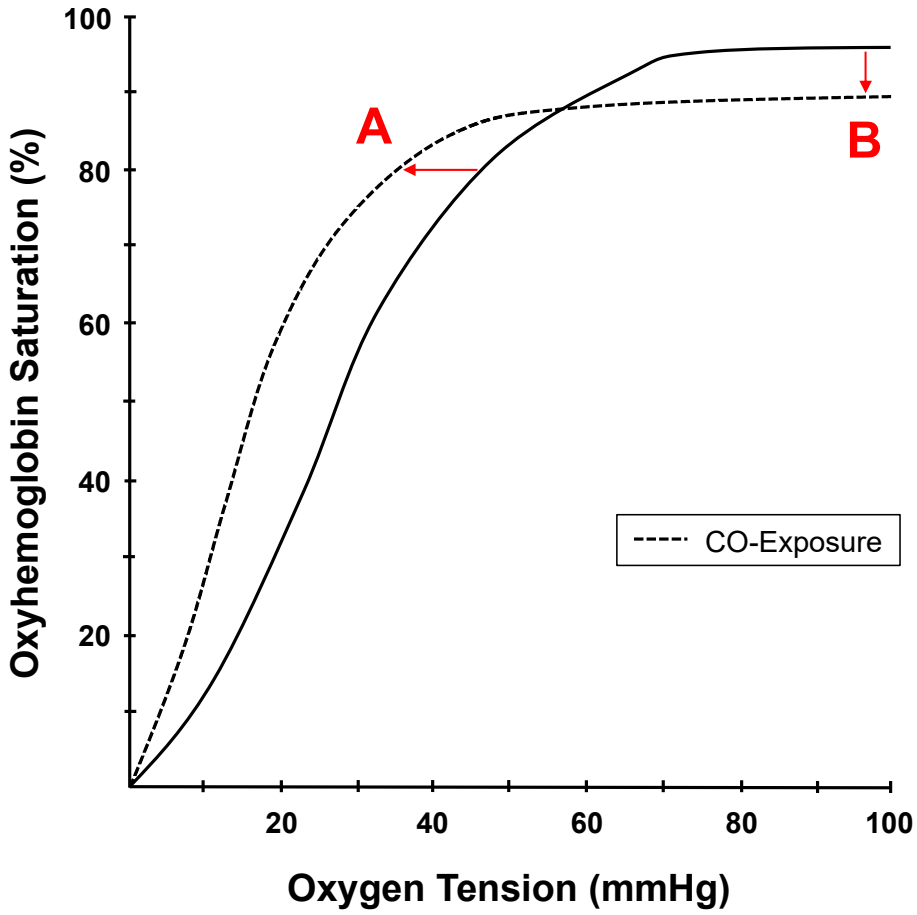

Figure 3. Oxyhemoglobin dissociation curve shifts with carbon monoxide. Solid curve represents the affinity of hemoglobin for oxygen in the absence of carboxyhemoglobin, increasing logarithmically with ambient oxygen tension as binding site saturation increases, augmenting hemoglobin affinity. Hatched curve represents hemoglobin affinity relationships with carbon monoxide (CO) exposure. Increasing carboxyhemoglobin results in progressive leftward shifts (A), indicating increased affinity of hemoglobin for substrates at progressively lower oxygen tensions, such as those at the level of tissue where oxygen extraction occurs. Displacement of oxygen via more stable carboxyhemoglobin formation reduces the maximal oxygen carrying capacity of hemoglobin proportionally to the level of carboxyhemoglobin present (B).

At rest, circulating COHb saturation is approximately 0.5-1.5% (Goldsmith & Landaw, 1968; Vreman *et al.*, 2006). Environmental exposure, such as inhalation of ambient pollution or cigarette smoke, elevates COHb modestly, with saturations of ~6% observed in habitual smokers (Goldsmith & Landaw, 1968). CO toxicity is a product of both severe restriction of oxygen delivery, as well as direct CO-mediated inhibition of aerobic metabolism and initiation of pro-inflammatory cascades (Palmeri & Gupta, 2021). Thus, COHb saturation is an incomplete marker of carbon monoxide toxicity. Notwithstanding, symptomology of acute CO toxicity is typically observed above approximately 20% COHb saturation, including headaches, dizziness and nausea, and becomes lethal above approximately 50% saturation (Vreman *et al.*, 2006; Palmeri & Gupta, 2021). Washout of COHb following acute exposure is biphasic, with an initial rapid washout period as CO simultaneously diffuses out of the body via the pulmonary system and into tissue to accumulate, followed by a more gradual washout back to baseline as tissue CO equilibrates back to homeostatic levels (Bruce & Bruce, 2006). The half-times of these two phases vary considerably between individuals, but typically reside between 200-300 minutes for the first phase, and 250-400 minutes for the second phase (Bruce & Bruce, 2006).

2.4 Vascular control by carbon monoxide

Heme oxygenase is expressed in the vascular smooth muscle (Christodoulides *et al.*, 1995) and endothelium (Zakhary *et al.*, 1996; Sammut *et al.*, 1998), implicating a role of the heme-oxygenase/CO axis in the regulation of vascular tone. The effects of CO within the vasculature may be examined through loss-of-function approaches involving inhibition of endogenous CO production via the inhibition of heme oxygenase, or through gain-of-function approaches involving administration of exogenous CO. The former aims to illustrate the role of CO as an endogenous signaling molecule in vascular control under standard physiological conditions. The breakdown of heme by heme oxygenase also produces biliverdin and iron; although, as these molecules have not been observed to exert significant vasoactive effects, inhibition of heme oxygenase may be used as an effective knock-out model of endogenous CO. However,

due to the integrative nature of vascular control and the numerous interactions and redundancies with different signaling mechanisms (such as NO), this knock-out approach may fall short in highlighting the direct effects of CO. The latter approach, elevating CO concentrations through exogenous administration, is more effective to tease out specific effects of the gasotransmitter, albeit with the limitation that the effects observed are a product of supra-physiological concentrations.

2.4.1 Carbon monoxide-mediated vasodilation

As a vascular signaling molecule, CO is primarily recognized for its vasorelaxant properties. In isolated rat gracilis muscle artery preparations, inhibition of heme oxygenase (*i.e.*, inhibition of the effects of endogenous CO) resulted in vasoconstriction, and was reversed upon exogenous CO administration, implicating a functional role of CO-mediated vasodilation at rest (Kozma *et al.*, 1999). Induction of CO production has also been implicated in the rescue of vasodilatory function when the effects of NO have been inhibited. Following inhibition of vasodilatory NO via infusion of nitric oxide synthase inhibitors or NO scavenging molecules, an up-regulation of the inducible HO-1 isoform was associated with compensatory recovery of vasodilation: an effect which was abolished via pharmacological inhibition of HO-1 activity (Motterlini *et al.*, 1998). Administration of exogenous CO via infusion of CORMs produces similar vasodilatory effects (Lamon *et al.*, 2009; Failli *et al.*, 2012). Interestingly, the investigation by Lamon et al. (2009) produced conflicting results, observing a vasoconstrictor effect of CO as a result of CO-mediated superoxide production. This constrictor effect is abolished with sufficient antioxidant activity, such as from co-produced biliverdin, allowing the HO/CO axis to self-regulate the effects of increased oxidative stress (Lamon *et al.*, 2009). Despite these potentially conflicting effects of CO, a review of the influence of exogenous CO on vascular tone found that net vasodilation was predominantly observed in muscular, aortic, coronary, hepatic, mesenteric, cerebral, pulmonary, and renal arteries (Ndisang *et al.*, 2004). This suggests that the vasoconstrictor effects of CO-mediated oxidative stress are well controlled in most conditions, and exert a minimal impact on the overall effects of CO on vascular tone.

Akin to its close analog NO, CO exerts its vasodilatory influence primarily through sGC, promoting myosin dephosphorylation and activating BKca in the vascular smooth muscle membrane, hyperpolarizing the cell and closing voltage-gated calcium channels (Wang *et al.*, 1997*b*, 1997*a*). However, these similar gasotransmitters differ considerably in their efficacy in these roles, with NO demonstrating a thousandfold greater potency for sGC activation (Furchgott & Jothianahdan, 1991). Yet, biological NO is highly unstable and short-lived, whereas CO is more stable and is thereby capable of exerting its effects over a longer period, emphasizing the divergent roles of these two signaling molecules.

2.5 Insights from hypoxia

2.5.1 Oxygen kinetics and delivery

Oxygen transport through the cardiorespiratory system is dependent on the movement of oxygen along its partial pressure gradient. Starting at its relatively high partial pressure in atmospheric air, oxygen diffuses along its pressure gradient into the bloodstream through pulmonary gas exchange in the alveoli, where it then binds to hemoglobin in the blood to then be circulated through the systemic circulation and ultimately delivered to tissue, where its partial pressure is lowest. This oxygen pressure cascade is depicted in **Figure 4**. A reduction in the partial pressure of oxygen (PO_2; *i.e.*, hypoxia) at the higher levels of this cascade reduces diffusion and transport of oxygen at each subsequent level, ultimately resulting in a reduction of oxygen delivery to tissue. The peripheral vasculature responds to hypoxia by vasodilating, increasing blood flow to tissue to compensate for the reduced oxygen content of the blood to maintain consistent oxygen delivery. The complete mechanism of this hypoxic vasodilation is unclear, but combined vasodilatory actions of NO and PGs appear to mediate this response entirely in resting tissue (Markwald *et al.*, 2011).

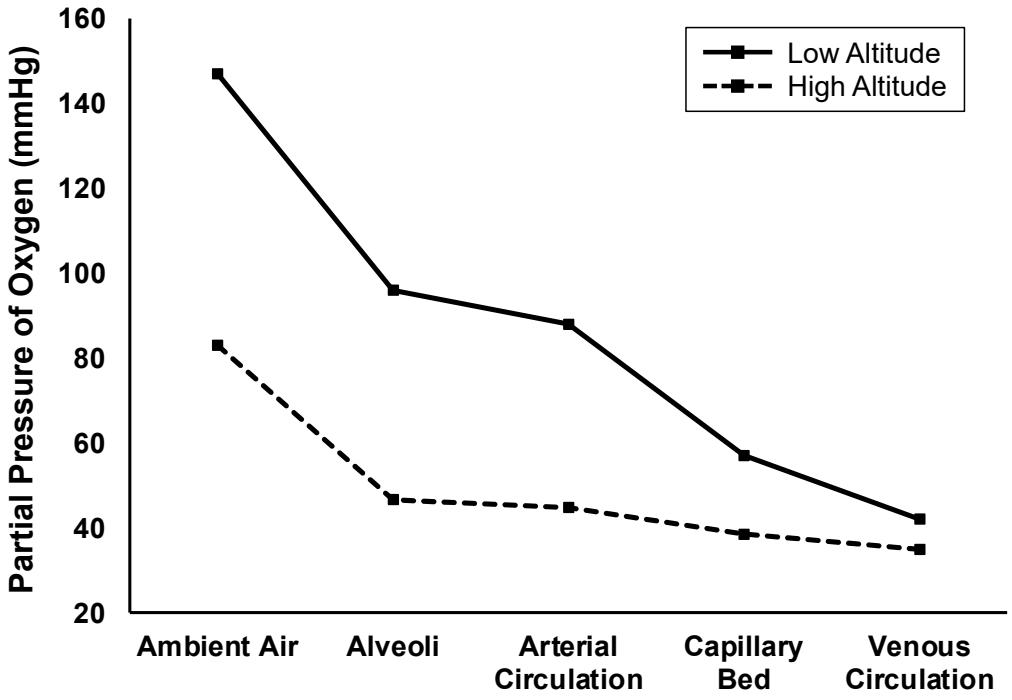

Figure 4. Incremental oxygen tensions along the cardiopulmonary system. Oxygen moves through the systemic circulation along its partial pressure gradient, from the relatively high pressures of atmospheric air to the low pressures within tissue and ultimately draining via the venous circulation. Reductions in the partial pressure of inspired oxygen such as with ascent to high altitude (hatched line) reduce each step of this cascade, reducing oxygen tension at the level of the capillaries. Figure based on the findings of Beall (2007).

2.5.2 Hypoxic exercise vasodilation

During exercise, the oxygen requirements of tissue are increased, theoretically creating a similar oxygen-mismatching situation to that of local hypoxia. Accordingly, exercise in hypoxia results in augmented hyperemic responses compared to normoxic exercise (Casey & Joyner, 2012). However, the same combined inhibition of NO and PGs that abolished hypoxic vasodilation in resting tissue does not fully account for this increased vasodilation during hypoxic exercise, implicating additional vasodilatory interactions between hypoxia and exercise at play (Crecelius *et al.*, 2011).

Several mechanisms have been proposed for this mystery vasodilatory candidate. While a reduction in arterial oxygen tension (PaO_2) is the prototypical definition of hypoxia, more precisely, local vascular responses to hypoxic exercise are governed by the degree of oxygen saturation of hemoglobin irrespective of PaO_2 (Roach *et al.*, 1999; González-Alonso *et al.*, 2006). Thus, the additional mechanism(s) involving in hypoxic exercise hyperemia must be sensitive to the oxygenation state of hemoglobin. The first and most widely appreciated of such mechanisms is ATP-mediated vasodilation. Upon binding to substrates (*e.g.*, oxygen loading during pulmonary gas exchange), hemoglobin undergoes a conformational shift, which is reversed upon substrate dissociation (*e.g.*, oxygen extraction by tissue) (Alpert *et al.*, 1974). This conformational shift triggers the release of ATP from erythrocytes into the intravascular space, where it binds to P2Y receptors on the vascular endothelium and triggers the release of NO, PGs, and EDHF (Dinenno, 2016; Ellsworth *et al.*, 2016). The degree of ATP release in this manner is directly proportional to the level of hemoglobin deoxygenation, effectively balancing vasodilatory simulation against oxygen demand (Sprague & Ellsworth, 2012). However, due to an unavailability of selective P2Y antagonists, whether intravascular ATP is obligatory for hypoxic exercise hyperemia in humans has yet to be determined. Adenosine, formed through the catabolism of intravascular ATP and binding to receptors to activate adenylyl cyclase has also been suggested as a mechanism of hypoxic exercise hyperemia (Leuenberger *et al.*, 1999), although this mechanism is also not obligatory (Casey *et al.*, 2009).

2.5.3 Carbon monoxide hypoxemia

By occupying available binding sites on hemoglobin and impairing oxygen dissociation, acute CO exposure elicits a hypoxic stimulus. The systemic effects of CO-mediated hypoxia bear considerable overlap with those of reduced inspired PO_2, such as in respiratory disease and ascent to high altitude, albeit with several notable exceptions. Reductions in PaO_2 stimulate peripheral chemoreceptor afferents in the carotid body, driving increases in efferent SNA and ventilation (Marshall, 1994). Challenges of (1) PaO_2 of ~40 mmHg, reducing oxygen saturation to ~80% and (2) acute CO exposure of ~20% COHb (also resulting in ~80% oxygen saturation) both increase efferent SNA by similar magnitudes (Hanada *et al.*, 2003). However, ventilation and heart rate are only increased with reductions in PaO_2; this suggests that the pressor effect of CO exposure is mediated by a non-chemoreflex pathway (Roughton & Darling, 1944; Hanada *et al.*, 2003). Lower level CO exposure of ~8% COHb fails to increase SNA, suggesting a requisite threshold level of COHb to elicit a pressor response (Hausberg & Somers, 1997).

The effects of CO-mediated hypoxia on vascular function are akin to those mediated by reductions in PaO_2. In both instances, the reduction in available oxygen to tissue necessitates compensatory measures to maintain adequate oxygen delivery for healthy tissue function, including increased volumetric blood flow to tissue. During single-leg knee extension exercise performed in (1) normoxia, (2) reduced inspired PO_2 (47 mmHg; ~80% oxyhemoglobin saturation), (3) competitive saturation with ~20% COHb in normoxia, and (4) ~20% COHb with hyperoxia (538 mmHg inspired PO_2), blood flow to the working muscle was augmented similarly in all three of the latter conditions (González-Alonso *et al.*, 2001). Notably, the degree of exercise vasodilation was tightly correlated to arterial oxyhemoglobin content rather than the PaO_2, supporting that augmented exercise vasodilation in hypoxia is mediated by oxyhemoglobin content, even when PaO_2 is greatly elevated. Additionally, oxygen extraction was slightly reduced with CO administration irrespective of PaO_2, potentially as a product of a leftward shift in oxyhemoglobin dissociation curve impairing oxygen release (González-Alonso *et al.*, 2001). A small sample of 2 participants underwent the same protocol following inhibition

of NO, and exhibited similar hyperemic responses; while these limited findings must be interpreted with caution, this aligns with later findings that NO and PGs are involved, but not obligatory for the augmented hyperemic response to exercise in hypoxia (González-Alonso *et al.*, 2001; Markwald *et al.*, 2011).

While an obligatory oxygen-sensor has yet to be characterized in the regulation of skeletal muscle blood flow, erythrocyte-mediated ATP release has received considerable attention for this capacity, with reductions in oxygenation correlating to ATP release into the intravascular space (Sprague & Ellsworth, 2012). Crucially, this process is disrupted in the presence of elevated COHb. CO administration significantly blunts ATP release in whole blood during deoxygenation in isolated conditions (Jagger *et al.*, 2001). These findings were later replicated *in vivo* from venous samples from exercising muscle in humans with acute CO exposure, whereas ATP release during exercise was well preserved during low-PO_2 hypoxia despite conspicuously similar hyperemic responses to exercise in both conditions (González-Alonso *et al.*, 2002). The precise mechanisms by which CO impairs ATP release from erythrocytes are unclear, but may be due to the increased stability of hemoglobin when partially bound by CO limiting deoxygenation-mediated conformational shifts requisite for ATP release, as well as impaired glycolytic metabolism within the erythrocyte itself (Jagger *et al.*, 2001; Tyunina & Artyukhov, 2018). These findings provide several key mechanistic insights: (1) Exercise hyperemia is augmented consistently during reductions in arterial oxygen content via CO exposure and reduced PaO_2, and (2) exercise hyperemia during CO exposure is mediated by ATP- and NO-independent mechanisms.

2.6 Vascular reactivity with carbon monoxide

Beyond its ability to mediate vasodilation, CO has also demonstrated capacity to mitigate adrenergic vasoconstrictor signaling. Inhibition of endogenous CO release increased vasoconstrictor responses to the α_1 adrenergic agonist phenylephrine in arterial segments of some, but not all vascular beds in rats, with mixed results between gastrocnemius and gracilis artery preparations (Kaide *et al.*,

2001; Koçer *et al.*, 2018). Similarly, up-regulation of HO-1 activity and CO production in isolated rat aortas was associated with depressed phenylephrine-mediated contractility (Sammut *et al.*, 1998); however, other reports found that this sympatholytic effect was only observed following up-regulation of HO-1 as a product of chronic hypoxia, and not in control tissue with normal HO-1 expression (Caudill *et al.*, 1998; Gonzales & Walker, 2002). Whether this is unique to adrenergic constriction is uncertain, with similar anti-constrictor responses observed in response to phenylephrine as well as U-46619, a non-adrenergic vasoconstrictor which directly invokes intracellular calcium release in the vascular smooth muscle (Wang *et al.*, 1997*a*).

Exposure to acute hypoxia does not attenuate adrenergic constriction at rest (Dinenno *et al.*, 2003) or during handgrip exercise (Wilkins *et al.*, 2006). However, given the discrepancies in mechanistic underpinnings of vasodilation between CO exposure and hypoxia such as inhibition of sympatholytic ATP (González-Alonso *et al.*, 2001, 2002; Kirby *et al.*, 2008), it is unclear whether these previous results are generalizable to CO-mediated hypoxia. The basic signaling cascades altered with CO are illustrated in **Figure 5**.

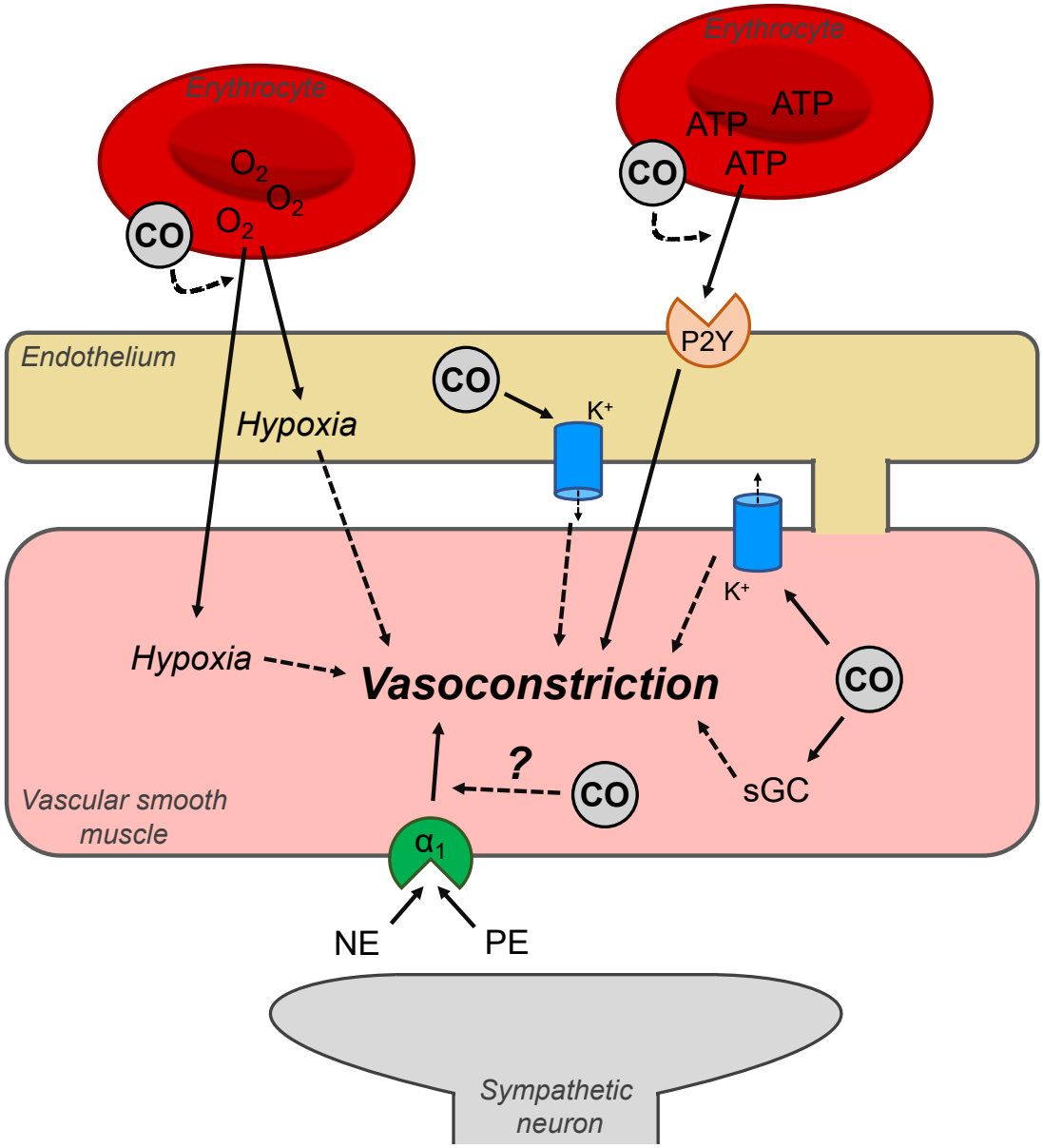

Figure 5. Vascular signaling with carbon monoxide. Carbon monoxide (CO) alters vascular signaling by restricting oxygen delivery to tissue, creating a hypoxic environment, and acting through direct signaling mechanisms. These include inhibition of ATP release from erythrocytes, mitigating the vasorelaxant effects typically exerted by ATP, as well as activation of large conductance calcium-activated K^+ channels and soluble guanylyl cyclase (sGC), promoting vasodilation. Whether CO exerts direct inhibitory effects on α_1 adrenoreceptor signaling cascades following agonist activation, such as via norepinephrine (NE) or phenylephrine (PE), is unclear.

2.7 Summary

Arterial vascular smooth muscle integrates responds to an array of vasodilatory and vasoconstrictor signals to modulate blood flow and match convective oxygen delivery to metabolic demand (Laughlin & Korzick, 2001). Efferent SNA stimulates vasoconstrictor signaling to restrain blood flow at rest, but this effect is blunted during exercise by local mechanisms within the working muscle (Remensnyder *et al.*, 1962; Shoemaker *et al.*, 2015). Signaling cascades of CO, predominantly favouring vasodilation, are involved in the regulation of vascular tone at rest (Ndisang *et al.*, 2004). Additionally, CO exposure augments the hyperemic response to exercise in a manner similar to hypoxia, but with key mechanistic differences that are independent of ATP and NO, suggesting additional mechanisms at play (González-Alonso *et al.*, 2001, 2002). Evidence from isolated models suggests a role for CO in the inhibition of sympathetically mediated vasoconstriction (Sammut *et al.*, 1998; Caudill *et al.*, 1998; Kaide *et al.*, 2004; Koçer *et al.*, 2018). Whether elevated CO is capable of disrupting α_1 adrenergic vasoconstriction within resting and exercising muscle in intact humans is unknown.

The present study sought to determine the impact of moderately elevated CO, similar to levels experienced through environmental exposure and targeting COHb levels of ~5%, on the hemodynamic responses to exercise and α_1 adrenergic stimulation in humans. We hypothesize that CO will augment the vasodilatory response to exercise, and attenuate α_1 adrenergic vasoconstriction.

Chapter 3: Methods

3.1 Research Design

This research was conducted as part of a larger study investigating various aspects of vascular regulation following CO exposure. The methods and data listed herein encapsulate those relevant to the present research, and are not an exhaustive list of methods conducted in the study. Adequate washout periods were incorporated throughout the study to avoid confounding influences of other measures performed. This study abided by the Canadian Government Tri-council Policy on Research Ethics Policy Statement (TCPS2) and the declaration of Helsinki. Ethical approval was obtained in advanced through the University of Alberta Biomedical Research Ethics Board (Pro00096251; See appendix 1), and clinical trial approval for the use of pharmacological agents was obtained through Health Canada (HC6-24-c241154; See appendix 2). Participants were briefed with in-depth study information and provided written consent prior to enrolment (see Appendix 3).

Participants included were between the ages of 18-50 years, with a body mass-index of <30 kg/m^2. Participants were recruited by word of mouth. Participants completed a health history questionnaire to screen for pre-existing cardiovascular, respiratory, nervous system, or metabolic diseases, and participants who were regular smokers, taking monoamine (MAO) inhibitors, or tricyclic antidepressants, or who were pregnant or post-menopausal were excluded from the study, as these are known confounding factors to the outcomes of this study. A total of 19 participants were included in this study (10 females, 9 males; age, 24 ± 3 years; height, 1.73 ± 0.08 m; weight, 69.4 ± 11.8 kg; body mass index, 23.1 ± 2.8 kg/m^2). Participants were tested on two consecutive days, at the same time of day. Female participants were all pre-menopausal, testing was not standardized by phase of menstrual cycle, and 7 females were taking hormonal contraceptives (Lo Loestrin, n = 2; Kyleena, n = 1; Mirena, n = 3; Marvelon 21, n = 1). All participants were moderately active (*i.e.,* not sedentary), and none were self-described elite athletes.

3.2 Experimental overview

Participants were tested in the supine position. Prior to testing, participants were fasted for at least 2 hours, abstained from alcohol and caffeine for at least 12 hours, and from vigorous physical exercise for at least 24 hours. Testing was conducted in a temperature-controlled room at a consistent 22°C.

Figure 6 depicts a schematic representation of the experimental protocol for each testing day, each consisting of two identical experimental trials. Fasted blood samples were collected via an intravenous catheter (IV) into heparinized vacutainer tubes and were analyzed immediately for the determination of COHb. As part of a separate research hypothesis, a flow-mediated dilation assessment was performed on the participant's right arm first. This procedure involved temporary forearm blood flow occlusion, measuring the return to flow following occlusion release as a measure of endothelium-mediated vasodilation. This assessment took approximately 15 minutes, after which a break was taken to adjust and re-calibrate equipment. This intermission ensured that sufficient washout time was given to avoid confounding influence of the post-occlusion response on subsequent vascular reactivity (Harris *et al.*, 2010). Commencing protocols of the present study, resting blood pressure measurements were obtained, after which the participants maximum voluntary contraction (MVC) force was determined for the standardization of exercise intensity, and at least 5 minutes were given to allow the participant to return to a quiet resting state. Heart rate, blood pressure, forearm blood flow (FBF) and forearm vascular conductance (FVC) were measured continuously throughout the remainder of the trial. A 2-minute resting baseline period was recorded, after which participants began rhythmic handgrip exercise. After 3 minutes of exercise to allow forearm vasodilation to reach steady-state hyperemia and match blood flow to the increased metabolic demand of the working muscle, an investigator rapidly infused a bolus dose of phenylephrine, an α_1 adrenergic agonist, while the participant continued to exercise. The resulting vascular responses were measured over the following 2 minutes, and the trial was concluded.

After the first experimental trial, the participant was given 10 minutes to stand up and walk. Participants were reinstrumented, and the rebreathe procedure was conducted with either CO or room air

(sham). After 10 minutes to allow COHb to stabilize, the second experimental trial was then conducted identically to the first, and the testing day was concluded. The second testing day was conducted identically to the first day, but with the alternate rebreathe condition. The order of experimental conditions were randomized, following an order created by a random number generating software.

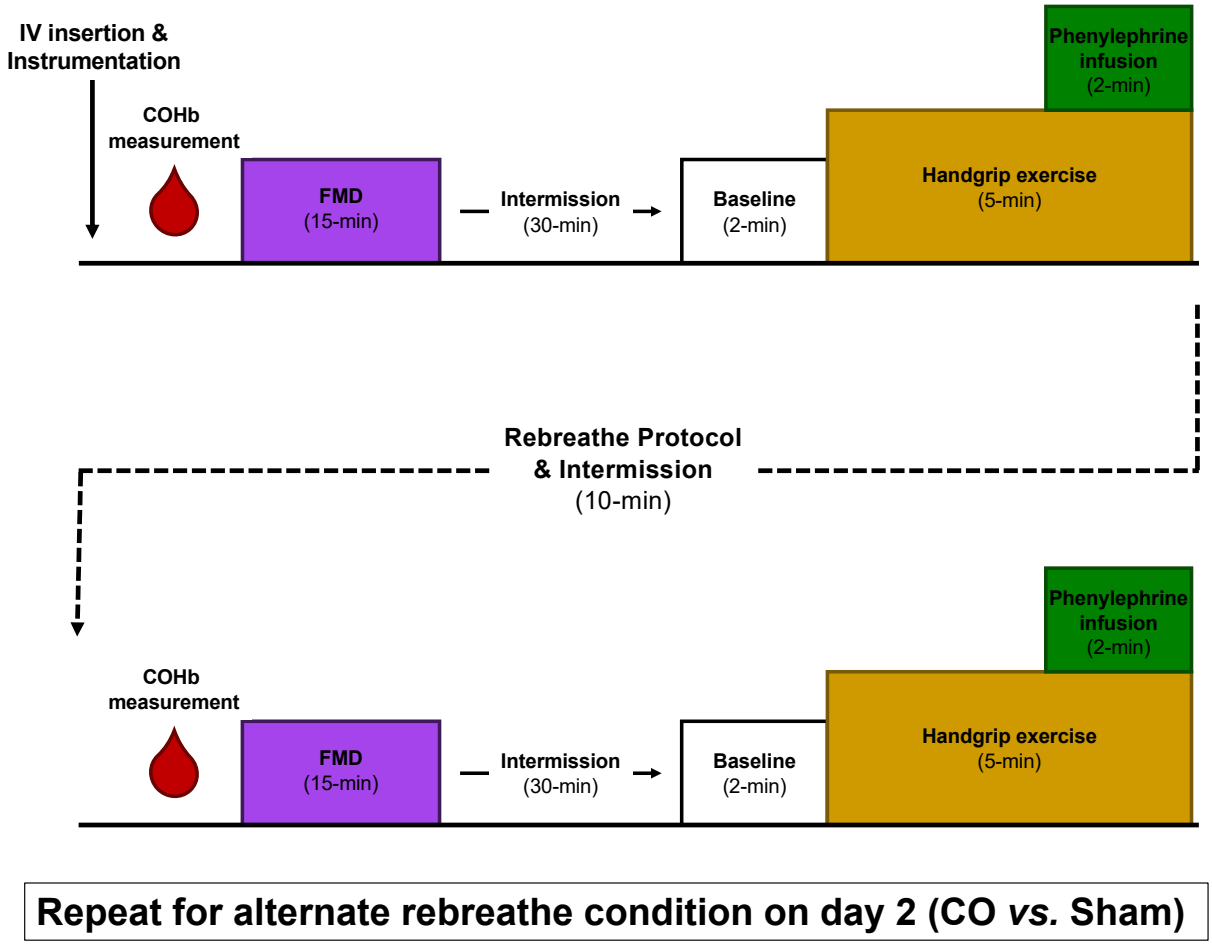

Figure 6. Protocol schematic. Following initial intravenous catheter (IV) insertion and instrumentation, resting venous blood samples were taken for the immediate determination of carboxyhemoglobin (COHb). The flow-mediated dilation (FMD) assessment was conducted first, followed by a 30-minute intermission to adjust and recalibrate instrumentation for the handgrip procedure, as well as providing adequate washout time following the FMD. The handgrip procedure consisted of a 2-minute baseline period, followed by 5 minutes of rhythmic handgrip exercise at 15% of participants' maximal voluntary contraction. Phenylephrine was infused in the final 2 minutes of handgrip exercise, and the first trial was complete. The rebreathing procedure was conducted with either carbon monoxide (CO) or room air (sham), and the procedures were repeated for a second post-rebreathe trial. Participants returned the second testing day and repeated the full protocol, receiving the alternate rebreathing condition.

3.3 Instrumentation and protocols

3.3.1 Venous catheterization, blood gas sampling and vasoactive drug infusion

A trained phlebotomist inserted a 22-gauge IV (BD, USA) into the antecubital vein of one arm for blood sampling and pharmacological infusions. Blood samples were collected using a multi-sampler into heparinized vacutainer tubes (**Figure 7)**, and were immediately processed using an automated blood gas analyzer for the determination of COHb (ABL-80 FLEX blood gas analyzer; Radiometer, Mississauga, ON, Canada). Dr. Craig Steinback, Dr. Michael Tymko, Emily Vanden Berg, and Andrew Steele assisted with IV placement and pharmacological infusions.

To assess the sensitivity of α_1 adrenoreceptor-mediated vasoconstriction, a bolus dose of the selective α_1 adrenergic agonist phenylephrine hydrochloride was injected intravenously (**Figure 6)**, followed immediately by 2ml of saline solution to ensure that the full dose was cleared from the line and administered. Bolus intravenous phenylephrine infusions have been utilized extensively in this manner to elicit a dose-dependent rise in mean arterial pressure for the assessment of baroreflex sensitivity by our lab and others (Rudas *et al.*, 1999; Simpson *et al.*, 2019), and recently within our lab for the specific assessment of α_1 adrenoreceptor reactivity within the spleen (Purdy *et al.*, 2019) and lower limb (*unpublished*). The dosage was standardized at 60 μg per litre estimated blood volume (BV), determined using the Nadler formula which incorporates the participant's height (H), body mass (W) and sex (**Equation 4)** (Sharma & Sharma, 2021).

Equation 4. Nadler formulae:

$$Males: BV = (0.3669 \times H\text{^}3) + (0.03219 \times W) + 0.6041$$

$$Females: BV = (0.3561 \times H\text{^}3) + (0.03308 \times W) + 0.1833$$

This dosage was chosen to elicit a ~20-30 mmHg increase in MAP, providing an observable and reproducible effect against which to detect potential differences in α_1 vasoconstrictor sensitivity. This

infusion protocol produces a dynamic systemic vascular response; representative traces of this response in the systemic arterial circulation and in exercising skeletal muscle vasculature in the two minutes following bolus phenylephrine infusion are depicted in **Figure 8**.

Previous observations from our lab have demonstrated that as the infusion disperses throughout the systemic circulation, an initial peak vasoconstrictor response is typically observed within the first ~30 seconds, and is then briefly restrained by robust baroreflex-mediated sympathetic withdrawal. Vascular tone then continues to increase to a second peak, before gradually returning to baseline as phenylephrine is catabolized. The precise timing of these peaks vary slightly between vascular beds as phenylephrine disperses throughout the systemic circulation. Pilot work and previous studies from our lab have indicated that the initial peak and the later stabilized linear decline of the vasoconstrictor response are reliably reproducible phenomena (Purdy *et al.*, 2019; Simpson *et al.*, 2019). Thus, (1) the magnitude and timing of the initial peak response within the first ~60 seconds, prior to significant baroreflex restraint, and (2) the late response between 90- and 120-seconds post-infusion, following baroreflex stabilization, can be used as indices of α_1 adrenoreceptor sensitivity.

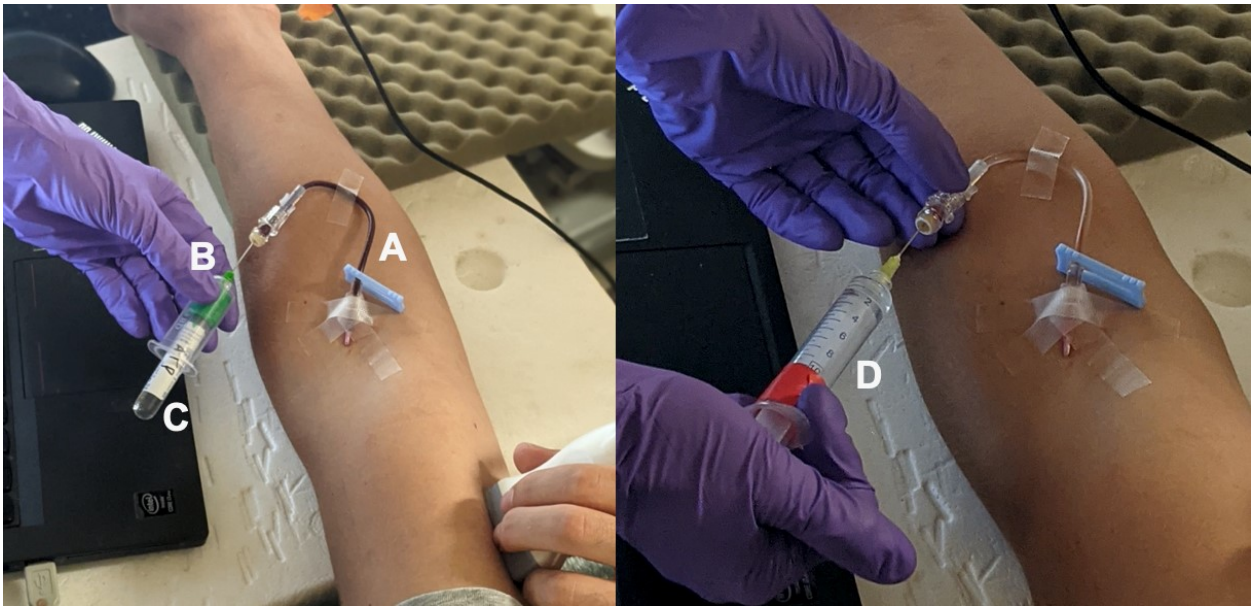

Figure 7. Intravenous catheter setup. Venous blood samples were collected via an intravenous catheter (A) using a multi-sampler (B) into heparinized vacutainer tubes (C) (Left). Phenylephrine was pre-loaded into sterile syringes (D), dosage determined accordingly using the Nadler formula, and infused via the intravenous catheter (Right).

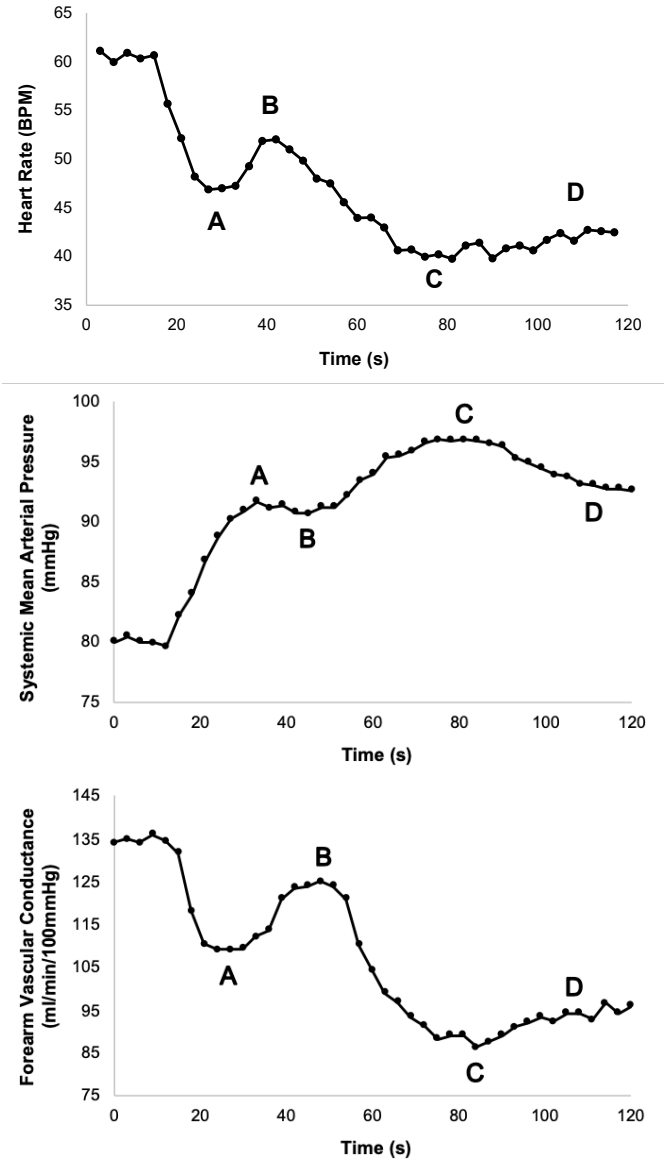

Figure 8. Representative systemic and local vascular responses to bolus infusion of phenylephrine. Heart rate (top), mean arterial pressure (middle) and vascular conductance in exercising forearm tissue (bottom) responses over 2 minutes following phenylephrine infusion at T = 0s. Stimulation of α_1 adrenoreceptors increases systemic blood pressure and decreases vascular conductance, with concomitant baroreflex-mediated decreases in heart rate. Typical responses exhibit (A) an initial "peak" constrictor response, (B) transient baroreflex-mediated blunting of the response, (C) further vasoconstriction to a secondary "peak", and (D) response stabilization and a gradual return to homeostatic levels.

3.3.2 Rhythmic handgrip exercise

Handgrip exercise was performed using a handheld force dynamometer (MLT004/ST; ADInstruments) in the left arm for all participants on account of practical limitations of the experimental setup. The left arm will henceforth be referred to as the exercising arm, and the right (resting) arm as the control arm. Participants' MVC force was determined prior to each trial as the highest of three maximal effort contractions, each effort separated by at least one minute. Rhythmic handgrip exercise was performed at a 1:2 duty cycle, involving 1 second of contraction at 15% of their determined MVC followed by 2 seconds of relaxation, timed to a metronome. Participants received immediate visual feedback for grip force and were coached throughout the protocol to ensure consistent handgrip intensity. This exercise intensity has been shown to invoke a moderate hyperemic response in the forearm vasculature with minimal effects on systemic hemodynamics and sympathoexcitation, as well as invoking a moderate (but incomplete) sympatholytic effect within the exercising muscle (Wilkins *et al.*, 2006; Hearon *et al.*, 2016, 2020). Moderate hyperemic and sympatholytic effects were pursued for this study to provide a response that could be measurably augmented or inhibited by the intervention.

3.3.3 Cardiac and vascular measures

Heart rate (HR) (Electrocardiogram, lead II configuration) and mean arterial pressure (NIBP; ADInstruments, Colorado Springs, CO, USA) were collected continuously at 1 KHz (LabChart Pro v8.1.17). Resting blood pressure was obtained during quiet rest using an automatic sphygmomanometer (model BP786n; Omron, San Ramon, CA, USA), with an average of three readings taken at least one minute apart, and were used to calibrate continuous blood pressure measurements.

Common brachial artery diameter and blood flow velocity Doppler signal were measured using a 12 MHz linear probe for the exercising arm (Vivid Q, General Electric, Milwaukee, WI, USA), and using an 8MHz linear probe for the control arm (Vivid 7, General Electric, Milwaukee, WI, USA). Nicholas Cheung assisted with vascular sonography of the control arm. The probes were held manually over the brachial arteries just proximal to the elbow, and positions were marked to ensure the same section of the

artery was insonated on subsequent trials/days. Blood flow velocity was measured with an insonation angle of 60°. Sample ultrasound images are depicted in **Figure 9**. Brachial artery diameters were analyzed using automated edge-detection software (Brachial Analyzer; Medical Imaging Applications LLC, IA, USA). Mean blood flow velocities (MBV) were analyzed for the exercising arm using a validated open-source spectral analysis software (Coolbaugh *et al.*, 2016), and for the control arm using a Doppler audio translator (Herr *et al.*, 2010). Experimental conditions (CO *vs.* sham) were blinded during analyses of brachial artery diameter and flow velocity. Different velocity analysis modalities were used for each arm on account of equipment constraints. Ultrasound analysis was performed blinded to the experimental conditions (CO *vs.* sham) and the sex of the participants. The present analysis sought to compare vascular responses within each arm across time, rather than between arms, limiting the confounding influence of potential variability between the MBV determination modalities. FBF and FVC were calculated as per **Equation 5** and **Equation 6** and expressed in units of ml min^{-1} and ml min^{-1} $(100\ mmHg)^{-1}$ respectively.

Equation 5. Forearm blood flow:

$$FBF = MBV \times \pi \times radius^2 \times 60s$$

Equation 6. Forearm vascular conductance:

$$FVC = \frac{FBF}{MAP}$$

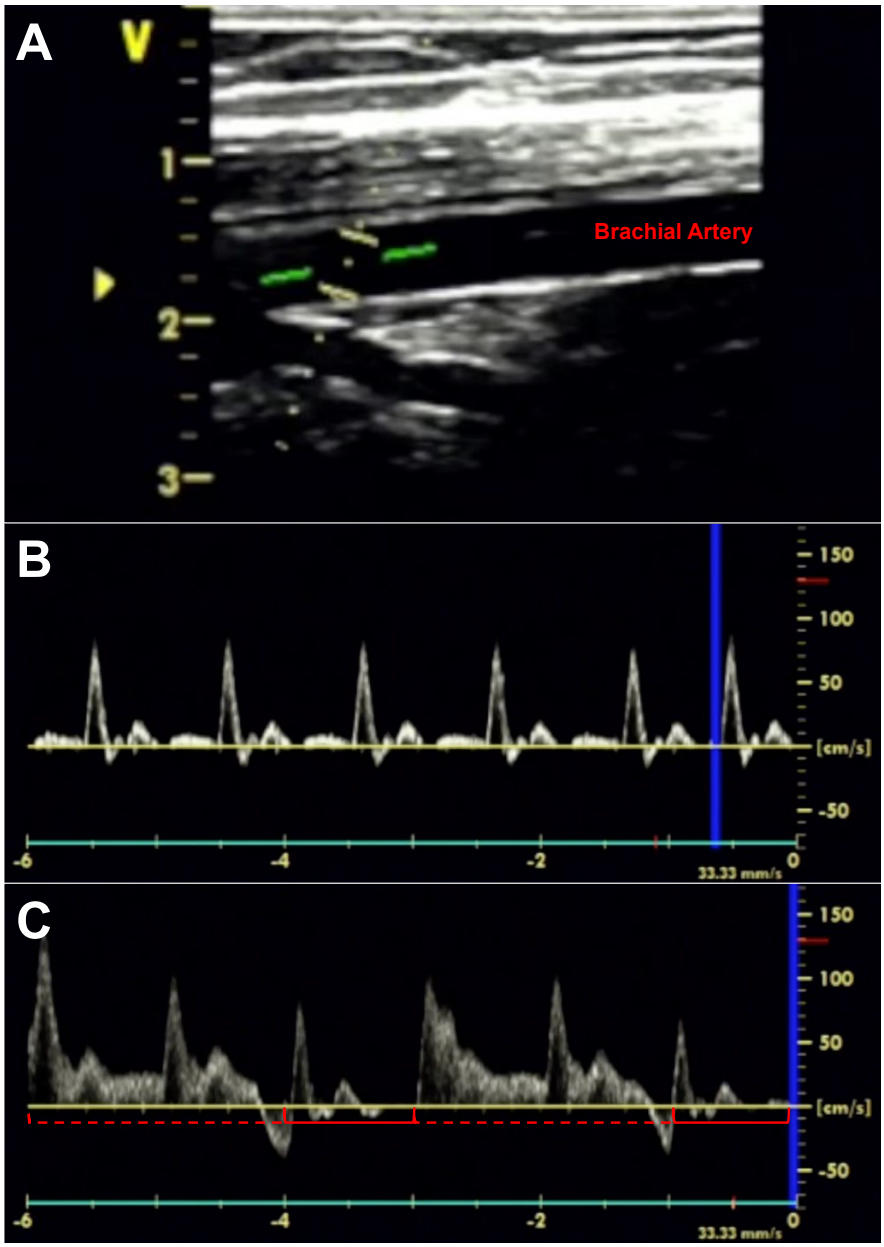

Figure 9. Brachial artery ultrasound images and Doppler velocity signals. B-mode image highlighting a longitudinal section of the brachial artery, as utilized for diameter analysis (A), with associated Doppler flow velocity signals over 6-second periods during quiet rest (B) and rhythmic handgrip exercise (C). Rhythmic handgrip exercise produces epochs of low flow as increased intramuscular pressure compresses the vasculature during muscular contractions (solid red brackets) between epochs of high flow during relaxation intervals (hatched red brackets).

3.3.4 Carbon monoxide administration

CO was administered as an inhaled bolus via a custom closed-circuit rebreathing apparatus (**Figure 10)**. The apparatus was comprised of a mouthpiece and filter, connected to a 3-litre balloon containing 100% oxygen by a three-way valve to swap the participant between breathing room air (open-circuit) and breathing from the balloon (closed circuit), and a valve-sealed port and line for the injection of either CO or room air (sham). Testing days were randomized and participants were blinded to conditions on each testing day. Investigators were not blinded to the CO condition for participant safety. A nose clip was used to ensure the participant did not breath ambient room air, and soda lime was added to the filter to scrub accumulating carbon dioxide. After breathing for several moments on the apparatus to acclimate to tidal breathing on the mouthpiece, participants were instructed to hold momentarily at the end of a normal expiration, at which point the three-way valve was swapped to the closed-circuit loop. Pausing breathing momentarily in this manner avoided mixing of room air with the 100% oxygen in the closed circuit, also avoiding overfilling the rebreathing balloon during expiration. After several breaths to ensure the circuit was sealed and the participant was breathing regularly from the 3-litre balloon, the participant was instructed once again to hold momentarily at the end of a normal expiration, the CO/room air bolus was rapidly administered into the circuit, and the participant then inhaled maximally and held an end-inspiratory apnea for 10 seconds. After breaking the breath-hold, the participant breathed normally on the closed circuit for an additional 2 minutes to allow for complete uptake of CO into the systemic circulation. This procedure has been validated previously for rapid and consistent administration of CO, and has been detailed elsewhere (Schmidt & Prommer, 2005). The dosage administered was standardized to the participant's body mass at a dosage of 1 ml CO per kg body mass, targeting COHb levels of ~5-6%.

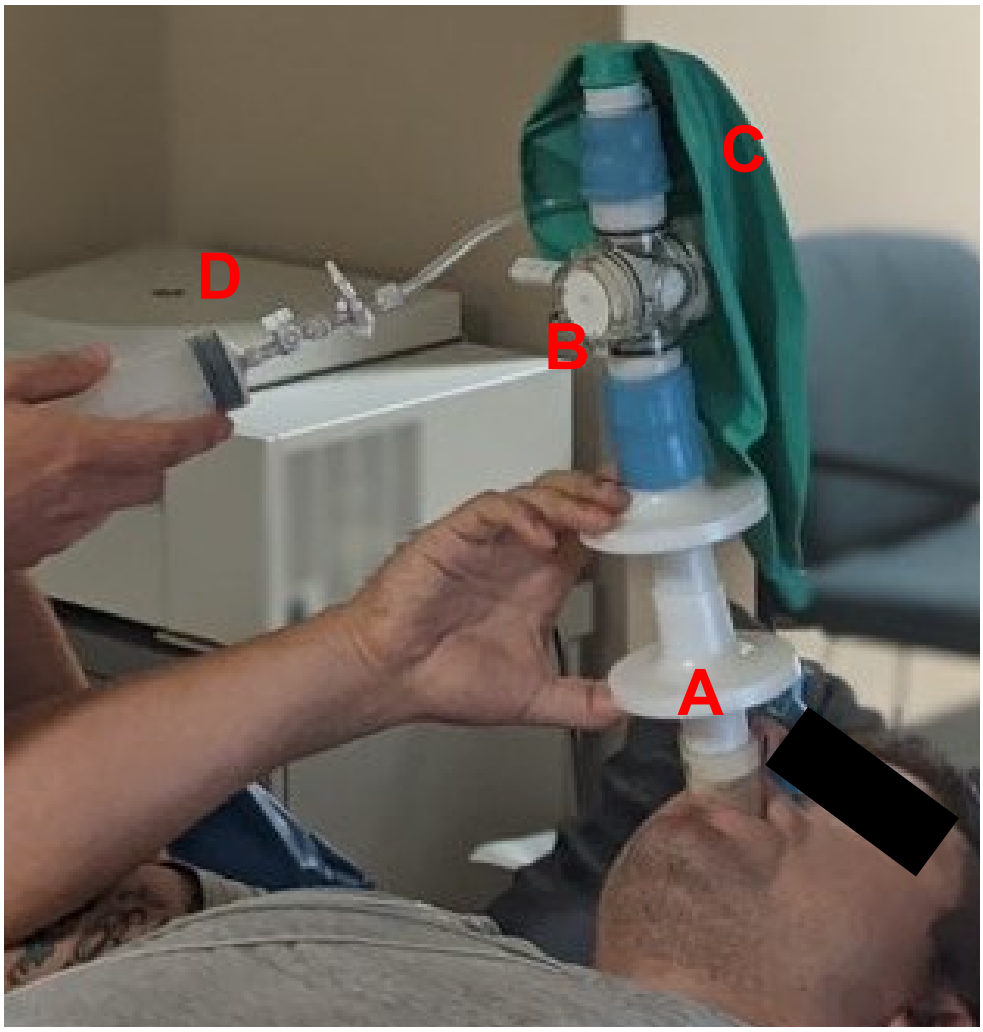

Figure 10. Rebreathing apparatus. Participants breathe through the circuit via a mouthpiece and filter (A) containing soda lime to scrub accumulating carbon dioxide. A three-way valve (B) alternates between open-circuit (breathing room air) and closed-circuit configurations (breathing from the 3-litre balloon containing 100% oxygen; C). Carbon monoxide or room air (sham), pre-measured into a 100ml syringe and connected via a valve-locked line (D), is introduced to the system immediately distal to the three-way valve on the apparatus.

3.4 Analysis of cardiovascular outcomes

Heart rate, blood pressure, FBF and FVC were averaged from thirty second bins during baseline and the final minute of steady-state handgrip exercise. 30-second bins were used to select periods with optimal ultrasound images within the 2-minute baseline period and the final minute of handgrip exercise. The initial peak vasoconstrictor response to phenylephrine infusion was determined as the point at which FVC was at its lowest within the first minute following infusion, or FBF if blood pressure measures were not available for calculation of FVC at that point due to technical issues. 3-second bins of heart rate, blood pressure, and FBF and FVC were measured from that time point, which will henceforth be referred to as the peak phenylephrine response. Averaging over 3-second bins normalized the dynamic hyperemic response to rhythmic handgrip exercise, whereby flow is restrained during the 1s contraction and augmented during the 2s relaxation. As the bolus infusion of phenylephrine disperses throughout the systemic circulation from the point of injection, different vascular beds may reach their respective peak vasoconstrictor responses asynchronously. As local forearm vascular reactivity was the primary outcome variable of interest in this study, timing of the nadir FVC (or FBF if FVC unavailable) within the first minute was used as the point of synchronization for the determination of HR, MAP, and FBF for this time point, rather than the respective "peak" responses in each of these parameters independently. This approach more precisely reflects the contributions of systemic hemodynamics on local vascular reactivity as the vasculature reaches its peak vasoconstrictor response. Moreover, it is unlikely that independent assessment of each variable would have yielded appreciably different values. Tracking the dynamic response to phenylephrine, HR, MAP, FBF and FVC were measured again as an average over the final 30 seconds of the recorded response (*i.e.*, between 90 and 120s post-infusion), which will henceforth be referred to as the late phenylephrine response. Whereas the peak response illustrates vascular reactivity prior to maximal baroreflex engagement, this second time point reflects vascular reactivity after baroreflex activation has stabilized against the dynamic vasoconstrictor stimulus.

To further investigate potential differences in vascular responses to handgrip exercise and phenylephrine infusion, differences in MAP, FBF and FVC were calculated in terms of relative (%) and absolute changes. Percent differences allow for a more nuanced interpretation of results when differences in individual baselines may be present; however, both values are reported for a more comprehensive analysis.

3.5 Statistical analysis

Venous COHb levels from initial resting samples and from ~10 minutes post-rebreathe for both experimental days were compared using a repeated measures two-factor analysis of variance (ANOVA) [Factor A: Condition (CO, SHAM); Factor B: Stage (pre-rebreathe, post-rebreathe)]. Absolute values for heart rate, mean arterial pressure, FBF and FVC were compared between resting baseline, steady-state handgrip exercise, peak phenylephrine response and late phenylephrine response using mixed methods repeated measures two-factor ANOVAs [Factor A: Condition (CO pre-rebreathe, CO Post-rebreathe, SHAM Pre-rebreathe, SHAM Post-rebreathe); Factor B: Time (baseline, handgrip exercise, peak phenylephrine response)]. The time elapsed following phenylephrine infusion to initial peak responses, as well as changes in heart rate, MAP, FBF and FVC between baseline and handgrip exercise, and between handgrip exercise and peak phenylephrine responses were also measured using mixed methods repeated measures two-factor ANOVAs [Factor A: Condition (CO, SHAM); Factor B: Stage (pre-rebreathe, post-rebreathe)]. Mixed methods tests were used in lieu of repeated measures tests to better handle patterns of missing data as a result of technical challenges during data acquisition. Tukey's post-hoc tests were conducted to test for pair-wise comparisons. Statistical analyses were conducted using Prism 9 (Graphpad Software, San Diego, CA, USA). All data in tables and figures were expressed as mean values ± SD. Significant differences were assumed at $P < 0.05$.

Chapter 4: Results

4.1 Hematology and hemodynamics – absolute values

Hematological data from venous blood samples are reported in **Table 1.** COHb was significantly elevated post-rebreathe on the CO intervention day compared to all other time points ($P < 0.0001$).

Hemodynamic data during resting baseline, steady-state handgrip exercise, and during peak and late phenylephrine responses are reported for each experimental trial (*i.e.,* pre-rebreathe *vs.* post-rebreathe) of both experimental days (*i.e.,* CO intervention *vs.* room air sham intervention) in **Table 2**. In some trials, technical issues and insufficient ultrasound image quality resulted in lost data points; the number of participants analyzed for each stage and condition are denoted in square brackets in the respective values.

No differences in the timing of the initial peak responses to phenylephrine were observed ($P = 0.7383$), occurring at 28 ± 8 and 26 ± 9 seconds post-infusion for the pre- and post-rebreathe conditions on the CO intervention day, and 29 ± 9 and 26 ± 10 seconds post-infusion for the pre- and post-rebreathe conditions on the sham day.

Due to an unexpected vasodilatory interaction in the control arm during the initial peak period following infusion, these responses were deemed unsuitable as indices of α_1 adrenergic sensitivity, and thus were not included in the analysis for this stage. **Figure 11** illustrates a comparison of simultaneous representative vascular responses over the 2 minutes following phenylephrine infusion between the exercising and control arms. As previously detailed, FVC in the exercising arm follows a dynamic response, declining to an initial nadir, which is then transiently attenuated by robust baroreflex activation before declining further to a second, lower nadir, before gradually ascending back towards pre-infusion levels. However, the response observed was markedly different in the control arms of participants, instead initially resembling an inversion of the exercising arm response. In the control arm, FVC began increasing promptly following infusion, pausing briefly near the time of the initial nadir in exercising arm FVC before continuing to rise to higher peak. From this point, the responses resynchronized, with control

arm FVC declining rapidly to fall below pre-infusion values. Thus, the initial rise in FVC in the control arm is not reflective of α_1 adrenergic vasoconstriction, likely instead to be the product of alternate interactions within the vasculature favouring vasodilation. However, as this unexpected effect appears to resolve in the second minute to emulate the reduced FVC values seen in the exercising arm at this time point, late phenylephrine responses between 90- and 120-seconds post-infusion were analyzed in the control arm for each condition.

Figure 12 illustrates hemodynamic responses during resting baseline, the final minute of steady-state handgrip exercise, and peak and late phenylephrine responses. Main effects of stage were observed for all parameters. Handgrip exercise resulted in a modest increase in heart rate, whereas mean arterial pressure and CTRL FBF/FVC were unchanged. This suggests that the selected exercise intensity may have evoked mild systemic sympathoexcitation, but was insufficient to increase peripheral vasoconstrictor signaling appreciably. EX FBF/FVC increased significantly with handgrip exercise, illustrating a robust reactive hyperemia response.

Rapid vasoconstriction following phenylephrine infusion caused an initial increase in mean arterial pressure and decrease in EX FBF and FVC from pre-infusion levels during the first peak response, with simultaneous reductions in heart rate due to baroreflex activation. These effects persisted through the dynamic 2-minute response to systemic phenylephrine infusion in heart rate and mean arterial pressure during all conditions, but persistent reductions in FBF and FVC in both arms failed to reach significance under some conditions. Specifically, sustained vasoconstriction during late phenylephrine responses reached significance only for EX FBF in the pre-rebreathe condition on the CO day, for EX FVC in both pre- and post-rebreathe conditions on the CO day in the pre-rebreathe condition on the sham day, and for CTRL FBF and FVC both pre- and post-rebreathe on the CO day. Importantly, no main effects of condition nor interaction effects were detected in any variables. As such, these varied results of post-hoc comparisons between conditions in FBF and FVC must be interpreted with caution.

Table 1: Hematological parameters

	CO		SHAM	
Parameter	Pre	Post	Pre	Post
Carboxyhemoglobin (%)	1.5 ± 0.3	**5.6 ± 1.2***	1.5 ± 0.2	1.4 ± 0.2
Oxyhemoglobin (%)	60.8 ± 17.2	57.6 ± 14	65.8 ± 16.7	61.9 ± 14.5
Deoxyhemoglobin (%)	36.9 ± 17.3	35.9 ± 13.4	31.8 ± 16.8	35.7 ± 14.6
Methemoglobin (%)	0.9 ± 0.2	0.9 ± 0.2	1 ± 0.2	1.1 ± 0.2
Total hemoglobin (g/dl)	13.4 ± 1.3	13.3 ± 1.3	13.5 ± 1.2	13.6 ± 1.2
PO_2 (mmHg)	36 ± 7	34 ± 8	41 ± 11	37 ± 8
PCO_2 (mmHg)	47 ± 4	45 ± 4	45.3 ± 5	44.4 ± 3
pH	7.4 ± 0.05	7.4 ± 0.04	7.4 ± 0.03	7.4 ± 0.03

Values are means ± standard deviations. * significance *vs.* control conditions ($P<0.05$).

Table 2: Forearm and systemic hemodynamic parameters

Parameter	Condition	Baseline	Handgrip exercise	Peak phenylephrine response	Late phenylephrine response
EX Brachial artery diameter (mm) [n]	CO Pre	3.69 ± 0.62 [19]	3.77 ± 0.68 [19]	3.86 ± 0.69 [19]	**3.37 ± 0.59 *** [19]
	CO Post	3.78 ± 0.64 [19]	3.78 ± 0.57 [19]	3.88 ± 0.55 [19]	**3.52 ± 0.47 *** [19]
	SHAM Pre	3.77 ± 0.7 [18]	3.75 ± 0.64 [18]	3.80 ± 0.58 [18]	**3.42 ± 0.57 *** [18]
	SHAM Post	3.74 ± 0.64 [19]	3.71 ± 0.63 [19]	3.77 ± 0.66 [19]	**3.38 ± 0.63 *** [19]
CTRL Brachial artery diameter (mm) [n]	CO Pre	3.97 ± 0.89 [15]	3.91 ± 0.87 [15]	---	**3.54 ± 0.82 *** [13]
	CO Post	4.00 ± 0.86 [13]	4.06 ± 0.92 [13]	---	**3.66 ± 0.9 *** [13]
	SHAM Pre	4.07 ± 0.94 [14]	4.00 ± 0.89 [14]	---	**3.68 ± 0.96 *** [12]
	SHAM Post	3.99 ± 0.79 [14]	3.93 ± 0.76 [14]	---	**3.61 ± 0.75 *** [12]
Heart rate (beats/min)	CO Pre	57 ± 10 [19]	**60 ± 10** † [19]	**47 ± 11 *** [17]	**42 ± 7 *** [16]
	CO Post	57 ± 10 [19]	**61 ± 10** † [19]	**47 ± 10 *** [17]	**42 ± 7 *** [15]
	SHAM Pre	57 ± 8 [19]	**62 ± 10** † [19]	**46 ± 12 *** [17]	**42 ± 7 *** [15]
	SHAM Post	57 ± 9 [19]	**61 ± 12** † [19]	**49 ± 12 *** [17]	**41 ± 7 *** [15]
Mean arterial pressure (mmHg) [n]	CO Pre	81 ± 9 [16]	82 ± 9 [16]	**95 ± 14 *** [13]	**97 ± 13 *** [13]
	CO Post	80 ± 9 [17]	81 ± 9 [17]	**90 ± 11 *** [15]	**96 ± 11 *** [15]
	SHAM Pre	76 ± 7 [16]	79 ± 7 [16]	**89 ± 8 *** [14]	**89 ± 8 *** [14]
	SHAM Post	80 ± 7 [16]	82 ± 7 [16]	**89 ± 10 *** [14]	**95 ± 9 *** [14]
EX Forearm blood flow (ml/min)	CO Pre	42 ± 20 [19]	**96 ± 35** † [19]	**64 ± 23 *** [17]	**81 ± 28 *** [16]
	CO Post	34 ± 18 [19]	**101 ± 34** † [19]	**67 ± 23 *** [17]	95 ± 26 [16]
	SHAM Pre	54 ± 26 [18]	**106 ± 45** † [18]	**69 ± 34 *** [16]	90 ± 33 [15]
	SHAM Post	37 ± 27 [19]	**97 ± 45** † [19]	**62 ± 28 *** [17]	84 ± 28 [16]
EX Forearm vascular conductance (ml/min/100mmHg)	CO Pre	52 ± 28 [16]	**116 ± 47** † [16]	**66 ± 26 *** [13]	**82 ± 29 *** [12]
	CO Post	43 ± 24 [16]	**126 ± 33** † [17]	**78 ± 23 *** [15]	**98 ± 17 *** [14]
	SHAM Pre	71 ± 39 [17]	**138 ± 55** † [16]	**74 ± 33 *** [14]	**102 ± 45 *** [12]
	SHAM Post	50 ± 41 [16]	**122 ± 57** † [16]	**71 ± 30 *** [14]	89 ± 31 [13]
CTRL Forearm blood flow (ml/min)	CO Pre	29 ± 25 [15]	30 ± 24 [15]	---	**13 ± 11 *** [13]
	CO Post	25 ± 22 [13]	27 ± 26 [13]	---	**14 ± 14 *** [11]
	SHAM Pre	37 ± 24 [14]	37 ± 30 [14]	---	23 ± 20 [10]
	SHAM Post	39 ± 37 [14]	35 ± 25 [14]	---	15 ± 13 [10]
CTRL Forearm vascular conductance (ml/min/100mmHg)	CO Pre	32 ± 28 [13]	31 ± 28 [13]	---	**10 ± 11 *** [11]
	CO Post	23 ± 27 [12]	26 ± 32 [12]	---	**8 ± 12 *** [10]
	SHAM Pre	46 ± 27 [12]	40 ± 34 [12]	---	19 ± 22 [8]
	SHAM Post	44 ± 50 [12]	37 ± 24 [12]	---	14 ± 13 [9]

Values are means ± standard deviations. EX, exercising arm; CTRL, control arm; Pre, pre-rebreathe condition; Post, post-rebreathe condition. † significance *vs.* baseline ($P < 0.05$). * significance *vs.* handgrip exercise ($P < 0.05$). Sample sizes denoted in square brackets.

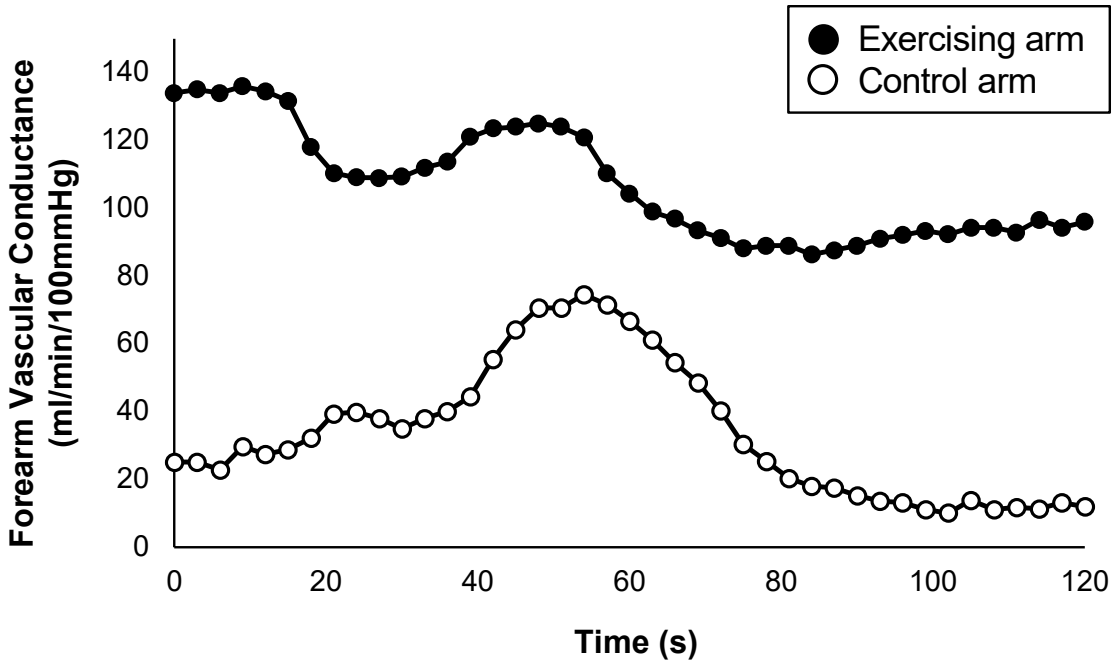

Figure 11. Representative exercising *vs.* control arm vascular responses to bolus phenylephrine infusion. Following infusion of phenylephrine, forearm vascular conductance in the exercising arm (filled circles) declines to an initial nadir, pausing and increasing slightly as baroreflex activation increases, then declining further to a secondary nadir before gradually returning to baseline levels. In the control arm, vascular conductance initially inverts this response, rising to an initial peak, pausing, then rising further to a secondary peak before declining to sub-baseline values.

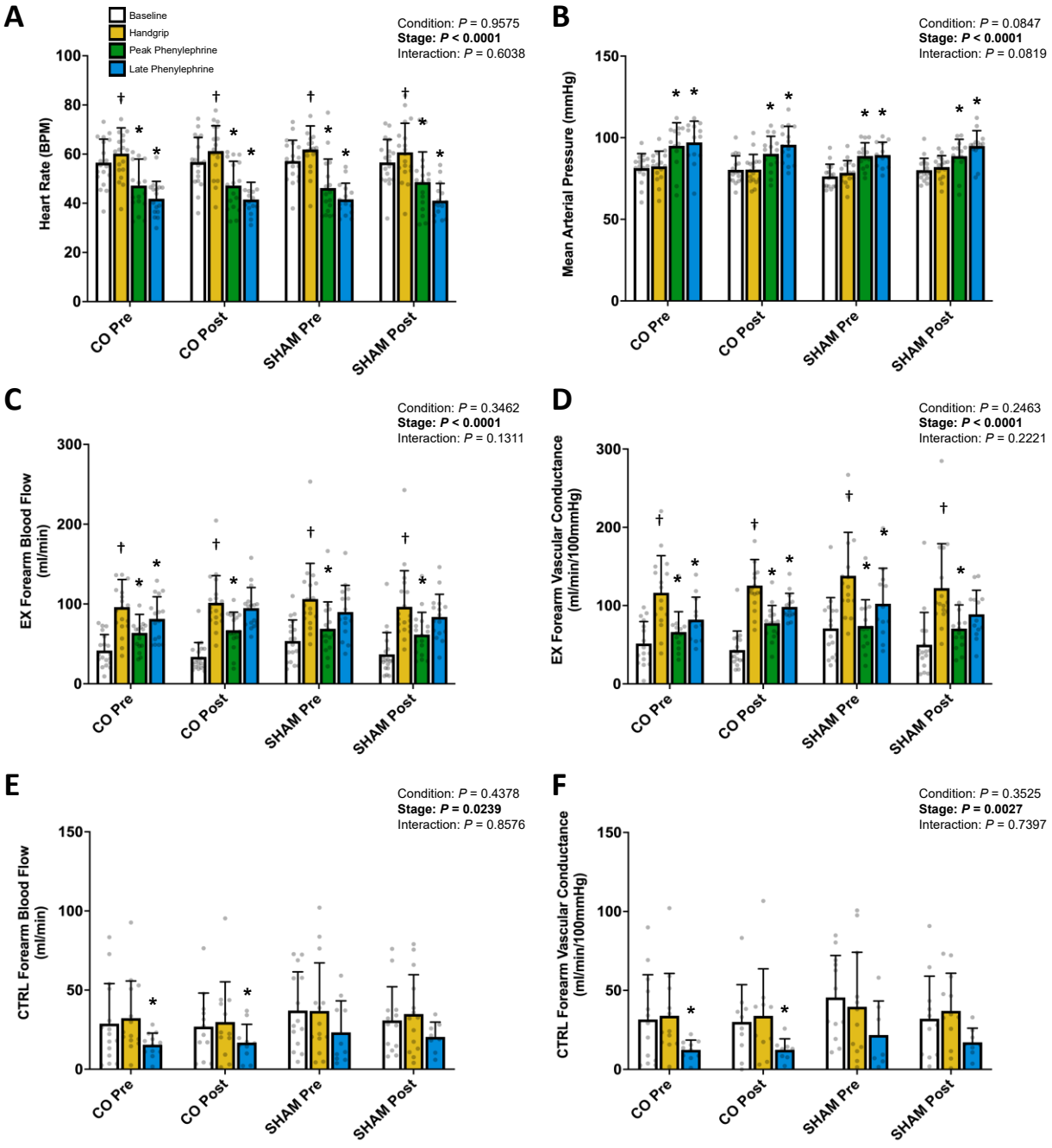

Figure 12. Absolute values for heart rate (A), mean arterial pressure (B), exercising forearm blood flow (C) and vascular conductance (D), and control forearm blood flow (E) and vascular conductance (F) during baseline (white), steady-state handgrip exercise (yellow), peak phenylephrine response (green), and late phenylephrine response (blue). Peak responses are omitted for CTRL data. Individual participant data is represented by grey circles. * P<0.05 compared to handgrip. † P<0.05 compared to baseline.

4.2 Hemodynamics – exercise response

Figure 13 illustrates hemodynamic responses to handgrip exercise, determined as percent and absolute changes between resting baseline and steady-state handgrip exercise responses in MAP, EX FBF/FVC. A main effect of stage was detected only in percent changes in FBF ($P = 0.0042$), where post-rebreathe values were significantly greater than pre-rebreathe values, irrespective of CO or sham condition. However, this effect was not mirrored in post-hoc analyses, with no pairwise differences observed between stages in either condition. In consideration of the outlier-nature of this effect *vs.* other hemodynamic responses to handgrip exercise observed, this finding should be interpreted with caution. These findings suggest that hemodynamic responses to exercise were not altered with CO exposure.

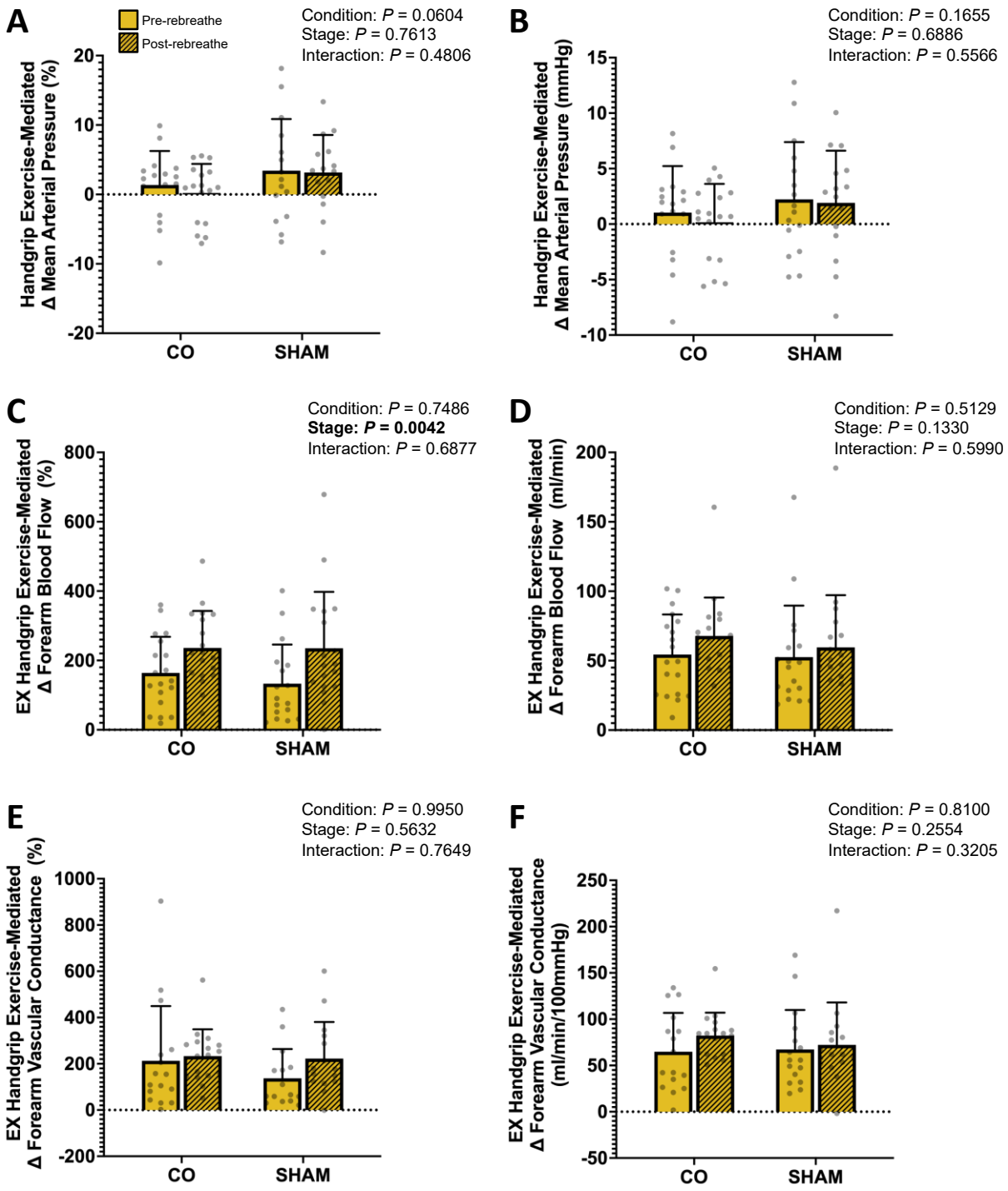

Figure 13. Percent (left panels) and absolute (right panels) changes in mean arterial pressure (A; B) and exercising forearm blood flow (C; D) and vascular conductance (E; F) from resting baseline to steady-state handgrip exercise. Open bars denote pre-rebreathe stage, and hatched bars denote post-rebreathe stage.

4.3 Hemodynamics – phenylephrine response

Figure 14 illustrates peak hemodynamic responses to phenylephrine infusion, determined as percent and absolute changes between steady-state handgrip exercise and peak phenylephrine responses in MAP, EX FBF/FVC. A main effect of stage was detected only in the percent changes in mean arterial pressure ($P = 0.0489$), where post-rebreathe values were significantly lower than pre-rebreathe values, irrespective of CO or sham condition. Once again, this effect stands as an outlier against other hemodynamic measures where no pre- vs. post-rebreathe differences were detected, and should be interpreted with caution.

Figure 15 illustrates late hemodynamic responses to phenylephrine infusion, determined as percent and absolute changes between steady-state handgrip exercise and late phenylephrine responses in MAP, EX FBF/FVC, and CTRL FBF/FVC. No significant effects were observed in any parameter. Although EX and CTRL values were not directly compared, mean relative reductions in CTRL FBF/FVC with phenylephrine were greater than those in EX FBF/FVC in all conditions. This supports the expected partial sympatholytic effect of exercise blunting α_1 adrenergic constriction (Wilkins *et al.*, 2006; Hearon *et al.*, 2016, 2020).

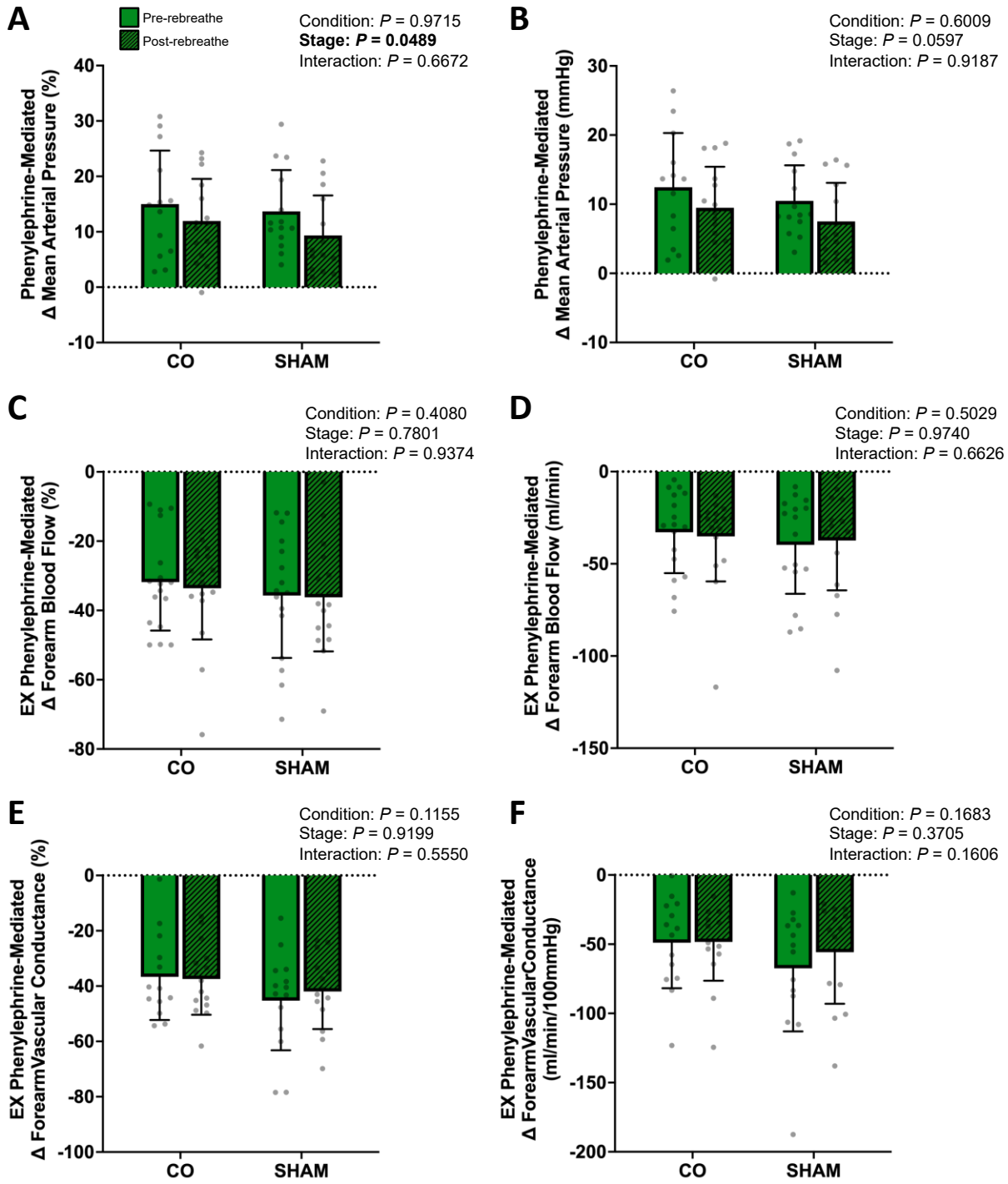

Figure 14. Percent (left panels) and absolute (right panels) changes in mean arterial pressure (A; B) and forearm blood flow (C; D) and vascular conductance (E; F) from steady-state handgrip exercise to peak phenylephrine responses. Open bars denote pre-rebreathe stage, and hatched bars denote post-rebreathe stage.

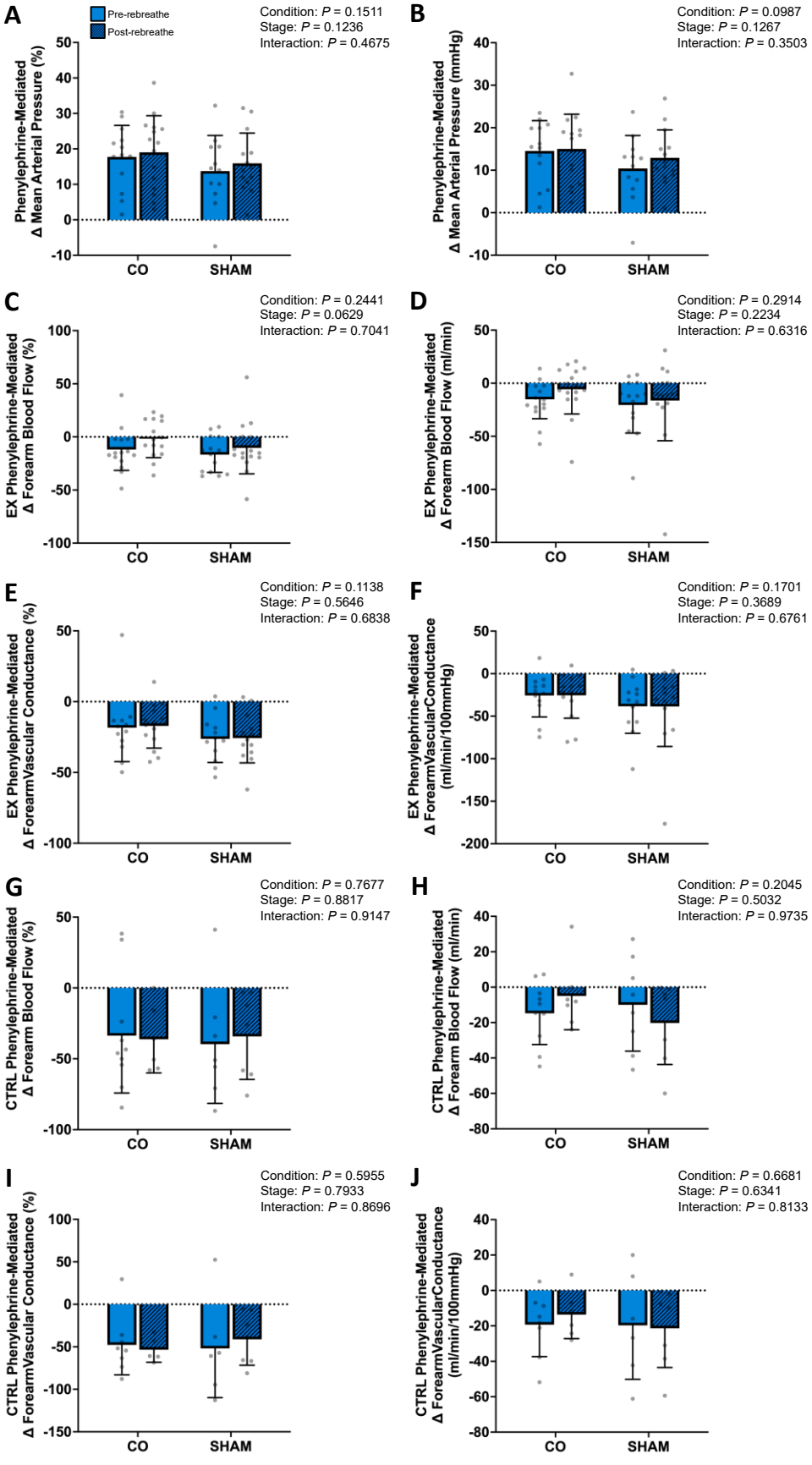
A
Pre-rebreathe
Post-rebreathe
Condition: P = 0.1511
Stage: P = 0.1236
Interaction: P = 0.4675
Phenylephrine-Mediated Δ Mean Arterial Pressure (%)
CO
SHAM
B
Condition: P = 0.0987
Stage: P = 0.1267
Interaction: P = 0.3503
Phenylephrine-Mediated Δ Mean Arterial Pressure (mmHg)
C
Condition: P = 0.2441
Stage: P = 0.0629
Interaction: P = 0.7041
EX Phenylephrine-Mediated Δ Forearm Blood Flow (%)
D
Condition: P = 0.2914
Stage: P = 0.2234
Interaction: P = 0.6316
EX Phenylephrine-Mediated Δ Forearm Blood Flow (ml/min)
E
Condition: P = 0.1138
Stage: P = 0.5646
Interaction: P = 0.6838
EX Phenylephrine-Mediated Δ ForearmVascular Conductance (%)
F
Condition: P = 0.1701
Stage: P = 0.3689
Interaction: P = 0.6761
EX Phenylephrine-Mediated Δ ForearmVascularConductance (ml/min/100mmHg)
G
Condition: P = 0.7677
Stage: P = 0.8817
Interaction: P = 0.9147
CTRL Phenylephrine-Mediated Δ Forearm Blood Flow (%)
H
Condition: P = 0.2045
Stage: P = 0.5032
Interaction: P = 0.9735
CTRL Phenylephrine-Mediated Δ Forearm Blood Flow (ml/min)
I
Condition: P = 0.5955
Stage: P = 0.7933
Interaction: P = 0.8696
CTRL Phenylephrine-Mediated Δ ForearmVascular Conductance (%)
J
Condition: P = 0.6681
Stage: P = 0.6341
Interaction: P = 0.8133
CTRL Phenylephrine-Mediated Δ ForearmVascularConductance (ml/min/100mmHg)

Figure 15. Percent (left panels) and absolute (right panels) changes in mean arterial pressure (A; B), exercising forearm blood flow (C; D) and vascular conductance (E; F), and control forearm blood flow (G; H) and vascular conductance (I; J) from steady-state handgrip exercise to late phenylephrine responses. Open bars denote pre-rebreathe stage, and hatched bars denote post-rebreathe stage.

4.4 Post-hoc statistical assessments

Post-hoc sample size calculations were performed using the observed effect sizes of percent and absolute changes in FVC between pre- and post-CO conditions to determine the theoretical number of participants required to find significant differences ($P < 0.05$; power = 0.80) (**Table 2**). The small observed effect sizes support that CO did not exert a physiologically relevant impact on the magnitude of exercise hyperemia, nor to vasoconstrictor reactivity at peak or late phenylephrine response points. The large theoretical sample sizes required to reach significance support that the lack of significant effects detected in the present statistical analyses are not attributable to insufficient sample sizes.

Table 2: Post-hoc analysis of sample size

Assessment	Comparison	n obtained (Pre-CO)	n obtained (Post-CO)	Effect size	Tukey's multiple comparisons test adjusted P value	n required for significance
Handgrip hyperemia	Ex FVC %	16	17	0.100	0.980	792
	Ex FVC Δ	16	17	0.478	0.431	37
Peak phenylephrine response	Ex FVC %	17	13	0.052	0.940	2918
	Ex FVC Δ	14	15	0.018	0.950	24586
Late phenylephrine response	Ex FVC %	12	14	0.060	0.866	2189
	Ex FVC Δ	12	14	0.007	0.962	165875
	CTRL FVC %	8	5	0.184	Ins.	234
	CTRL FVC Δ	8	5	0.072	Ins.	1497

Post-hoc comparisons of observed effect sizes, multiple comparisons test P values, and theoretical n required to attain statistical significance ($P < 0.05$; power = 0.80) for percent and absolute changes in primary vascular outcomes between pre- and post-carbon monoxide rebreathe conditions (Pre-CO and Post-CO respectively). EX, exercising arm; CTRL, control arm; FVC, forearm vascular conductance; Ins., insufficient matching for multiple comparisons test.

Chapter 5: Discussion

In this study, we aimed to assess the influence of acute, mild carbon monoxide exposure on vascular reactivity to moderate-intensity exercise and α_1 adrenergic sensitivity. Contrary to our *a priori* hypotheses, we did not observe differences in forearm or systemic hemodynamics with acute carbon monoxide exposure in response to either moderate-intensity rhythmic handgrip exercise, or α_1 adrenergic-mediated vasoconstriction. In support of these conclusions, we compared responses with carbon monoxide exposure against a second control day (sham) to control for any potential time-of-day or protocol repeatability effects and isolate the influence of carbon monoxide. To further explore potential interactions of carbon monoxide, hemodynamic responses were quantified in terms of both percent and absolute (delta) changes to account for differences in inter-individual baseline values, and α_1 adrenergic sensitivity was quantified across two distinct phases of the dynamic response to systemic phenylephrine infusion to assess the influence of varied degrees of reflexive baroreceptor activation. Nonetheless, this comprehensive approach found no interactions of carbon monoxide on vascular function.

5.1 Carbon monoxide and exercise hyperemia

Our first aim was to determine the impact of mild CO exposure on the hyperemic response to moderate-intensity rhythmic handgrip exercise. Blood flow and vascular conductance increased several-fold in the exercising muscle, reflecting the vasodilatory response to moderate-intensity exercise. Concurrently, modest increases in heart rate were observed, indicating that exercise induced mild sympatho-excitation; however, that mean arterial pressure and control arm hemodynamics remained unchanged indicates that the pressor response to this moderate-intensity exercise was small, and insufficient to appreciably increase sympathetic vasoconstrictor signaling.

Exercise increases the oxygen demand of tissue, exacerbating any insufficiencies in oxygen delivery. To that end, we found no differences in exercise-mediated increases in FBF or FVC between CO and control conditions in either percent or absolute changes, indicating that oxygen delivery was

sufficient to meet metabolic demand during handgrip exercise with CO exposure. However, it should be noted that without measurements of arterio-venous oxygen extraction, we cannot conclude with certainty that CO exposure did not mildly impair oxygen uptake in tissue (Ayres *et al.*, 1969). In contrast, previous reports on the effects of more severe CO exposure (~20% COHb) demonstrated increased blood flow responses to progressive knee-extension exercise following CO administration, with the magnitude of said increases ranging from ~15 to 44% (González-Alonso *et al.*, 2001, 2002). Notably, the degree of these augmented responses were inversely related to exercise intensity, most prominent at lower intensities (~27% of max), with the disparity relative to normoxic responses diminishing approaching maximal intensity (González-Alonso *et al.*, 2001, 2002). These diminishing impacts of CO may have been reflective of the vasculature approaching a maximally vasodilated state, as blood flow responses were similar at maximal intensities regardless of condition, despite the lower absolute intensity achieved with CO exposure (González-Alonso *et al.*, 2002). The present study investigated hyperemic responses during rhythmic handgrip exercise at 15% MVC, pursuing a moderate, sustainable intensity of exercise. While direct comparisons of exercise intensity against the previous works are limited by differences in upper *vs.* lower body muscle mass and composition, our exercise intensity is broadly more comparable to the lower intensities in the previous works. While the greater CO stimulus in these studies accounts for a portion of the discrepancy, a gradation of augmented hyperemia would still have been expected, particularly at our lower exercise intensity where this effect appears to be more pronounced. Taken together, these previous findings and those of the present study suggest that a threshold level of CO exposure is requisite for an augmented hyperemic response to exercise, and that oxygen delivery and vasodilatory signaling cascades are not functionally perturbed with common environmental exposure levels of COHb (~5-6%).

5.2 Carbon monoxide and α_1 adrenergic reactivity

Our second aim was to assess the effects of CO on α_1 adrenergic sensitivity. This was determined by quantifying various aspects of systemic and local vascular responses to a bolus infusion of the α_1 adrenergic agonist phenylephrine during the initial peak response within the first minute after infusion, and the delayed response between 90- and 120-seconds post-infusion after baroreflex engagement has stabilized. Phenylephrine elicited anticipated increases in heart rate and mean arterial pressure, and reductions in FBF and FVC in both the exercising and control arms. Although vascular responses were not directly compared between the exercising and control arms on account of the vastly differing baseline levels of blood flow and flow analysis modalities, the substantially greater relative reductions in FBF and FVC in the control arm *vs.* the exercising arm (reductions of ~40-50% *vs.* ~10-20% respectively) support that our exercise stimulus successfully elicited a partial sympatholytic effect, allowing for comparisons of the effects of CO in resting tissue as well as in conjunction with sympatholytic mechanisms in working muscle. While reductions in exercising arm FBF and FVC were greater during the initial peak response and tapering back to pre-infusion levels into the late response, brachial artery diameter declined progressively and was only significantly reduced at the late response point, highlighting temporal differences in conduit artery *vs.* microvascular α_1 adrenergic regulation with conduit arteries constricting in a slower, more sustained manner. No CO-mediated differences in vascular reactivity were determined in FBF, FVC, or conduit diameter in the exercising or inactive forearms, nor in systemic mean arterial pressure, in either percent or absolute changes. To our knowledge, this is the first such investigation of CO on vascular reactivity in humans, and suggests that α_1 adrenoreceptor sensitivity is unchanged in both macro- and microvascular circulations during mild CO exposure. This was unexpected, as previous findings from animal research have broadly illustrated anti-constrictor effects of CO. These disparate findings warrant exploration of the caveats of isolated models to better understand their translation to integrative *in vivo* models.

Anti-constrictor effects of CO have been documented at physiological concentrations through inhibition of heme oxygenase and endogenous CO production (Wang *et al.*, 1997*a*; Kaide *et al.*, 2001, 2004; Koçer *et al.*, 2018), as well as elevated concentrations through up-regulation of heme oxygenase activity (Sammut *et al.*, 1998; Caudill *et al.*, 1998; Gonzales & Walker, 2002) and administration of exogenous CO (Wang *et al.*, 1997*a*). A major limitation when comparing the effects of exogenous CO administration between isolated and integrative models is the ability to precisely quantify the CO stimulus present at the level of the vascular smooth muscle *in vivo*; while an increase in COHb from 1.4% to 5.6% implies a 4-fold increase in CO available to interact with intracellular signaling cascades, the majority of CO being left bound to hemoglobin in the blood and the progressive diffusion of CO into skeletal muscle over time may skew this ratio, further compounded by variable rates of diffusion into different tissue types (Vreman *et al.*, 2006). For example, Wang *et al.* (1997*a*) found a ~50% reduction in vasoconstriction with 300 μM exogenous CO, but without an arterial biopsy and direct measurement of CO from *in vivo* models, it is unclear how comparable the magnitude of this stimulus is to a given increase in circulating COHb. In a similar series of experiments, Kaide *et al.* (2001) found that after reducing endogenous CO production by 54% (resulting in significant increases in vasoconstrictor sensitivity), administration of a mere 0.1-1 μM CO was sufficient to restore anti-constrictor effects. These studies illustrate a potent anti-constrictor effect of CO that is dose-dependent at very low concentrations. That a several-fold increase in COHb in the present study failed to replicate this effect is indicative of compensatory interactions with alternate vasodilators, not otherwise present in isolated models.

Recent evidence has suggested that the mechanisms involved in α_1 adrenergic sympatholysis are both highly integrative and specific. Endothelial stimulation via acetylcholine or ATP has shown a considerable capacity to blunt α_1 adrenergic vasoconstriction (Kirby *et al.*, 2008; Hearon *et al.*, 2016). Reductionist efforts to isolate the downstream mechanisms responsible for this indicate that this sympatholytic effect is preserved during combined inhibition of NO, PGs, inwardly-rectifying K^+ channels, and Na^+/K^+ ATPase channels (Hearon *et al.*, 2017). Moreover, it is not replicated with

administration of endothelium-independent vasodilators such as sodium nitroprusside (an NO donor), KCl, or adenosine, nor do these vasodilators produce additive sympatholytic effects when combined with acetylcholine or ATP (Kirby *et al.*, 2008; Hearon *et al.*, 2016; Terwoord *et al.*, 2021). The particularities of endothelium-dependent signaling responsible for sympatholysis are not fully understood, but have been suggested to involve activation of IKca and SKca channels in the endothelium – sensitized by an influx of IP_3 from the vascular smooth muscle during α_1 adrenergic activation – initiating hyperpolarization which is then transmitted back to the vascular smooth muscle via EDH (Hearon & Dinenno, 2019). The vasodilatory mechanisms of CO have been strongly likened to those of NO, albeit with varied efficacies in some of their shared pathways (Furchgott & Jothianahdan, 1991; Wang *et al.*, 1997*b*). In spite of the accumulated evidence in favour of anti-constrictor effects of CO, the null finding of the present study aligns with the inability of direct activation of sGC and BKca channels (via sodium nitroprusside) to blunt adrenergic vasoconstriction *in vivo*, and emphasizes the critical role of endothelial stimulation in modulating sympathetic constriction (Hearon *et al.*, 2016; Terwoord *et al.*, 2021).

5.3 Considerations

The findings of the present study indicate that functional vascular reactivity is well preserved with mild CO exposure. Post-hoc sample size calculations were performed, aiming to determine the theoretical sample size required to reach statistically significant differences ($P < 0.05$; power = 0.80) between the pre- and post-CO rebreathe conditions in primary vascular outcomes for handgrip hyperemia and phenylephrine responses (**Table 2**). The calculated requisite sample sizes ranged from moderate (n = 37 required for absolute changes in handgrip hyperemia) to immense, requiring hundreds or thousands of samples, and in conjunction with the small effect sizes observed, we assert that our statistical analysis was sufficiently powered, and our null findings were not the result of insufficient sample sizes. Caution may be warranted in the interpretation of control arm vascular responses on account of the lower sample size, which was insufficient for post-hoc pairwise comparisons. However, as main effects of condition were

not significant across all parameters, these pairwise comparisons are not integral to the interpretation of our results, and are highlighted for illustrative purposes. Further supporting of the lack of CO-mediated influence observed in control arm vascular reactivity, mean arterial pressure responses with CO were also not significantly different from control conditions. Mean arterial pressure provides an additional metric of vascular responses in quiescent tissue, albeit as a pooled index of global vascular function, rather than one specific to skeletal muscle.

Inclusion and randomization of a control day, whereby participants were tested identically and at the same time of day on both days, controlled for diurnal variability in vascular responsiveness (Bau *et al.*, 2008), participant acclimation to the testing environment, as well as demonstrating within-day test-retest reliability. Handgrip exercise intensity and phenylephrine dosages were determined daily prior to testing from participants' maximum voluntary contractions and anthropometrics respectively to ensure standardized testing conditions.

Intraclass correlation coefficients of brachial artery diameter and FBF measurements were calculated as a measure of sonographer consistency, comparing CO and sham day pre-rebreathe conditions during both resting baseline and handgrip exercise. Correlation coefficients obtained for brachial artery diameter were ≥0.9, indicating strong repeatability of these measurements. Correlation coefficients obtained for FBF were between 0.2 and 0.4, indicative of substantially greater variability between these values. However, as these measures are highly sensitive to changes in blood flow velocity, regulated by changes in microvascular tone throughout the arm, a greater degree of variability is expected in these measures. With respect to the standardized scanning techniques applied and the high degree of repeatability in diameter measures, we support that the vascular assessments in the present study were accurate.

To determine the variability implicit in measures of FBF and how it may contribute to obscuring effects of CO on vascular reactivity, standard errors of measurement were calculated in FBF, comparing CO and sham day pre-rebreathe conditions at rest and during exercise. These errors were found to be 20

and 32 ml/min respectively. As these errors constitute approximately half of the effect size in handgrip hyperemia and most of the effect size in phenylephrine responses, it is possible that a very modest effect of CO on vascular reactivity may have been overshadowed by this variability. However, on account of the randomized control trial design of the present study, wherein responses in the CO condition showed no trends deviating from the abundance of control data, we believe this interpretation is unlikely.

Some contention exists as to the respective significance of relative *vs.* absolute changes in vascular outcomes, whereby the former controls for differences in individual baseline value and helps standardize analysis between the sexes, where males tend towards higher volumetric flow, and thus greater absolute differences compared to females. For transparency, we have reported both, and found no influences of CO in either metric.

Finally, secondary analyses were performed to investigate potential impacts of sex (see below), correlations between COHb levels and vascular responses, and the relationship of the magnitude of initial peak phenylephrine responses to the time-to-peak; all of which detected no effects of CO on hemodynamic responses and supported our primary conclusions, and have thus been omitted for brevity.

With the above considerations in mind, we assert that our conclusions that mild CO exposure does not influence functional vascular reactivity are reflective of integrative physiological compensation to the CO stimulus, not due to analytical omissions or a lack of experimental controls.

5.3.1 Signaling interactions of carbon monoxide

Investigations of the effects of CO on cardiovascular outcomes have incorporated a range of CO exposure levels, including sub-physiological levels via inhibition of endogenous CO production (Koçer *et al.*, 2018), mild exposure of ~3-9% COHb (Ayres *et al.*, 1969), moderate/severe exposure of ~20% COHb (González-Alonso *et al.*, 2001), up to critical exposure of ~50% COHb (Villeneuve *et al.*, 1986), each level of exposure exerting unique effects on different physiological processes and systems. In the present study, CO was administered using the optimized CO rebreathing technique developed by Schmidt &

Prommer (2005), producing an anticipated, mild CO stimulus of 5.6 ± 1.2% COHb. COHb levels of 5.6% provide a marked increase in CO *vs.* endogenous production, and have been shown to alter vascular and neural reactivity, including impairment of oxygen delivery in the coronary circulation (Ayres *et al.*, 1969) and augmented post-occlusion hyperemia (McRae *et al.*, 2019). However, in the latter investigation and others, these levels of COHb did not evoke changes in basal vascular tone, nor in efferent SNA (Hausberg & Somers, 1997; McRae *et al.*, 2019). In conjunction with the present study, whereby no differences in baseline cardiovascular parameters were observed with CO exposure, these findings suggest that vasoactive effects of CO of ~3-9% are well tolerated, likely through compensatory shifts in alternate vasodilatory mechanisms. While the present study is not equipped to address such interactions in intracellular vasodilatory cascades, their potential influence should be considered when interpreting results.

Control of vascular tone is an integrative process with numerous layers of redundancy in vasoactive signaling, responding to changes in the level of one vasodilator by counterbalancing the production of others. For example, selective inhibition NO or PGs stimulates an up-regulation of the alternate vasodilator to maintain steady vascular tone (Boushel *et al.*, 2002). Similar such interactions have been observed between CO and NO, although the relationship between their respective concentrations and the synthesis of the other remains obscure (Choi & Kim, 2021). As such, when compared to loss-of-function investigations (inhibiting endogenous CO) which highlight the effects of CO by observing vascular responses in its conspicuous absence, it is unclear to what extent the vasodilatory capacities of CO may be further augmented through supra-physiological exposure before interactions with alternate vasodilators compensate against them, such as through down-regulation of NO. Moreover, interactions with signals of extravascular origin *in vivo* present additional confounding factors, such as CO-mediated inhibition of ATP release from erythrocytes – blunting ATP's vasodilatory and sympatholytic effects – directly opposing similar effects of CO itself (Jagger *et al.*, 2001; González-Alonso *et al.*, 2002).

In addition to the interactions of CO with the production of other vasodilators, overlapping downstream signaling cascades may also lead to unexpected results when translating isolated animal research into *in vivo* models. The thorough characterization of CO-mediated vasorelaxation by Wang *et al.* (1997*a*, 1997*b*) supports that its vasoactive effects are mediated entirely through sGC and BKca activity, and are independent of an intact endothelium and NO. This mechanism is expanded to include a double-negative CO-mediated inhibition of the endogenous BKca-inhibitor, 20-hydroxyeicosatetraenoic acid (Kaide *et al.*, 2004; Rocic & Schwartzman, 2018). Arteries *in vivo* receive vasoactive signaling from the surrounding tissue and humoral factors which may also act via similar pathways, such as NO produced in the endothelium via local hypoxic vasodilation and activation of β_2 adrenoreceptors by epinephrine (Queen & Ferro, 2006; Markwald *et al.*, 2011). Prior activation of sGC and BKca pathways in this manner may dilute the functional significance of increased CO.

CO has been previously utilized experimentally as a means of inducing hypoxemia, with the assumption that subsequent vascular effects are due rather to a local hypoxic response. To this end, studies have aimed to match COHb levels against similar reductions in oxygen saturation via reductions in inspired PO_2 to control for the hypoxemic stimulus (González-Alonso *et al.*, 2001, 2002). A limitation of this approach is the assumption that matching COHb levels against reductions in arterial oxygen saturation produce equal degrees of hypoxia in tissue, neglecting the impact of the leftward shift in the oxyhemoglobin dissociation curve and potentially underestimating the level of tissue hypoxia with CO. Limited evidence of this has been demonstrated by Ayres *et al.* (1969), finding a 20% reduction in mixed venous PO_2 (as an index of tissue PO_2) with only a 9% saturation of COHb, suggesting that increasing uptake of unbound oxygen in the blood compensates for impaired oxygen extraction from erythrocytes. Not only does this result in an underestimation of the hypoxemic stimulus, this approach also neglects other CO-mediated interactions: critically, its ability to inhibit the release of ATP from erythrocytes. A central tenet arising from the work of González-Alonso *et al.* (2001, 2002, 2006) and others has been the recognition of intravascular ATP as a key mediator of hypoxia- and exercise-mediated vasodilation

(Crecelius *et al.*, 2015). However, previous works incorporating CO as a means of inducing hypoxemia have been unable to explain the preserved – even augmented – vasodilation observed with CO, while further suggesting that the mechanisms responsible are also independent of NO (González-Alonso *et al.*, 2001, 2002). Although the use of CO as a hypoxemic stimulus has fallen somewhat out of favour as the role of hemoglobin and the erythrocyte have been characterized in increasing detail, returning to explore this fascinating interaction may yield novel insights into the integrative mechanisms vascular control, and the role CO – either of endogenous origin or exogenous exposure – may play.

5.3.2 Influence of sex

As a secondary analysis aim, potential sex differences in the impact of CO on vascular reactivity to exercise and α_1 adrenergic stimulation were assessed using 3-way ANOVAs [Factor A: Condition (CO, SHAM); Factor B: Stage (pre-rebreathe, post-rebreathe); Factor C: Sex (male, female)]. These analyses found that, although females typically exhibited lower absolute responses than males, no differences were observed in relative measures where differences in baseline values were controlled for. This was expected, as previous reports have indicated that vascular responses to this exercise modality and α_1 adrenergic activation are comparable between the sexes, and that sex differences in vascular reactivity stem primarily from differences in β_2 adrenoreceptor activity (Limberg *et al.*, 2010; Hart *et al.*, 2011). Moreover, no interactions of sex and CO exposure were detected, indicating that neither the direct influence of CO, nor any CO-mediated interactions with alternative vasodilatory pathways differentially regulated vascular reactivity between the sexes.

Menstrual cycle phase was not controlled for in female participants, constituting a potential source of variability. Estrogens and progesterone exert potent vasoactive effects and are known to fluctuate across the menstrual cycle; however, the functional significance of menstrual phase timing on micro- and macrovascular function is complex, and potentially overstated given the substantial variability present even when controlling for ovarian phase (Knowlton & Lee, 2012; Williams *et al.*, 2020).

Moreover, the prevalence of hormonal contraceptives in females obscures the implications of phase timing further, as testing was not standardized to the placebo phase, nor do all hormonal contraceptive protocols entail a placebo phase. Investigations aiming to control for the effects of sex hormones should aim to measure their serum concentrations directly for covariate analysis.

5.3.3 Study limitations

The level of CO exposure was determined through point-measurements of venous carboxyhemoglobin at discrete intervals during the experimental protocol. This approach modestly underestimates the degree of exposure at the point of testing, as a small amount of CO is gradually eliminated via the respiratory system between the time of measurement and initiation of the experimental protocols. COHb reversion to mean homeostatic levels is biphasic, undergoing an initial "rapid" period with an elimination half-time of 200-300 minutes as CO diffuses into tissue as well as out of the body via the lungs, followed by a "slow" period after equilibration with a half-time of 250-400 minutes (Bruce & Bruce, 2006). As part of a larger investigation into the effects of CO on vascular function, our post-rebreathe vascular assessments took place approximately 45 minutes after CO administration. Accordingly, we approximate an error in COHb levels of ~10% between the point-measure and the time of testing, which was supported by COHb measures taken immediately following study completion in a small subset of participants (See appendix 6). Of note, due to the equilibration of CO into tissue during the initial rapid elimination phase, COHb measurements modestly underestimate the level of CO available for intracellular signaling within the vasculature, reducing the impact of this ~10% reduction.

Bolus infusions of phenylephrine evoke systemic cardiovascular effects which must be considered when assessing α_1 adrenergic sensitivity. Systemic vasoconstriction triggers baroreflex-mediated sympathetic withdrawal, reducing vasoconstrictor tone and heart rate (Rudas *et al.*, 1999; Simpson *et al.*, 2019). Blunted endogenous NE release from pre-infusion levels reduces the degree of α_1 adrenergic activation, and may result in modest underestimation α_1 adrenoreceptor sensitivity to a given

dosage of phenylephrine. While our results indicate that heart rate is reduced uniformly with phenylephrine infusion, implying consistent sympathetic withdrawal, the cumulative effect on cardiac output is more complex. While reductions in heart rate and increased afterload from arterial vasoconstriction may reduce cardiac output, α_1 adrenergic venoconstriction increases venous return, resulting in an increase in cardiac output (Cannesson *et al.*, 2012). The integrative result of these conflicting influences is contentious, however recent evidence from anaesthetized animals and perioperative patients suggests that phenylephrine infusion increases cardiac output when the heart is preload-dependent (*e.g.*, under resting conditions) (Kalmar *et al.*, 2018; Wodack *et al.*, 2019). Increased cardiac output in the present study may have augmented the increases in mean arterial pressure and lessened the reductions in local blood flow; however, confounding influences of these effects are reconciled through the calculation of vascular conductance as an index of vascular tone. Moreover, the hemodynamic responses observed in the present study may reflect a combination of both arterial and venous α_1 adrenergic sensitivity, potentially extending the implications of carbon monoxide exposure to its effects on underappreciated venoconstrictor reactivity. An alternative, albeit more invasive approach to mitigate these systemic effects is the use of low-dose local intra-arterial infusions of phenylephrine or tyramine (evoking endogenous NE release) (Wilkins *et al.*, 2006; Hearon *et al.*, 2016).

Phenylephrine is considered a selective α_1 adrenergic vasoconstrictor, and is utilized frequently in clinical settings for prevention of acute hypotension and in experimental settings for the assessment of α_1 adrenoreceptor sensitivity (Hearon *et al.*, 2016; Kalmar *et al.*, 2018). Unexpectedly, our phenylephrine infusion resulted in dramatic increases in FBF and FVC specifically in the control arm, persisting for the first minute post-infusion (**Figure 11**). This response was observed consistently and reproducibly in nearly all (>90%) participants irrespective of experimental condition, despite marked reductions in FBF and FVC in the exercising arm and increases in mean arterial pressure, indicative of systemic vasoconstriction. The underlying mechanisms responsible for this are unclear, although there have been select reports finding vasodilatory effects of phenylephrine under specific conditions. Stemming from a

chance observation, Torp *et al.* (2001) found that administration of large doses of phenylephrine during α_1 adrenergic blockade via phentolamine resulted in marked vasodilation within the first minute following infusion, and that this effect was abolished when combined with β adrenergic blockade via propranolol. Common between this observation and the current study are (1) the time course of this vasodilatory response, peaking ~30 seconds post-infusion and resolving after roughly 1 minute, while constrictor effects persisted beyond this point, (2) the dosages administered were substantially greater than those typically used in other clinical and experimental settings when scaled for affected tissue mass, and (3) the effect was observed in quiescent forearm tissue in humans (Torp *et al.*, 2001). These commonalities suggest that β_2 adrenergic activation by phenylephrine may be threshold-dependent, occurring only after α_1 adrenoreceptors are sufficiently saturated, and resolving as the dose is catabolized below this super-saturation point. It is puzzling why this response was only observed in the control arm, while all other systemic hemodynamics parameters indicated prevailing vasoconstriction; this may be due to differential distribution of adrenoreceptor subtypes in different tissues, and the co-activation of alternate vasodilatory pathways in the exercising arm limiting the degree to which additional β adrenoreceptor activation could contribute, as has been noted during hypoxic exercise (Casey *et al.*, 2011). Additionally, limited evidence has been presented for α_{1D} adrenoreceptor-mediated vasodilation in longitudinally-oriented vascular smooth muscle, although this novel mechanism is poorly characterized to date (Marconi *et al.*, 2020). To the extent that this may occur in humans, the high dosage implemented in the present study may have stimulated this effect as well.

As an additional consideration, it is possible that the brief attenuation of vasoconstriction during bolus phenylephrine infusions after ~30 seconds, attributed up to this point to mismatched baroreflex engagement briefly overcompensating for the highly dynamic vasoconstriction, may be mediated in part by transient β adrenergic activation at a systemic level restraining α_1 constriction. While this would weaken the implications of the initial peak vasoconstrictor responses as an index of α_1 adrenergic sensitivity, the inclusion of the late response between 90-120 seconds is independent of this confounding

influence. Future clarification of the capacity for phenylephrine to stimulate β_2 adrenoreceptors may shed light on this complication. However, as no CO-mediated differences in local or systemic vascular responses were observed at either the peak or late time points, we maintain that mild CO exposure does not alter α_1 adrenergic sensitivity in intact humans in either resting or exercising tissue.

Chapter 6: Conclusion

6.1 Main findings

Our findings indicate that mild CO exposure does not influence vascular reactivity to moderate exercise, nor to α_1 adrenergic sensitivity. Irrespective of CO condition, moderate handgrip exercise augmented steady-state blood flow and conductance in the working muscle by similar degrees, and no effects of CO were observed on systemic hemodynamics. As previous reports have demonstrated augmented hyperemic responses with greater degrees of CO exposure, this suggests a requisite threshold level of CO exposure to impact functional vasodilation outcomes (González-Alonso *et al.*, 2001, 2002). Similarly, indices of α_1 adrenergic sensitivity determined across the dynamic vasoconstrictor response to systemic phenylephrine infusion were unchanged with CO exposure. These findings contrast against those from a series of studies which have shown anti-constrictor properties of CO in isolated models, highlighting differences between isolated *vs.* integrative models (Wang *et al.*, 1997*a*; Sammut *et al.*, 1998; Caudill *et al.*, 1998; Kaide *et al.*, 2001, 2004; Koçer *et al.*, 2018), but are in agreement with previous work in humans that α_1 adrenergic sensitivity is only altered by particular vasodilatory cascades, primarily those involving endothelial stimulation and hyperpolarization (Hearon *et al.*, 2016; Terwoord *et al.*, 2021).

6.2 Future directions

Translation of reductionist mechanistic research in isolated models towards intact humans is complicated by the numerous compensatory mechanisms acting to maintain consistent vascular tone and blood flow during perturbations in vasoactive signaling. Future investigations aiming to clarify the role of CO on vascular function in humans may address this through the inhibition of alternate vasodilators, such as through the use of NG-monomethyl-L-arginine and indomethacin for the inhibition of NO and PGs respectively. As an extension of this, the null findings of the present study – indicating no impact of CO

on functional sympatholysis – may allude to a novel opportunity for the continued investigation of this elusive phenomenon. To date, it is uncertain whether ATP is obligatory for sympatholysis due to the unavailability of selective P2Y antagonists in humans. However, a top-down approach, harnessing the capacity of CO to inhibit ATP from erythrocytes upon deoxygenation, may prove invaluable to address this mechanistic question. While the administration of CO comes with expected confounders due to its direct effects on enhancing sGC and BKca signaling and indirect effects as a hypoxemic stimulus, these particular pathways do not appear to be involved in sympatholysis, making CO a tempting candidate for an ATP knock-out model for the exploration of the role of this critical and diverse vasodilator.

Exposure to CO is common in day-to-day life, with individuals frequently exposed to smoke typically exhibiting elevated COHb levels akin to those seen in the present study. Our findings improve our understanding of how this environmental agent may alter vascular function during exercise, a topic of considerable importance for individuals such as firefighters who are required to perform physically during considerable exposure to CO. Moreover, following in the well-documented footsteps of NO, CO has demonstrated an array of cardioprotective properties, and its therapeutic applications are a topic of considerable interest, including for the prevention of preeclampsia during pregnancy (Wikström *et al.*, 2010; Venditti *et al.*, 2014; Kim & Choi, 2018). Our findings support that mild administration of CO in controlled settings is well tolerated, with minimal impact on vascular function.

6.*3 Conclusion*

To conclude, in this study we have demonstrated that mild carbon monoxide exposure does not alter vascular reactivity to exercise, both in terms of vasodilation to maintain oxygen delivery and in sympathetically mediated vasoconstriction to balance the distribution of blood flow to critical tissues. These findings add to the growing body of knowledge surrounding the mechanistic properties of this gasotransmitter molecule, and highlight novel opportunities for future investigation of its applications in both research and clinical settings.

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Appendices

Appendix I: Ethics Approval for Human Subjects (University of Alberta)

Approval Form

Date:	February 05, 2021
Principal Investigator:	Craig Steinback
Study ID:	Pro00096251
Study Title:	The Impact of Carbon Monoxide and Altitude on Vascular Function
Protocol Number:	N/A
Approval Expiry Date:	February 4, 2022

Date of Informed Consent:	Approval Date	Approved Document
	2/5/2021	Consent Form (lowlander version)
	2/5/2021	Consent Form (highlander version)
	2/5/2021	Optional Biobanking Consent Form
Funding/Sponsor:	NSERC - Natural Sciences And Engineering Research Council	

Thank you for submitting the above study to the Health Research Ethics Board - Biomedical Panel, which was reviewed by special sub-committee in March 2020. All issues arising from the reviews have been addressed. The study is now approved. The following documentation forms part of this approval:

- Protocol Version 4 (07 Jan 2021)
- Product Monograph for Sodium Nitroprusside (25 Jan 2011)
- Phenylephrine Label (Revised 11/2019)
- Consent Form_lowlander Version 4 (dated 04 Feb 2020, revisions uploaded 04 Feb 2021)
- Optional Biobanking Consent Form Version 3 (-2 Jul 2019)
- Consent Form_highlander Version 4 (dated 04 Feb 2020, revisions uploaded 04 Feb 2021)
- Epworth_Sleepiness_Scale
- Medical screening form
- lake louise and ESQ

We acknowledge receipt of the Health Canada No Objection Letter re: Protocol # PRO00096251 Revision 3 (25 Aug 2020)

Any proposed changes to the study must be submitted to the REB for approval prior to implementation. A renewal report must be submitted next year prior to the expiry of this approval if your study still requires ethics approval. If you do not renew on or before the renewal expiry date (February 4, 2022), you will have to re-submit an ethics application.

The membership of the Health Research Ethics Board - Biomedical Panel complies with the membership requirements for research ethics boards as defined in Division 5 of the Food and Drug Regulations and the Tri Council Policy Statement. The HREB - Biomedical Panel carries out its functions in a manner consistent with Good Clinical Practices.

Inquiries regarding administrative approval, and operational approval for areas impacted by the research should be directed to the Alberta Health Services Research Administration office (Edmonton Zone) at nactrc.contracts@albertahealthservices.ca or Covenant Health Research Administration (research@covenanthealth.ca) as applicable.

Approval by the Research Ethics Board does not encompass authorization to recruit and/or interact with human participants at this time. Researchers still require operational approval as applicable (eg AHS, Covenant Health, ECSD etc) and where in-person interactions are proposed, institutional and operational requirements outlined in the** Resumption of Human Participant Research - June 24, 2020 **must be met.

Sincerely,

Donald W. Morrish, MD, PhD, FRCPC
Associate Chair, Health Research Ethics Board – Biomedical Panel

Note: This correspondence includes an electronic signature (validation and approval via an online system).

Appendix II: No Objection Letter (Health Canada)

Health Canada Santé Canada

Therapeutic Products Directorate
5th Floor, Holland Cross, Tower B
Address Locator # 3105A
OTTAWA, Ontario
K1A 0K9

25 August 2020

Dr. Craig Steinback
Associate Professor
The Governors of the University of Alberta
1-059A Li Ka Shing Centre for Research
11203-87 Ave NW
EDMONTON, Alberta
T6G 2H5
(780) 492-5553

Your file Votre référence
HC6-24-c241154
Our file Notre référence

No Objection Letter RE: Protocol # PRO00096251 (Revision 3)

Dear Dr. Craig Steinback:

I am pleased to inform you that the information and material to support your Clinical Trial Application for **PHENYLEPHRINE HYDROCHLORIDE / SODIUM NITROPRUSSIDE / CARBON MONOXIDE**, control number **241154**, received on July 20, 2020, have been reviewed and we have no objection to your proposed study. I would remind you of the necessity of complying with the *Food and Drug Regulations,* Division 5, in the sale of this product for clinical testing. In addition, the regulations impose record keeping responsibilities on those conducting clinical trials. You are also reminded that all clinical trials should be conducted in compliance with the Therapeutic Products Directorate's *Guideline for Good Clinical Practice*.

Please note that Health Canada has implemented electronic reporting of adverse drug reactions and is currently in pilots with some sponsors. Those sponsors who have an established electronic connection with Canada Vigilance Production stream should submit their reports using the distribution rules provided to them by Health Canada, and reporting to multiple directorates is no longer required. For the sponsors who have not yet established this connection, they should continue submitting their reports to the applicable directorate by fax or by courier. The following website provides further clarification on Health Canada's adverse drug reactions reporting requirements for clinical trials: https://www.canada.ca/en/health-canada/services/drugs-health-products/drug-products/health-canada-clinical-trials-database.html

Consistent with Health Canada's Notice - *Registration and Disclosure of Clinical Trial Information* of November 30, 2007, sponsors are encouraged to register their clinical trials within 21 days of the trial's onset, using a publicly available registry that conforms with international standards for registries such as: Clinicaltrials.gov (www.clinicaltrials.gov); Current Controlled Trials (www.controlled-trials.com).

Should you have any questions concerning this letter, please contact the Office of Clinical Trials (613) 941-2132.

Yours sincerely,
This document has been signed electronically using the Health Canada docuBridge system.

Larissa Lefebvre
Acting Manager, Submission Management Division
Office of Clinical Trials

LL/mw

Canada

Appendix III: Consent Forms for Participants

PARTICIPANT CONSENT FORM

Title of Research Study: The impact of carbon monoxide and altitude on vascular function

Principal Investigator: Dr. Craig Steinback, PhD craig.steinback@ualberta.ca

Research Coordinators: Nicholas Cheung, BSc nkcheung@ualberta.ca
Scott Thrall, BSc sthrall@ualberta.ca

This letter provides the information you need to make an informed decision as to whether you wish to take part in our study. Before you make a decision, one of the researchers will go over this form with you. Please ask questions if you feel anything needs to be made clearer. You will be given a copy of this form for your records.

Why am I being asked to take part in this research study?
You are being asked to participate in this research study because you are healthy and of lowlander descent. The aim of our study is to assess how carbon monoxide and high altitude alter blood vessel health. In addition, we are interested in comparing these responses between lowlanders and Sherpa highlanders, who have adapted to high altitude for several thousand years.

What is the reason for doing the study?
At high altitude you are exposed to reduced oxygen. Low oxygen reduces the health of your blood vessels. During travel to high altitude, we may also be exposed to carbon monoxide. We may be exposed to carbon monoxide through vehicle fumes, incense, and second-hand smoke exposure. Carbon monoxide is viewed as a life-threatening toxic gas, and may also reduce the health of your blood vessels. Study of low oxygen and carbon monoxide together may help us understand how carbon monoxide affects our cardiovascular system at altitude and as we travel.

For the experimental protocol we will measure how your blood vessels respond to several tests. A doctor will place an intravenous (IV) catheter into your arm where we will give you very small doses of safe drugs that are commonly used in hospitals. For the first test we will use a blood pressure cuff to restrict blood flow for 5 minutes, then measure your blood vessels after it is released. For the second test we will measure your blood vessels while you squeeze a handgrip device and with a very small dose of safe drugs. The way that your blood vessels respond to these tests will give us information on the health of your blood vessels. Then we will have you take one breath of a safe amount of carbon monoxide, similar to amounts experienced through smoking, and repeat the tests. Throughout the protocol we will be measuring blood flow through your arm using ultrasound. The protocol will be completed at sea level (Edmonton, AB; 645m), and at high altitude for those joining our high-altitude expedition (Khumbu valley, Nepal, 3800m).

Am I eligible to take part in this study?
You have already been pre-screened for general criteria making you eligible for this study. Following you giving consent, we will provide you with a questionnaire designed to gather more information on your current and previous health. If any current or previous health concerns are identified which exclude you from participating, we will tell you and the testing session will be cancelled.

What will happen in the study?
If you participate in this study, you will be asked to participate a total of two times. You will be asked to come into the Neurovascular Health Lab (VVC 4-269). The location of the lab is at the University of Alberta room 4-269 in the Van Vliet Centre building on 87 Ave and 114 St NW. It is accessible by city transit and near two train stations (University or Health Sciences/Jubilee). You will need to visit the lab twice in Edmonton, AB. Each testing session will take approximately three hours. For those joining our high-altitude expedition, the tests will be repeated at a high altitude research facility in Nepal.

To start, we will measure your weight and height. For females, you will be asked to take a urine pregnancy test. Then, we will ask you to sit in a comfortable chair where we will take measures of your heart and blood vessels.

Equipment:

- An IV catheter will be placed into a blood vessel in your arm. We will use this to infuse a small amount of fast acting drugs into and collect small (2ml) blood samples.

- Three small electrocardiogram (ECG) stickers will be used to monitor your heart rate throughout the experiment. One sticker goes on your left shoulder, one on the right shoulder, and one on your left side.

- An arm cuff will be placed around one arm for taking blood pressure in the same way that your doctor would. In addition, a small finger cuff will be placed on the middle finger of the same arm. This finger cuff will allow us to measure your blood pressure during every heartbeat.

- A small clip will be placed on the index finger of one hand to measure the oxygen in your blood.

- An ultrasound probe will be used on the inside of one of your elbows to measure changes in blood flow. In addition, a second probe will be used on the left side of your rib cage to measure your heart as it beats.

- A mouthpiece (similar to one used while snorkeling) and nose clip to allow us to measure the rate and depth that you breathe as well as the amount of oxygen and carbon dioxide you breathe in and out.

- A sensor will be placed on your forearm to measure oxygen in the muscle.

- Once equipment is set up, we will ask you to breathe through the mouthpiece. The mouthpiece, finger clip, and ECG leads must be worn throughout the experiment. If the equipment becomes uncomfortable during any part of the protocol, an investigator will help you readjust.

Protocol:
After being instrumented and comfortably seated, we will ask you to lay quietly and relax prior to starting testing. This will allow us to take normal values (baseline) for each measure we are recording. We will also collect small blood samples at rest via the IV. We will then measure the health of your blood vessels using several tests.

First, we will use a blood pressure cuff on the forearm to restrict blood flow for 5 minutes. Upon releasing the cuff, we will measure blood flow returning to the arm. This is called a flow-mediated dilation test. This test is safe, and may cause minor discomfort while the cuff is inflated.

We will then measure blood flow to the arm during light handgrip exercise, and infusion of phenylephrine via the IV. Phenylephrine makes your blood vessels constrict temporarily. After determining your maximal grip strength by squeezing a handheld handgrip device as hard as possible 3 times, we will have you squeeze the device rhythmically (1 second contraction per 2 seconds rest, timed to a metronome) at a light intensity for 5 minutes. During the last 2 minutes, we will infuse a dose of phenylephrine and measure the changes in blood flow in the arm. We will then have you squeeze the device at three increasing intensities for 2 minutes each. We will measure blood flow to the arm at each intensity.

Finally we will infuse a dose of sodium nitroprusside, which makes your blood vessels dilate temporarily, and measure blood flow in your arm.

For the second portion of the study, we will measure the effects of carbon monoxide on your cardiovascular system. We will ask you to breathe through a rebreathing apparatus containing a gas mixture of either safe levels of carbon monoxide, according to standard procedures, or room air for the control trial. The gas mixture you are given will be randomized, meaning that you may be given either carbon monoxide or room air (control) on the first day, then the other gas mixture on the second day. It will also be blinded, meaning you will not be told which gas mixture you were given on either day. You are unlikely to experience noticeable symptoms on the carbon monoxide testing day. After you have breathed the gas mixture given, we will then repeat the flow-mediated dilation test, the handgrip tests, and the sodium nitroprusside infusion test.

What will I be asked to do while I am in the study?
You will be asked to not eat anything for 2 hours before coming to the lab. We ask you not have any caffeine before your test or alcohol the night before. Finally, please do not go to the gym or do any physical activity more than normal walking / stair climbing the morning of your visit.

What are our COVID-19 safety procedures?
All participants and researchers are screened for COVID-19 symptoms before entering the lab, and all equipment and surfaces have been sanitized prior to your arrival. We will also be wearing personal protective equipment (PPE), including a face mask and a face shield. We will regularly sanitize our hands, and hand sanitizer and face masks will be provided for you as well while you are in the lab.

What are the benefits to me?
You are not expected to benefit directly from being in this research study.

What are the risks and discomforts?

Venous catheter: Placement of a needle into your blood vessel may cause mild pain or bruising. Some people may experience light headedness or fainting. While the catheter is in place, you should not feel much discomfort, but if you do, please tell our doctor. There is a very small risk that the vein can become damaged or infected. We do our best to avoid this small risk by using highly sterile and proven techniques, and employing highly experienced staff.

Carbon monoxide: The concentration of carbon monoxide used should not cause noticeable symptoms. There is a rare possibility of mild headaches or nausea.

Pharmacological infusions: The risks associated with the drug administration include increases or decreases in blood pressure and heart rate, and flushing or warm sensations at the site of the infusion. It is possible for you to have a local allergic reaction to a pharmacological infusion, but we are not aware of any cases in which this has been reported.

***Ultrasound*:** The ultrasound used to image your blood vessels has no known risks.

Blood Pressure Monitors: The finger and/or arm cuff measuring blood pressure may cause some discomfort including numbness, tingling, or discoloration (bruising). These will return to normal soon after the cuff is removed.

Abnormal findings: Within this study, we take many different measurements of your heart and blood vessels. It is possible, but rare, that we may find abnormalities that require further consultation from a medical professional. If any abnormal findings are identified during your participation in the study, we will provide you with full details and contact your chosen medical professional (e.g. your family doctor or obstetrician). With your permission, we will refer you to a responsible medical doctor.

If you experience any abnormal and ongoing problems as a result of any of the study procedures, we ask that you inform the researchers immediately. We will ensure that you receive necessary medical treatment, at no additional cost to you. Again, we will provide you with full details of the study and our measurements, contact your chosen medical professional (e.g. your family doctor) and refer you to a responsible medical doctor. If you suffer any ongoing problems, please call Dr. Craig Steinback at 780-492 5553. Should you need urgent medical care, please go to the hospital.

Other: If we find out anything new during the course of this research which may change your willingness to be in the study, we will tell you about these findings.

Do I have to take part in this study?
Being in this study is your choice. If you decide to be in this study, you can change your mind and stop being in the study at any time, and it will in no way affect the care or treatment you are entitled to.

Can my participation in the study end early?
You are free to withdraw from this study at any time for any reason. You can do this by contacting the investigators. If after participating in the study you wish to remove your information or blood samples from the study, you have until December 31, 2023 to do so. After this time all information will be used. We may request that you withdraw from the study during the protocol if we are at all worried about your general health (i.e. high blood pressure, irregular heart rhythm etc.). We will notify you of our reason should this occur.

Will I be paid to be in the research?
You will not be paid for participation in this study, nor should you incur any expenses by participating. If needed, we have two parking spots reserved in a covered lot nearby. If you travel to the lab via public transit, we will reimburse the cost up to $15.00 CAD. Expenses will be reimbursed by the study coordinator through petty-cash; no additional information will be required on your part.

Privacy and Confidentiality
During this study we will be collecting information (or "study data") about you. We will use the data to help answer research questions and we will share (or "disclose") your information with others such as the study sponsor and other researchers. Your study data may also be shared with government departments involved in the approval of drugs for sale in a country. These departments are often called "regulatory authorities". An example of a regulatory authority is Health Canada.

Below we describe in more detail how your data will be collected, stored, used and disclosed.

What data will we be collecting?
During this study we will be collecting data about you. Examples of the types of data we may collect includes your name, where you live, your ethnic background, your date of birth, your age, your health conditions, your health history, your medications and results of tests or procedures that you may have had. We will only look for and collect the information that we need do the research. We will get this information by asking you questions and doing the tests outlined in this form. We will also look at your medical chart (paper or electronic) held by the study doctor or other doctors you have seen (such as your family doctor).

How will the study data be stored?
The study data we collect which will include your name will be securely stored by the study doctor during and after the study. We will also put a copy of this consent form in your clinical record, so that doctors you see in the future will know you were in the study. In Canada, the law says we have to keep the study data stored for at least 25 years after the end of the study.

The study doctor will not release your name to anyone unless the law says that they have to.

How will the study data be used?
Your study data will be coded (with a number) so that it no longer contains your name, or anything else that could identify you. Only your study doctor will be able to link your coded study data to you. Your coded study data will be sent to the Sponsor. This coded study data will be kept by the Sponsor in a secure manner and will be used now and in the future to learn more about how the study drugs (and possibly similar drugs) works and how safe they are.

This coded study data may also be shared with people who work with the Sponsor and with regulatory authorities. The Sponsor and/or the people they work with may be located outside of Canada, in countries that do not have the same privacy laws as in Canada. However, because nothing that is sent to the Sponsor will contain your name, no one who uses this information in the future will be able to know it came from you. The risk to your privacy, then, should be very small.

When the study is done, the Sponsor may place your coded study data into a secure database. The coded data may then be used to answer other research questions in the future. Only researchers who have the training and experience to do the research (also known as "qualified researchers") will be allowed to use the data. The data will be de-identified before being sent to the database, so it cannot be linked to you. The data will be stored indefinitely.

Who will be able to look at my health data?
During research studies it is important that the data we get is accurate. Therefore, your study data and original medical records may also be looked at by people from: the study sponsor, the University of Alberta auditors and members of the Research Ethics Board, Health Canada, and/or other foreign regulatory authorities.

By signing this consent form you are saying it is ok for the study doctor/staff to collect, use and disclose information from your medical records and your study data as described above.

If you would like to see the study data collected about you, please ask the study doctor. You will be able to look at the study data about you and you can ask for any mistakes to be corrected. The study doctor may not be able to show you your study data right away and you may have to wait until the study is completed or another time in the future before you can see your study data.

If you leave the study, we will not collect new health information about you, but we will need to keep the data that we have already collected.

What happens if I am injured because of this research?
If you become ill or injured from being in this study, you will receive necessary medical treatment at no additional cost to you. By signing this consent form you are not releasing the investigator(s), institution(s) and/or sponsor(s) from their legal and professional responsibilities. If you suffer a research-related injury, please call Dr. Craig Steinback at 780-492 5553. Should you need urgent medical care, please go to the hospital.

What if I have questions?
If you have any questions about the research now or later, please contact Dr. Craig Steinback at 780-492 5553. If you have any questions regarding your rights as a research participant, you may contact the Health Research Ethics Board at 780-492 2615. This office is independent of the investigators.

This study is funded by the Natural Sciences and Engineering Research Council of Canada (NSERC).

Title of Study: The impact of carbon monoxide and altitude on vascular function

Principal Investigator: Dr. Craig Steinback, PhD Phone Number: 780-492 5553
Research Coordinators: Nicholas Cheung, BSc
Scott Thrall, BSc

	Yes	No
Do you understand that you have been asked to be in a research study?	☐	☐
Have you read and received a copy of the attached Information Sheet?	☐	☐
Do you understand the benefits and risks involved in taking part in this research study?	☐	☐
Have you had an opportunity to ask questions and discuss this study?	☐	☐
Do you understand that you are free to leave the study at any time, without having to give a reason and without affecting your future medical care?	☐	☐
Has the issue of confidentiality been explained to you?	☐	☐
Do you understand who will have access to your records, including personally identifiable health information?	☐	☐
Do you want the investigator(s) to inform your family doctor that you are participating in this research study? If so, give his/her name _____	☐	☐

Who explained this study to you? _____

I agree to take part in this study:

Signature of Research Participant: _____

(Printed Name) _____

Date: _____

I believe that the person signing this form understands what is involved in the study and voluntarily agrees to participate.

Signature of Investigator or Designee: _____

Date: _____

THE INFORMATION SHEET MUST BE ATTACHED TO THIS CONSENT FORM AND A COPY GIVEN TO THE RESEARCH PARTICIPANT

Appendix IV: Sample "Day Of" Sheet and checklists for protocol

Participant ID: Participant Information Form CARMA-2021

Date:

Age: _____ Height (m): _____Weight (kg): _____

Time of last meal: _____

Size of last meal (small/medium/large): _____

What day did you last start menstruation:_____ Pregnancy Test: + / -

Y / N Have you abstained from caffeine for the past 12 hours, if not how long: _____

Y / N Have you abstained from alcohol for the past 12 hours, if not how long: _____

Y / N Have you abstained from strenous exercise for the past 24 hours, if not how long: _____

Test Day Staff:

Computer: _____

FMD: _____

Drug Administration: _____

RH Handgrip: _____

Bloods:_____

Pretest Data:

Daily atmospheric Pressure (mmHg) _____ Temperature: _____

SNP/PE doses

Sodium Nitroprusside dosage (μg) :_____ SNP Dosage/ Blood Volume (ml):

_____

Phenylephrine dosage (μg) : _____ PE Dosage/ Blood Volume (ml): _____

CO DOSAGE:_____

BASELINE:

MANUAL BP			

Dose 1 SNP BP Pre:_____ Post:_____

COHb:_____

MANUAL BP			

Handgrip

MVC: _____ 15%: _____

Dose 1 phenylephrine BP Pre:_____ Post:_____

Rebreathe: SHAM / CO

PARTICIPANT STAND UP/ BATHROOM VISIT

MANUAL BP			

POST-REBREATHE:

COHb:_____

Handgrip

MANUAL BP			

Dose 2 phenylephrine _____, BP Pre:_____ Post:_____

Dose 2 SNP_____, BP Pre:_____ Post:_____

Appendix V: Health History Questionnaire

SECTION 1: PERSONAL DATA (please print)
Name: _____

Date of birth: _____

Age: _____

Where do you live (village / region)? _____

Have you descended from high altitude (Simien Mountain Park) in the last year?

Y / N

If yes, when and for how long?

Place of birth: _____

Place of parents' birth:

Mother: _____

Father: _____

Place of grandparents' birth:

Mother's side
Grandmother: _____

Grandfather: _____

Father's side
Grandmother: _____

Grandfather: _____

Occupation: _____

SECTION 2: MEDICAL HISTORY

1. Y / N Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?

2. Y / N Do you feel pain in your chest when you do physical activity?

3. Y / N In the past month, have you had chest pain when you were not doing physical activity?

4. Y / N Do you lose your balance because of dizziness or do you ever lose consciousness?

5. Y / N Do you have a bone or joint problem (e.g. back, knee, hip and or wrist) that could be made worse by a change in your physical activity?

6. Y / N Is your doctor currently prescribing drugs (e.g. water pills) for your blood pressure or heart condition?

7. Y / N Do you know of any other reason you should not do physical activity?

8. Do you have, or have you ever had, problems with any of the following?

	Yes	No
i. Heart or blood vessels (these might include but are not limited to: heart attack, stroke, heart murmur angina, coronary artery disease, high blood pressure, high cholesterol, congenital heart disease, any heart operation, bleeding or clotting disorders)	___	___
ii. Nerves or brain	___	___
iii. Breathing or lungs	___	___
iv. Hormones, thyroid, or diabetes	___	___
v. Muscles, joints, or bones	___	___

vi. Other (please list) _____

9. Please list the diagnosis or/or briefly describe any problems identified in #8

_____
_____
_____
_____

Are you presently taking any medications or supplementations? If yes, please list.

10. Do you have any allergies to medications, adhesive tape, latex, etc.?

11. Do you currently smoke? _____ **yes/no** Number of cigarettes/day_____

If previous smoker date of last cigarette_____ (year, month)

Men please proceed to signature section.

For women only: Hormonal fluctuations during the menstrual cycle can impact physiological function. Please answer the following questions (*i - vi*) regarding your menstrual cycle history:

i. Are you currently having menstrual periods?

____ *No*
____ Yes
Date of the start of last menstrual period _____

ii. Have you given birth in the last 12 months?

____ *No*
____ Yes

iii. Are you pregnant?

____ *No*
____ Yes

iv. Are you currently taking oral or any other form of hormonal contraceptives (e.g. intrauterine devices)?

_____ Yes Brand _____
_____ No

v. Currently, what is the average duration of your menstrual cycle (A full cycle goes from the start of menstrual flow [menses] to the start of the next menstrual flow [menses])? The average cycle length is 28 days.

____ *days.*

vi. How many days do you typically experience menstrual flow each cycle? Please check the correct response below:

- ❑ 0 days
- ❑ 1 day
- ❑ 2 days
- ❑ 3 days
- ❑ 4 days
- ❑ 5+ days

vii. Please estimate the number of menstrual cycles you have had in the past 12 months:

____ (number) menstrual cycles.

I acknowledge that the study investigators completed this form according to my specifications; this information is true to the best of my knowledge.

_____	_____
Participant Name	Participant Signature

Date (dd/mm/yyyy) : _____

Appendix VI: Carboxyhemoglobin stability

Table 3. Post-testing COHb stability

	COHb (%) – 10-min post rebreathe	COHb (%) – immediately post-testing	Δ COHb
i	6.7	6.2	0.5
ii	6.2	5.7	0.5
iii	5.9	5.1	0.8
Average	---	---	0.6

Venous carboxyhemoglobin (COHb) levels were tested in three individuals 10-minutes after the carbon monoxide (CO) rebreathe as normal, and again immediately following protocol completion (T + ~60 minutes) as an assessment of the stability of CO at the time of testing for the procedures of the present study. These results indicate that a limited degree of CO elimination occurs between the initial point-determination of COHb and the time of testing. These elimination rates are in accordance with previously reported elimination half-times for inhaled CO, indicating that the CO load utilized in the present study can be estimated reliably based on these previously reported findings (Bruce & Bruce, 2006).