Neurovascular Responses Following Acute Hypoxia

by

Rachel Skow

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University Of Alberta

Abstract

Introduction: The sympathetic nervous system is the primary regulator of the "fight or flight" response, with muscle sympathetic nerve activity (MSNA) increasing in response to various stressors, including low oxygen (hypoxia; HX). MSNA is an important regulator of cardiovascular function, whereby an acute increase in MSNA constricts blood vessels and increases arterial blood pressure (ABP). Previous work from our lab and others has shown that MSNA increases during HX and remains elevated following hypoxic exposure for a period beyond the original exposure. However, it is unknown if and how the elevation in MSNA affects vascular function following acute HX.

Hypothesis: We hypothesize that the increase in MSNA that persists following an acute HX exposure will be associated with acute changes in vascular function measured as increases in arterial stiffness and femoral vascular resistance (FVR).

Methodology: We recruited 16 healthy volunteers (8 female) who were 24 ± 3 years old and instrumented them to continuously measure ABP, heart rate, arterial stiffness (pulse wave velocity; PWV), FVR and MSNA. Participants breathed through a mask that allowed for changes in inspired gases (room air vs. HX). Following ten minutes of rest, participants underwent a ten minute poikilocapnic HX exposure (~80% oxygen saturation), followed by a twenty min recovery period

Results: ABP was not increased during HX, but systolic blood pressure was increased at 15 minutes of recovery. Cardiac output was increased during, but not following HX and, similarly, total peripheral resistance (TPR) was decreased during, but not following HX. Additionally, FVR was decreased during HX and remained decreased up to ten minutes

following HX. Neither central (aortic) nor peripheral (upper or lower limb) PWV were changed during or following HX. Lastly, MSNA burst frequency (burst per minute) and burst amplitude were increased following HX.

Discussion: This study adds to the current knowledge in that it is the first to comprehensively analyze both sympathetic and cardiovascular responses during and during and following acute poikilocapnic HX. Specifically, the augmented burst amplitude may imply that the peripheral chemoreflex gain is increased following poikilocapnic HX and that this may contribute to the overall increase in MSNA following HX. Further the differences in the TPR and FVR responses highlight the differential regulation of blood flow during and following acute HX. We believe these data are important for understanding the physiological consequences of environments, activities and clinical disorders associated with poikilocapnic HX (i.e. travel to altitude).

Preface

This thesis is an original work by Rachel Skow. The research project, of which this thesis is a part, received ethics approval from the University of Alberta Research Ethics board, Project Name "Cardiovascular regulation following acute exposure to reduced oxygen", Pro00040669, October 25, 2013. No part of this thesis has been previously published.

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List of Abbreviations & Symbols

ABP	arterial blood pressure
DBP	diastolic blood pressure
FBF	femoral blood flow
FBV	femoral blood velocity
FVR	femoral vascular resistance
HR	heart rate
HX	hypoxia
MAP	mean arterial blood pressure
MSNA	muscle sympathetic nerve activity
PaO_2	pressure of arterial oxygen
PO_2	pressure of oxygen
$P_{ET}CO_2$	partial pressure of end-tidal carbon dioxide
$P_{ET}O_2$	partial pressure of end-tidal oxygen
PWV	pulse wave velocity
Q	cardiac output
SaO_2	arterial oxygen saturation
SBP	systolic blood pressure
SNS	sympathetic nervous system

Chapter 1. Introduction

Oxygen (O_2) is critical to survival, as such, any perturbation which affects oxygen availability or utilization must be met with an appropriate response. The absence of an appropriate response may result in decreased physiological function, clinical illness, or death. There are many reasons why a person might experience low O_2 (hypoxia; HX) including ascent to high altitude, holding ones breathe while diving, and diseases such as obstructive sleep apnea (OSA).

Altitude results in decreased O_2 availability due to decreased atmospheric pressure. For example, the atmospheric pressure at Mt. Everest base camp in Nepal (5400m above sea level) is almost half of that at sea level (~400 Torr at base camp vs 760 Torr at sea level (West *et al.*, 1983); therefore, the partial pressure of O_2 (PO_2) is also half of that at sea level (~80 Torr PO_2 vs 160 Torr PO_2 at basecamp and sea level, respectively). Thousands of people ascend to similar altitudes (or higher) every year, if the availability of O_2 is reduced, it will affect the amount of O_2 that can be utilized by the tissues. **Figure 1** outlines the typical cascade of O_2 through the body at sea level (solid line) and at 4500m (dashed line; Hurtado, 1964). Specifically, arterial O_2 (PaO_2) at 4500m is similar to venous O_2 at sea level; the reduced availability of O_2 at each step in the cascade requires adaptations to be made in order to maintain sufficient O_2 delivery to the tissues.

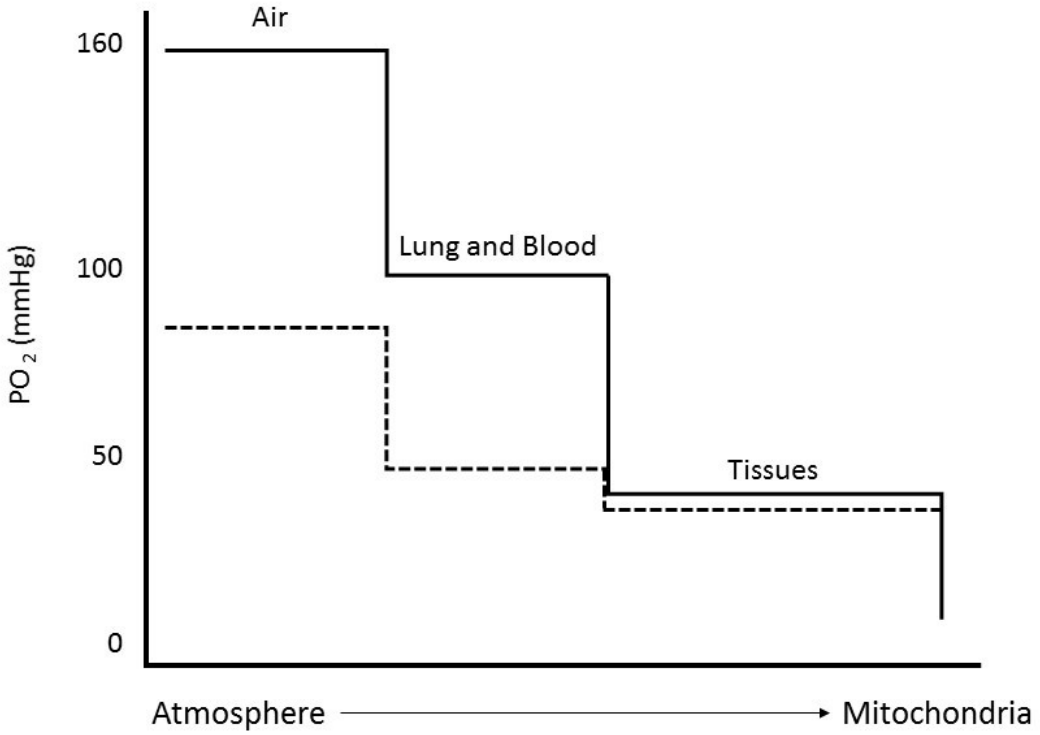

Figure 1. Oxygen cascade at sea level (solid lines) and at 4500m (dashed lines). There are the differences in the partial pressure of oxygen (PO_2) in the air between sea level and altitude that are directly translated to differences in arterial blood O_2 content. Modified with data from Hurtado *et al.* 1964.

A healthy human will adapt to acute HX environment by increasing their ventilation 2-fold by stimulating the chemoreceptors and cardiac output (Q) up to 30% by increasing their heart rate (HR) to ensure sufficient O_2 delivery (Guyton and Hall, 2006). There is also a significant increase in the activity of the sympathetic nervous system (SNS) to maintain blood vessel tone, arterial blood pressure (ABP), and blood flow to critical tissues. Acclimatization to longer HX exposures (days to weeks) involves renal excretion of bicarbonate (to counter-balance the alkalosis from hyperventilation) and increased hemoglobin concentrations and increased pulmonary pressure. Full acclimatization has been suggested to take 11.4 days for every vertical kilometer in altitude and is achieved when the increase in hemoglobin reaches a plateau (Zubieta-Calleja. *et al.*, 2007); full acclimatization at 4500 m would take approximately 51 days.

Not every person adapts well to HX, and even a brief exposure to HX (within a day) can have consequences (West, 2012). Maladaptation to HX can result in dangerous outcomes including acute mountain sickness, high altitude pulmonary edema, or high altitude cerebral edema. The risk of developing any of these conditions depends largely on the rate of ascent to altitude and the altitude (West, 2012). Both high altitude pulmonary edema and high altitude cerebral edema can be fatal, and the best treatment is O_2 supplementation and descent to lower altitude, indicating the importance of reduced O_2 availability in the pathogenesis of these disorders.

Acute (less than 20 minutes) exposure to HX results in increases in SNS activity (SNA) that are immediate and reflective of the strength of the stimulus (level of O_2), the duration of the stimulus and the control of carbon dioxide (CO_2). Specifically, muscle SNA (MSNA) is increased to a greater extent during HX exposures that mimic higher altitudes, are longer in

duration, and have higher CO_2 levels. Regardless of the perturbation, this increase in MSNA has been shown to persist following exposure for a time at least as long as the original exposure.

The increase in MSNA appears to be the result of a response to the vasodilation occurring during HX. As such, ABP is only increased during acute exposures with CO_2 levels greater than those which would occur if a person was freely breathing (increasing ventilation results in a decrease in CO_2 below eupneic/resting levels), or during long term exposures (see Appendix I). That is to say, ABP is increased when MSNA is increased to a greater extent. Further, ABP may only become increased after some delay and may occur during the time following HX exposure (see Appendix I).

Despite an increase in MSNA and ABP, vasodilation typically results in a decrease (or no change) in vascular resistance. There may be a regional response in changes in vascular resistance in that peripheral resistance (i.e. FVR) is maintained while TPR is decreased, however this has not been previously investigated during or following acute poikilocapnic HX (see Appendix I). This would suggest a re-distribution of blood flow during HX to favor oxygen delivery to central organs/brain. The decrease in TPR has been shown to persist into the recovery (or have a delayed response becoming apparent in the recovery) during short term isocapnic HX exposures.

Arterial stiffness may be decreased peripherally during HX exposure, but appears to return to baseline during long term exposure. However, central arterial stiffness (clinical index of cardiovascular health) may be increased following long term HX exposure. These changes may be the result of vascular dysfunction occurring as the result of HX exposure.

Understanding the differences in the results will help clarify for future studies the impact of exposure severity, duration and CO_2 control on the vascular outcomes.

Purpose and Hypothesis

The purpose of this study is to determine the influence of persistent MSNA following acute poikilocapnic hypoxia (ten minutes at SaO_2 ~80%) on vascular function. The literature is gapped in the investigation of acute poikilocapnic HX exposure in that no one has comprehensively investigated the effects on the SNS and cardiovascular function. Specifically, we will focus on the responses of MSNA, ABP, vascular resistance, and arterial stiffness during a ten-minute poikilocapnic HX exposure and twenty-minute recovery. To date, no study has investigated the MSNA response following poikilocapnic HX and no study has measured MSNA burst occurrence or amplitude during acute poikilocapnic HX exposure. Additionally, no study has assessed femoral blood flow, femoral vascular resistance or arterial stiffness (central or peripheral) during or following acute poikilocapnic HX. Lastly, no study has measured past ten minutes of recovery for the variables that have been previously assessed (HR, ABP, and forearm blood flow).

In order to comprehensively assess the SNS and cardiovascular responses following poikilocapnic HX, we will measure: 1) all aspects of MSNA (burst frequency, burst occurrence, burst amplitude and total MSNA, 2) all aspects of ABP (MAP, diastolic and systolic ABP; DBP and SBP, respectively), 3) large artery (femoral) vascular resistance to blood flow, and for the first time ever 3) both central and peripheral pulse wave velocity (PWV; measure of arterial stiffness). We hope to add to the literature in a meaningful way to

be able to clarify the effects of CO_2 on the responses observed during and following isocapnic HX exposures (majority of acute HX studies).

We hypothesize that the elevated MSNA during HX will be associated with no change in ABP or femoral vascular resistance (FVR), decreased total peripheral resistance (TPR), and no change in PWV. Further, we hypothesize that each ABP, FVR, TPR and PWV will be increased in the recovery period when MSNA is persistently increased.

Significance

By recording multiple cardiovascular measures simultaneously and continuously, more accurate conclusions regarding the responses of the blood vessels during increased MSNA following HX can be determined. If the augmented MSNA following HX changes vascular function acutely, this may suggest why longer duration exposures have similar or more pronounced effects (See Appendix I). We believe that these data will be important for understanding the physiological consequences on environments, activities and clinical disorders associated with HX.

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Chapter 2. Adaptations in neurovascular function during and following acute poikilocapnic hypoxia in humans

Introduction

The activation of the sympathetic nervous system (SNS) is a compensatory mechanism promoting adequate blood flow distribution to critical tissues during periods of reduced oxygen availability. Direct recording of muscle sympathetic nerve activity (MSNA) by microneurography is the gold-standard measure of activity of SNS output (see Appendix II). Using this method, MSNA has been shown to increase in response to various stressors, including low oxygen (hypoxia; HX), in healthy humans. The increase in MSNA correlates with the level of HX (or simulated altitude), increasing as arterial oxygen (O_2) saturation (SaO_2) decreases and persisting once the original stimulus has been removed (Saito *et al.*, 1988, Xie *et al.*, 2001). So far, HX is the only sympathetic stimulus that shows this pattern/persistence of MSNA (Mark *et al.*, 1985, Vissing *et al.*, 1989). Steinback and Shoemaker (2012) demonstrated that frequency but not amplitude of sympathetic bursts is increased during the recovery period suggesting differential regulation of MSNA following HX; this, and work by Querido *et al,* (2010, 2011) suggest that both baroreceptor and peripheral chemoreceptor (PCR) regulation of MSNA is changed following HX. However, the extent to which the increase in MSNA affects vascular function (i.e. blood pressure, vascular resistance, arterial stiffness) is relatively un-characterized.

Arterial blood pressure (ABP) increases during acute isocapnic (Somers *et al.*, 1989b, Xie *et al.*, 2001, Ainslie and Poulin, 2004, Lusina *et al.*, 2006, Steinback and Poulin, 2008,

Steinback *et al.*, 2009, Vedam *et al.*, 2009, Querido *et al.*, 2010, 2011, Steinback and Shoemaker, 2012) but not poikilocapnic HX exposure (Somers *et al.*, 1989a, Saito *et al.*, 1991, Ainslie and Poulin, 2004, Steinback and Poulin, 2008). However, studies have shown decreases in total peripheral resistance during both isocapnic and hypocapnic HX (Steinback *et al.*, 2009) with no change in peripheral (both upper and lower limb) vascular resistance (Morgan *et al.*, 1995, Xie *et al.*, 2001, Tamisier *et al.*, 2004b, Tan *et al.*, 2013) suggesting that the increase in MSNA is compensatory (i.e. to offset the vasodilation occurring directly by HX; Tamisier *et al,* 2004). Further, central arterial stiffness has been shown to not be changed during short duration HX exposure (Vedam *et al.*, 2009), while peripheral/global stiffness may be decreased during poikilocapnic HX exposure only (Wilkinson *et al.*, 2002, Sharman *et al.*, 2005).

During the recovery period where MSNA is accepted to remain increased, ABP has been shown to remain increased in some (Rowell and Blackmon, 1986, Xie *et al.*, 2001, Lusina *et al.*, 2006, Steinback and Poulin, 2008, Querido *et al.*, 2010, Querido *et al.*, 2011, Steinback and Shoemaker, 2012) but not all (Saito *et al.*, 1988, Morgan *et al.*, 1995, Tamisier *et al.*, 2004a, Cooper *et al.*, 2005, Steinback and Poulin, 2008, Vedam *et al.*, 2009) HX exposures. ABP is more likely to be increased following recovery during isocapnic HX exposures (when it was increased during HX), but is also increased following some poikilocapnic HX exposures (when ABP was not increased during HX; Rowell and Blackmon 1986, Steinback and Poulin 2008, Saito *et al.,* 1988, Cooper *et al.,* 2005). This indicates that the persistent MSNA occurring post-HX may have an effect on the vasculature in a way that is increasing ABP. Indeed, it has been shown that the baroreceptor response curve is shifted following HX exposure (Monahan *et al.*, 2006, Querido *et al.*, 2011) to higher MSNA for a

given DBP with no change in the sensitivity of the system (Halliwill and Minson, 2002). The increase in MSNA post-HX has been suggested to be the result of persistent vasodilation (Tamisier *et al,* 2004) and it may be that as the vasodilatory responses are slowly diminished, the re-setting of the baroreflex causes an over-compensation resulting in increased ABP, vascular resistance and arterial stiffness. Further, it may be that vascular resistance may increase during the recovery only (Tamisier *et al.*, 2004b, Cooper *et al.*, 2005) with no change in arterial stiffness (Thomson *et al.*, 2006, Vedam *et al.*, 2009). To date, no study has comprehensively investigated the vascular outcomes (e.g. changes in resistance or stiffness) following acute poikilocapnic HX exposure. This is important because it most closely mimics situations during which healthy humans would be exposed to HX (i.e. that CO_2 is not maintained).

The purpose of this study was to determine the influence of persistent MSNA following ten minutes of poikilocapnic HX on SNS and cardiovascular responses. Specifically, we focused on the vascular responses during the recovery period following acute HX. These responses included: 1) ABP, 2) femoral vascular resistance to blood flow, 3) central (carotid to femoral) and peripheral (carotid to finger and femoral to toe; upper and lower, respectively) PWV. This is the first study designed *a-prori* to comprehensively investigate the responses following acute piokilocapnic HX. We hypothesize that the elevated MSNA during HX will be associated with no change in ABP or femoral vascular resistance (FVR), decreased total peripheral resistance (TPR), and no change in PWV. Further, we hypothesize that each ABP, FVR, TPR and PWV will be increased in the recovery period when MSNA is persistently increased.

Methods

Participants

Sixteen (8 female) participants aged 24 ± 3 (mean ± SD) were recruited for this study. Subjects were all non-smoking, non-hypertensive, non-obese (BMI was 22.4 ± 1.7kg/m^2, range 18.7-26.0; see **Table 1**) and not taking medications other than contraceptives. Females were tested in their self-reported early-follicular phase (i.e. during menstruation) with the exception of those who did not cycle who were tested at their convenience, to minimize the risk of confounding results due to differences in cycling hormones on cardiovascular or nervous system variables (Minson *et al.*, 2000, Usselman *et al.*, 2013). Participants abstained from caffeine, alcohol, and exercise twelve hours prior to testing. Informed consent was obtained from all participants and this study was approved by the University of Alberta Human (Biomedical) Research Ethics Board (Pro 00040669).

Table 1. Participant characteristics by person (n=16, F=8).

ID#	Sex	Age	Height (cm)	Weight (kg)	BMI
3	F	22	161	56	21.6
4	F	26	158	55	22.0
5	F	21	160	59	23.0
7	M	26	175	67	21.9
8	F	21	168	62.3	22.1
10	M	22	185.5	76	22.1
11	M	22	175	64	20.9
14	F	28	166	60	21.8
16	M	25	168.5	60	21.1
23	F	21	162	49	18.7
28	M	23	193.5	92.5	24.7
29	M	23	180	73	22.5
38	M	23	188	79	22.4
41	F	25	167.5	73	26.0
51	M	32	170	70	24.2
55	F	30	170	68	23.5
Average		24.38	171.75	66.49	22.4
SD		3.07	10.44	11.12	1.7

BMI, body mass index.

Instrumentation

Participants were seated in semi-recumbent position (in a dentist chair) and were instrumented and tested in this position. As demonstrated in **Figure 2**, the participants were fitted to measure instantaneous heart rate (HR; ECG leads), and ABP (Finometer; Finometer Pro™; Finapres, Amsterdam, Netherlands). Cardiac output (derived model flow) was also determined using the Finometer. Participants were instrumented with bilateral blood pressure cuffs (one for the return to flow calculation done by the Finometer, the other (left side) for manual blood pressure measurements using a sphygmomanometer), a toe pressure transducer (TN1012; ADInstruments, Colorado Springs, USA), and finger pulse oximeter to measure SaO_2 (N-560; Nellcor, Covidien, Ireland).

MSNA was obtained in ten participants (5 female) and was assessed using multiunit microneurography (662C-3; Iowa University Bioengineering, Iowa). This included the use of a sterile, tungsten microelectrode (35 mm long, 200 μm in diameter; tapered to a 1-5μm un-insulated tip), inserted subcutaneously into the peroneal (fibular) nerve. The location of electrode was posterior to the fibular head with a reference electrode positioned subcutaneously 1-3cm away. MSNA was obtained by manually manipulating the electrode until a pulse-synchronous (characteristic) burst pattern was achieved (Delius *et al.*, 1972). This was confirmed by an increase in MSNA to voluntary apnea (end-expiration), and a lack of response to sudden loud noise or light touches on the skin of the lower leg and foot. Once a useable MSNA signal was obtained (maximal search time was limited to 10 minutes per site or 45 minutes total), the raw MSNA signal was amplified (10000x), band-pass filtered (700-2000Hz), rectified and integrated (0.1-s time constant) to obtain a mean voltage neurogram. Raw SNA data was sampled at 10,000 Hz and stored for off-line analysis (Lab Chart).

Once MSNA was obtained, the subject was fitted with a facemask (Hans Rudolph), providing a tight seal around the mouth and nose. The face mask was connected to a three-way-valve and had gas sampling at the mouth (ADI gas analyzer; ML206, ADInstruments, Colorado Springs, USA) to determine end-tidal PO_2 and PCO_2 ($P_{ET}O_2$ and $P_{ET}CO_2$, respectively). Superficial femoral artery blood flow (FBF; L/min) was determined using peripheral ultrasound (Vivid 7, General Electric) and collected continuously throughout the duration of the protocol. The pressure waveform of the carotid artery was determined using hand-held arterial tonometry (SPT-301; Miller Instruments, Houston, Texas, USA) and was used to calculate central and peripheral PWV. All variables were exported into Lab Chart 7 Pro (Lab Chart, ADI) via Powerlab 16/35 data acquisition system (Powerlab, PL3516, ADI) and stored for offline analysis.

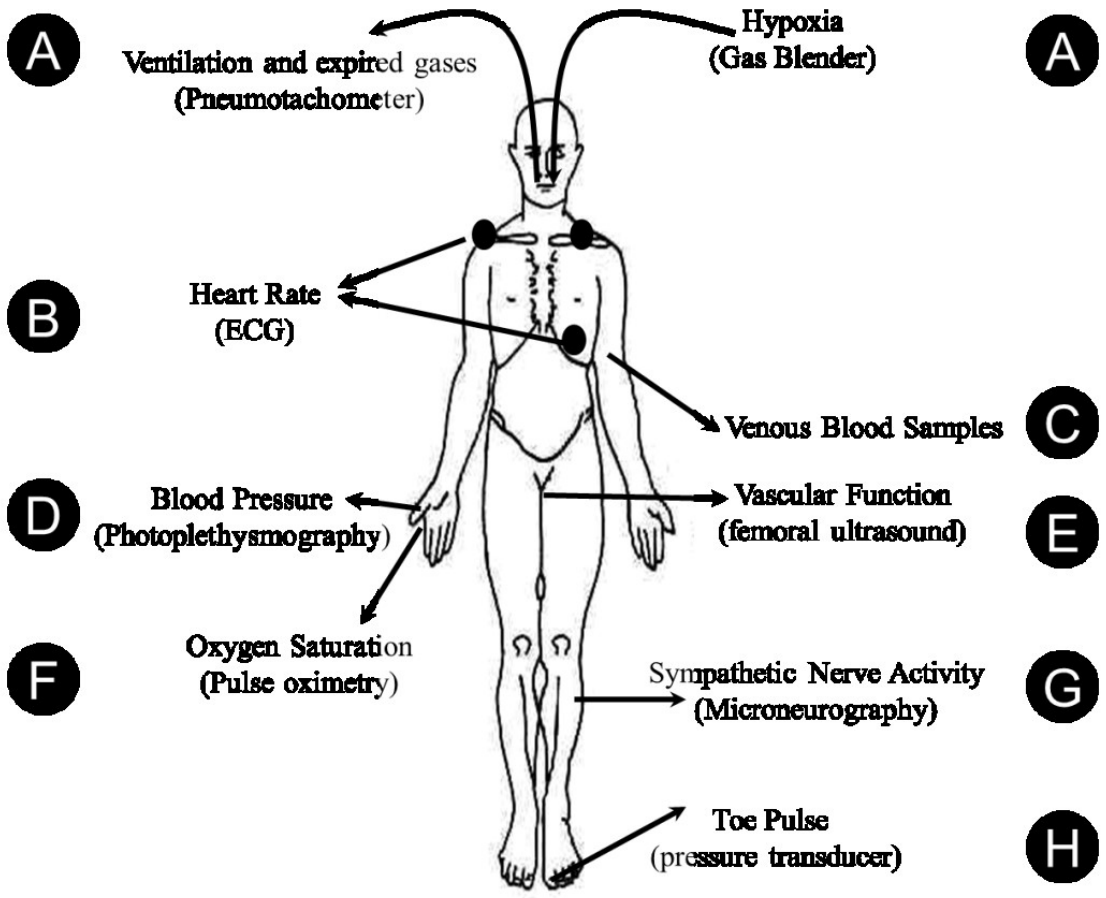

Figure 2. Instrumentation includes: A) a facemask attached to a heated pneumotachometer to determine minute ventilation. The facemask will also be connected to a three-way-valve that will allow for switching between breathing room air and breathing hypoxia (delivered from a gas blender) B) electrocardiograph recordings of heart rate C) venous catheter for sampling blood every ten minutes D) Finometer to determine beat-by-beat arterial pressure at the finger E) femoral ultrasound to determine arterial blood velocity and vessel diameter F) pulse oximeter on the finger to determine arterial blood hemoglobin saturation G) microneurography for recording multi-unit sympathetic nerve activity from the Peroneal (Fibular) nerve and H) pressure transducer to determine pressure waveform (pulse wave velocity) at the toe.

Cardiovascular variables (including carotid tonometry for PWV) were assessed beat-to-beat and taken as one minute averages during the second last minute of baseline and HX (minute 8-9), and every five minutes during the recovery (i.e. at minute 3-4, 8-9 etc.). Three ultrasound images for the determination of FBF were taken at the end of these one minute segments (indicated by the arrows in **Figure 3**). The $P_{ET}O_2$ and $P_{ET}CO_2$ and SaO_2 were measured breath by breath and interpolated for every heart beat and averaged over the same one-minute segments. MSNA was analyzed as a two-minute average overlapping the minute during which cardiovascular data is analyzed (shown by the grey bars in **Figure 3**).

Protocol

Following instrumentation, baseline values were recorded for ten minutes of quiet rest. Following baseline, the three-way valve was switched to a gas delivery system connected to an air blender initially delivering medical-grade air (21% O_2). The blender was first connected to a reservoir bag and then to the inspired port of the three-way-valve. Once the participant was comfortable breathing through this system, the mixture was changed to increase the amount of nitrogen delivered via the gas blender until 80% SaO_2 was reached (~45Torr $P_{ET}O_2$). Titration of nitrogen and medical air via the blender was done manually during the exposure to obtain and maintain SaO_2 measures of 78-82% (measured with a finger pulse oximeter). Following the establishment of steady-state SaO_2 (~2 minutes), participants continued with a ten-minute poikilocapnic hypoxic exposure after which they were switched back to breathing medical air for a twenty minute recovery.

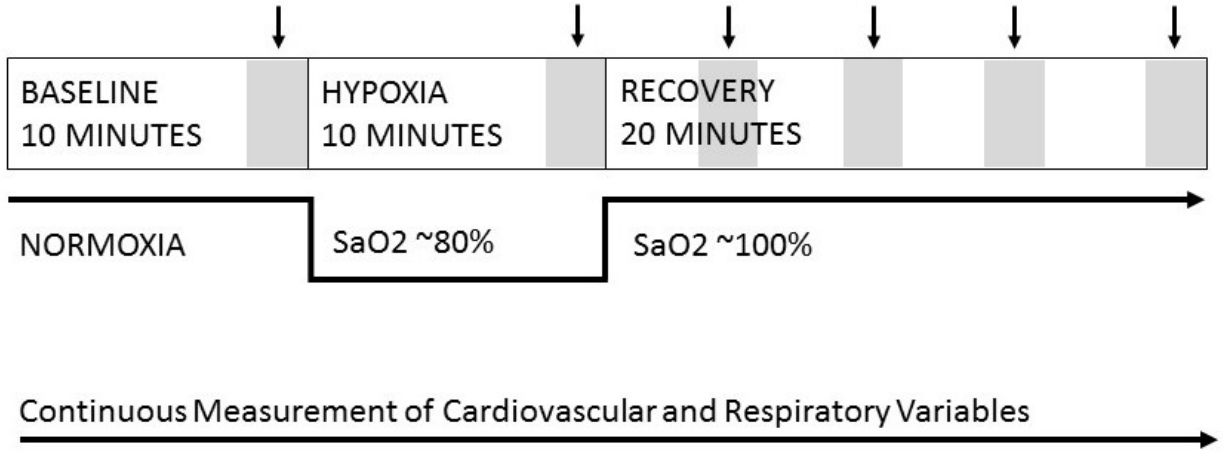

Figure 3. Protocol. Following ten minutes of baseline (normoxic), participants were exposed to ten minutes of poikilocapnic hypoxia at 80% arterial oxygen saturation (SaO_2) followed by a twenty minute recovery breathing room air (~100% SaO_2). The arrows indicate the time points where data was analyzed (one minute sections; data taken as an average measurement). Shaded regions represent the time points where muscle sympathetic nerve activity was analyzed (two minute sections)

Data Analysis

MAP, SBP, and DBP were calculated from the pressure waveform using Lab Chart peak analysis software. Q was derived from the model flow output from the Finometer and used to calculate TPR as

$$\mathbf{TPR = MAP/Q}$$

The femoral blood velocity (FBV) signal was analyzed in real-time via a 500M TCD (Multigon Industries Inc, New York) and the peak velocity trace was outputted to Lab Chart. Mean FBV (cm/s) was used in conjunction with changes in vessel cross sectional area (cm^2) to determine mean FBF (cm^3/s; ml/s) through the vessel. Cross sectional area was calculated by determining the blood vessel diameter during systole and diastole as

$$\mathbf{Mean\ cross\ sectional\ Area = 1/3\ Systolic\ Area + 2/3\ Diastolic\ Area}$$

Where cross sectional area is calculated as

$$\mathbf{Area = \prod\ x\ (radius)^2}$$

Where the radius is equal to half of the diameter of the blood vessel. Diameter was determined by three measurements during each systole and diastole for three ultrasound images (average of 9 measurements per time point). Mean FBF (ml/min) was determined using the following equation:

FBF= mean FBV x (mean cross sectional area).

Using MAP, femoral vascular resistance (FVR; mmHg/ml/min) was calculated as:

FVR = MAP (mmHg)/FBF (ml/min).

Carotid pulse transit time was determined in Lab Chart (using threshold detection) as the difference between the R-wave of the ECG and the foot (notch) of the carotid waveform. Finger, femoral and toe pulse transit time were assessed similarly, using time from R-wave to the foot of the systolic upstroke for each location. The pulse distance was determined by subtracting the distance from the sternal notch to the carotid artery from the distance of the finger, femoral artery or toe to the carotid notch. PWV (m/s) was calculated using

PWV = pulse distance (m)/time (s)

For each location (finger, femoral and toe) and corrected for changes in heart contractility by subtracting the pulse transit time and distance to the carotid artery from each measurement.

MSNA was quantified in terms of both burst frequency and amplitude changes from baseline (normoxia), during HX, and during the recovery period. Burst frequency was assessed in both absolute (bursts/minute) and relative (bursts/100 heart beats) terms and amplitude was expressed as a percent change from baseline. Baseline amplitude was determined as the average amplitude measured during a two-minute period following at least ten minutes of rest. Total MSNA was expressed as the product of the absolute frequency (bursts per minute) and average amplitude for each time point.

For more details on any specific methodology please refer to Appendix II.

Statistics

All values are shown as mean ± SD. For each variable, the null hypothesis was stated that there is no difference in between time points for an individual's response. Significance was determined using a-priori paired t-tests to compare the recovery data to the baseline and HX exposure. These were corrected for multiple comparisons using the following equations as previously described by Steinback and Poulin (2008).

$$p^1 = \alpha\ (p^0 / \alpha^1)$$

$$\alpha^1 = \alpha_e / c$$

$$\alpha_e = 1 - (1 - \alpha)^c$$

Where $\alpha^1 = 0.0452438$ to account for the multiple (five) comparison groups (*c*; baseline vs all other time points), $\alpha=0.05$, and p^0 and p^1 represent the original and corrected p-values, respectively (Steinback and Poulin, 2008). All statistical analysis was performed in Microsoft Excel. Significance was set at $p^1 < 0.05$. Data are expressed as mean ± SD.

Results

Hypoxic Stimulus

By design, both SaO_2 and $P_{ET}O_2$ decreased during HX (**Table 2**) and returned to baseline during the 20 minutes of recovery. The increase in ventilation during the ten minutes of poikilocapnic HX resulted in a reduction in $P_{ET}CO_2$ during HX that persisted during the 20 minute recovery.

Arterial Blood Pressure During and Following Hypoxia

During HX, none of SBP, DBP or MAP changed from baseline ($p>0.05$). Following HX, SBP was increased at 15 and 20 minutes ($p=0.017$ and 0.019, respectively; see **Table 2 and Figure 4**). MAP was increased at 15 minutes of recovery ($p= 0.046$) and trended towards increased at 20 minutes ($p=0.056$).

Table 2. Hemodynamics during and following ten-minute poikilocapnic hypoxia (HX) exposure.

	Baseline	*Hypoxia*	*5 min*	*10 min*	*15 min*	*20 min*
Heart Rate (bpm)	62.43 ± 10.1	76.69 ± 14.3*	61.77 ± 12.4	62.76 ± 12.1	64.06 ± 12.1	63.67 ± 11.8
SaO_2 (%)	96.98 ± 1.47	78.56 ± 4.5*	95.89 ± 2.4	97.41 ± 1.6	97.71 ± 1.6	97.43 ± 1.6
$P_{ET}O_2$ (Torr)	96.71 ± 4.9	47.90 ± 6.9*	93.75 ± 7.7	101.62 ± 8.0	103.10 ± 8.5	101.14 ± 8.0
$P_{ET}CO_2$ (Torr)	41.77 ± 7.2	36.56 ± 9.6*	39.39 ± 9.9	38.34 ± 11.5*	37.76 ± 10.2*	39.33 ± 9.9*
SBP (mmHg)	123.81 ± 15.8	127.48 ± 10.6	127.76 ± 9.7	128.72 ± 10.5	130.99 ± 10.9*	130.38 ± 11.1*
DBP (mmHg)	66.40 ± 10.7	67.39 ± 9.4	68.16 ± 7.0	69.35 ± 6.5	71.25 ± 6.9*	71.26 ± 7.0
MAP (mmHg)	88.53 ± 13.1	89.10 ± 9.5	90.51 ± 7.8	92.18 ± 7.3	94.21 ± 7.8	94.37 ± 8.7
Q (L/min)	5.69 ± 0.8	6.65 ± 1.5*	5.70 ± 1.2	5.88 ± 1.1	5.96 ± 1.1	5.92 ± 1.1

*$p < 0.05$ compared to baseline. HR, heart rate; SaO2, arterial oxygen saturation; PETO2, partial pressure of end-tidal oxygen; PETCO2, partial pressure of end-tidal carbon dioxide; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial blood pressure; Q, cardiac output.

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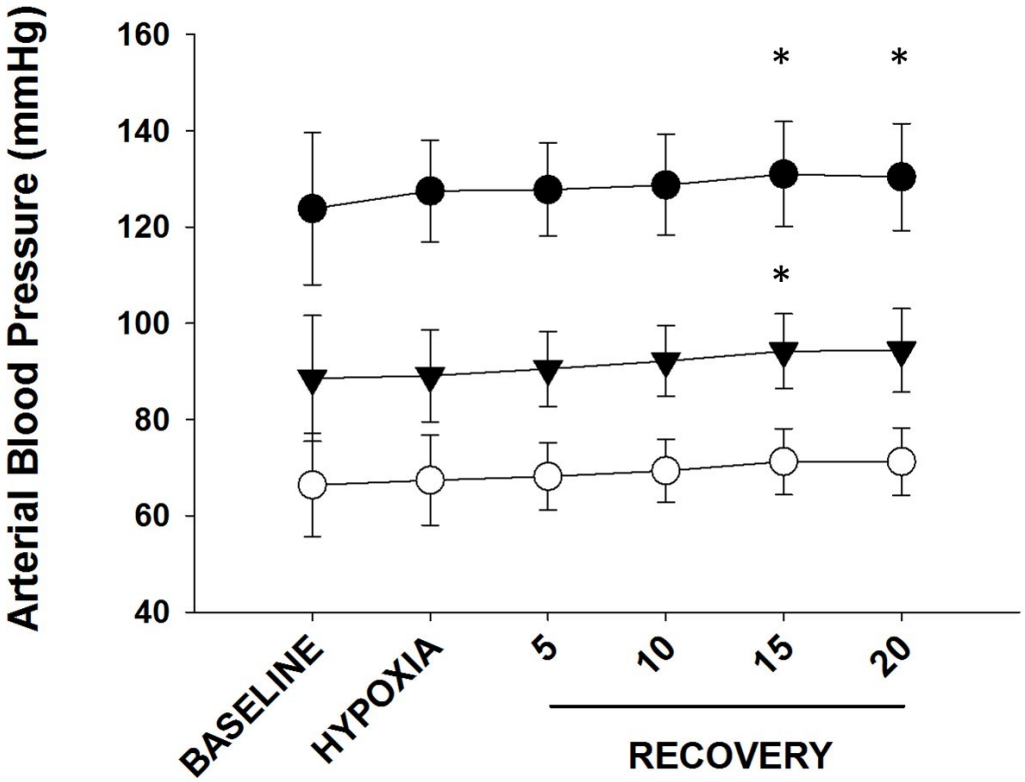

Figure 4. Effects of 10 minute poikilocapnic hypoxia (HX) on arterial blood pressure (BP). Systolic BP (filled circles) and mean arterial BP (triangles) are increased during the recovery only. Diastolic BP (open circles) is not changed during or following HX. *$p < 0.05$ compared to baseline.

Vascular Resistance During and Following Hypoxia

Femoral Vascular Resistance

We were only able to visualize the common femoral artery in 2 participants due to our experimental setup. However we were able to visualize the superficial femoral artery in the remaining 14. For consistency, data is reported for the superficial femoral artery hemodynamics (omitting those individuals in whom the common femoral artery was insonated). FBF was increased from 180.8 ± 83 ml/min during baseline to 253.9 ± 117 ml/min during HX ($p < 0.001$) and remained increased during the 20 minutes recovery ($p > 0.05$). FVR was decreased compared to baseline at ten minutes of recovery ($p = 0.02$). Specifically, FVR decreased from 0.038 ± 0.02 mmHg/ml/min/ during baseline to a nadir of 0.031 ± 0.01 mmHg/ml/min/ at ten minutes of recovery (shown in **Figure 5**). Inversely, femoral vascular conductance (FVC; not shown) was different from baseline at the same time points during the protocol (during HX and at ten minutes of recovery, $p<0.05$).

Total Peripheral Resistance

Q was increased during HX (from 5.69 ± 0.8 L/min to 6.65 ± 1.5 L/min, $p=0.002$), and MAP was not different ($p=0.93$). Therefore, TPR was decreased during hypoxia (from 15.8 ± 3.0 to 14.0 ± 3.4 L/min/mmHg; $p<0.001$) compared to baseline. The decrease in TPR did not persist into the recovery ($p>0.05$; shown in **Figure 6**).

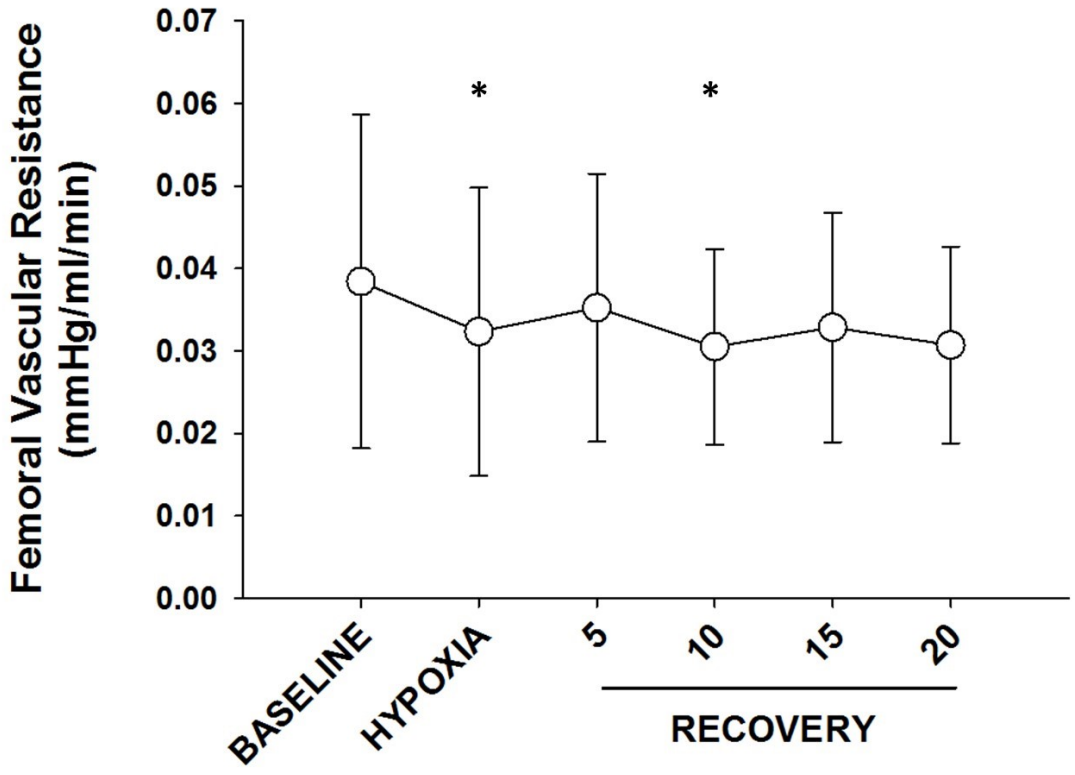

Figure 5. Changes in femoral vascular resistance (FVR; mmHg/L/min) during and following 10 minutes poikilocapnic hypoxic (HX) exposure. FVR is decreased during HX and at ten minutes into the recovery. *$p > 0.05$ compared to baseline.

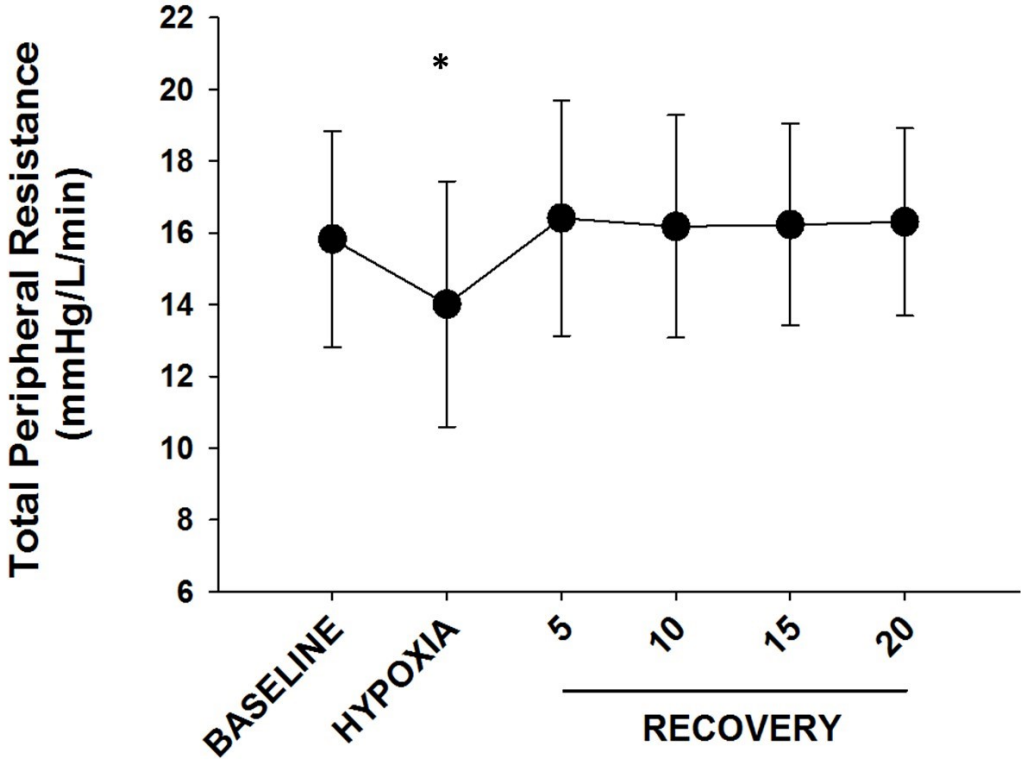

Figure6. Changes in total peripheral resistance (mmHg/L/min) during and following 10 minutes poikilocapnic hypoxic exposure. *p>0.05 compared to baseline.

Arterial Stiffness

Peripheral PWV was determined for both upper (finger-carotid) and lower (toe-femoral) limbs. Upper limb PWV (indicated by the closed circles in **Figure 7**) was 6.7± 0.7 m/s at baseline and did not change during or following HX ($p > 0.05$). Lower limb PWV (indicated by the open circles in **Figure 7**) was 9.0 ± 1.3 m/s at baseline and did not change during or following HX ($P > 0.05$). Lastly, central PWV (indicated by the triangles in the lower panel of **Figure 7**) was 9.0 ± 3.1 m/s during baseline and did not change during or following HX.

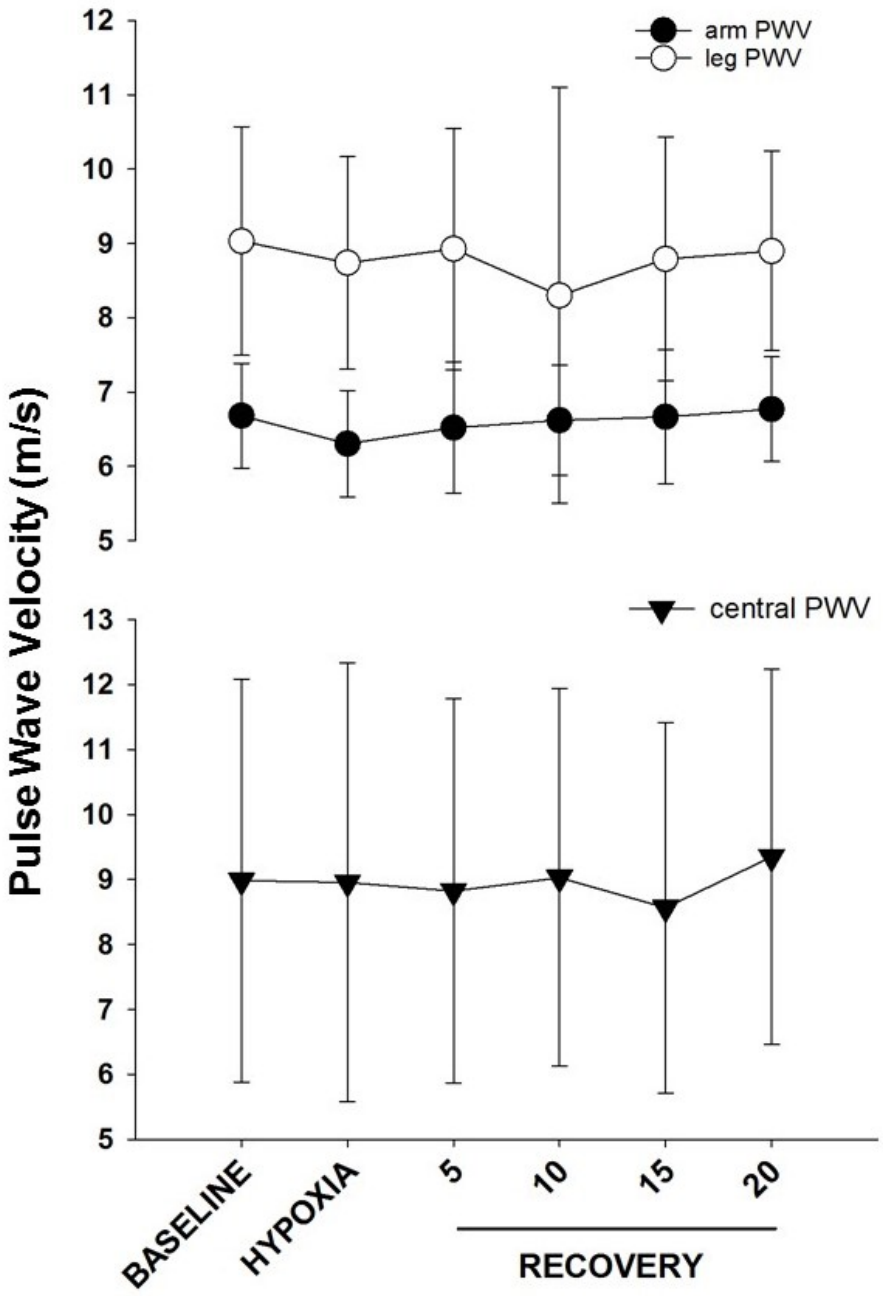

Figure 7. Changes in upper (filled circles), lower (open circles) and central (triangles) pulse wave velocity (PWV) during and following ten minutes poikilocapnic hypoxic (HX) exposure. None of upper, lower or central PWV is changed during or following HX. $P > 0.05$ at every time point compared to baseline.

Muscle Sympathetic Nerve Activity

MSNA burst frequency

MSNA burst frequency (n=10) was increased during HX and remained increased until 15 minutes of recovery following HX (**Figure 8**, $p<0.5$). MSNA burst frequency was increased from 20.7 ± 5.9 burst per minute to a maximum of 25.8 ± 5.7 bursts per minute during the 15^{th} minutes of recovery ($p=0.03$ compared to baseline). Burst frequency was not different from baseline at 20 minutes of recovery ($p=0.32$) and was not different at any point during recovery compared to during HX ($P>0.05$).

MSNA Burst Occurrence

MSNA burst occurrence (number of bursts per 100 heart beats; n=10) was not increased during HX (43.6 ± 13 and 42.7 ± 14 bursts per minute during baseline and HX, respectively; shown in **Figure 9**) due to the increase in HR from 62.4 ± 10 bpm to 76.7 ± 14 bpm ($p<0.001$ during HX, see **Table 2**). Burst occurrence was increased during the recovery, peaking at 55.0 ± 18 bursts per 100 heart beats at 15 minutes ($p=0.04$ compared to baseline) but returned to baseline by 20 minutes of recovery (47.8 ± 16 bursts per 100 heart beats; $p=0.46$ compared to baseline).

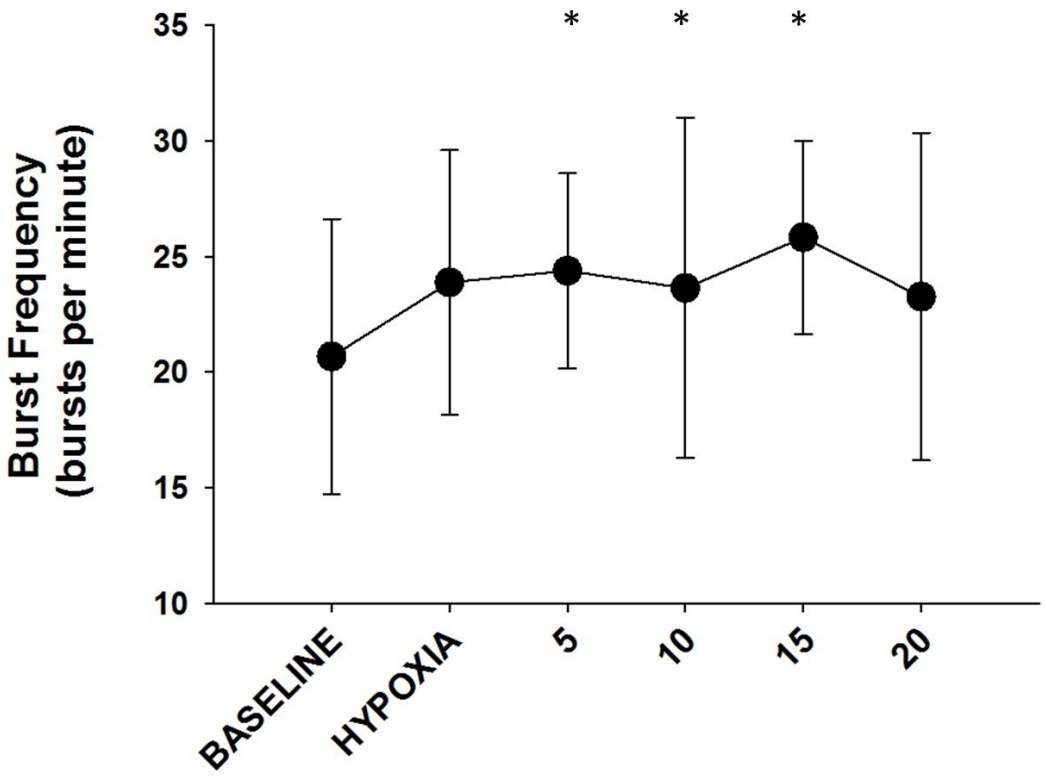

Figure 8. Changes in muscle sympathetic nerve activity (MSNA; n=10) burst frequency during and following ten- minutes of acute poikilocapnic hypoxia (HX). MSNA burst frequency is measured as number of bursts per minute and is increased following HX and remains increased for at least 15 minutes. * $p < 0.05$ compared to baseline.

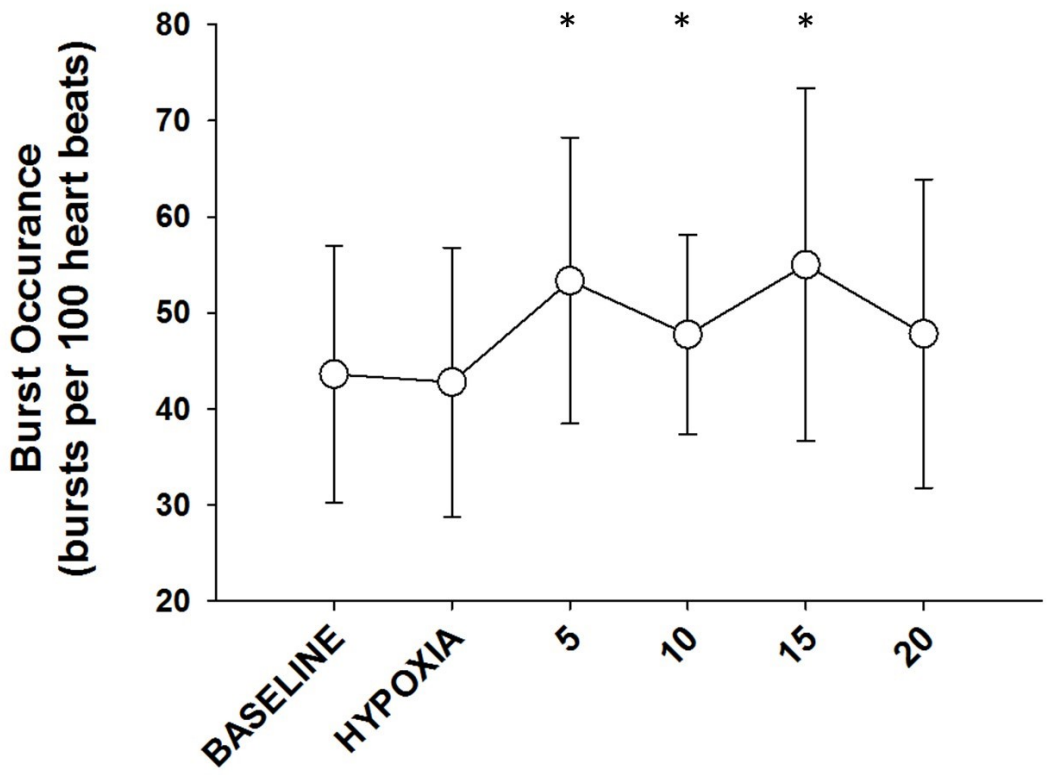

Figure 9. Changes in muscle sympathetic nerve activity (MSNA; n=10) burst occurrence during and following ten- minutes of acute poikilocapnic hypoxia (HX). MSNA burst occurrence is measured as number of bursts per 100 heart beats and is increased following HX and remains increased for at least 15 minutes. * $p < 0.05$ compared to baseline.

MSNA Burst Amplitude

MSNA burst amplitude (n=10) was measured as a percent change from baseline and was increased 121 ± 26% during hypoxia (p=0.02 compared to baseline, shown in **Figure 10**) and was only increased at 15 minutes during the recovery (137 ± 45% increase; p=0.03 compared to baseline).

Total MSNA

Total MSNA (n=10) was measured as a percent change from baseline and was increased 148 ± 58.6 % during HX (p= 0.03 compared to baseline, shown in **Figure 11**). Total MSNA remained increased up to 15 minutes following HX, peaking at 191 ± 102 % increase at 15 minutes (p= 0.02) and returning to baseline by 20 minutes (158 ± 120%, p= 0.17) following HX.

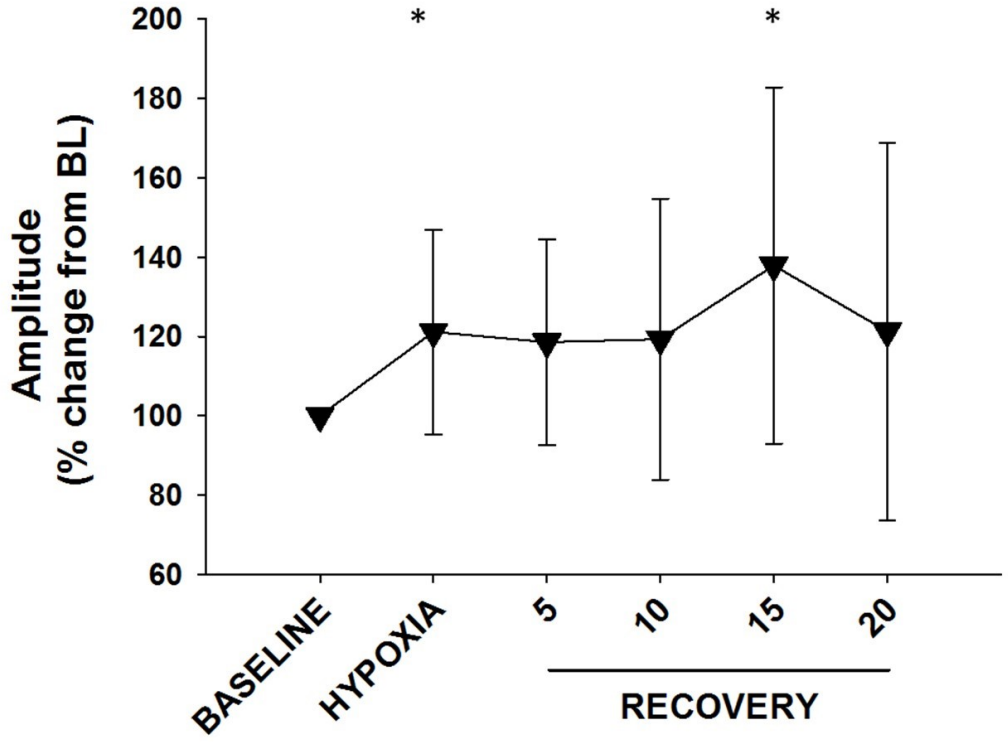

Figure 10. Changes in muscle sympathetic nerve activity (MSNA; n=10) burst amplitude during and following 10 minutes poikilocapnic hypoxic (HX) exposure. MSNA amplitude is measured in arbitrary units and expressed as a percent change from the average baseline (BL) amplitude. MSNA burst amplitude is increased during HX and at 15 minutes of recovery. *p>0.05 compared to baseline.

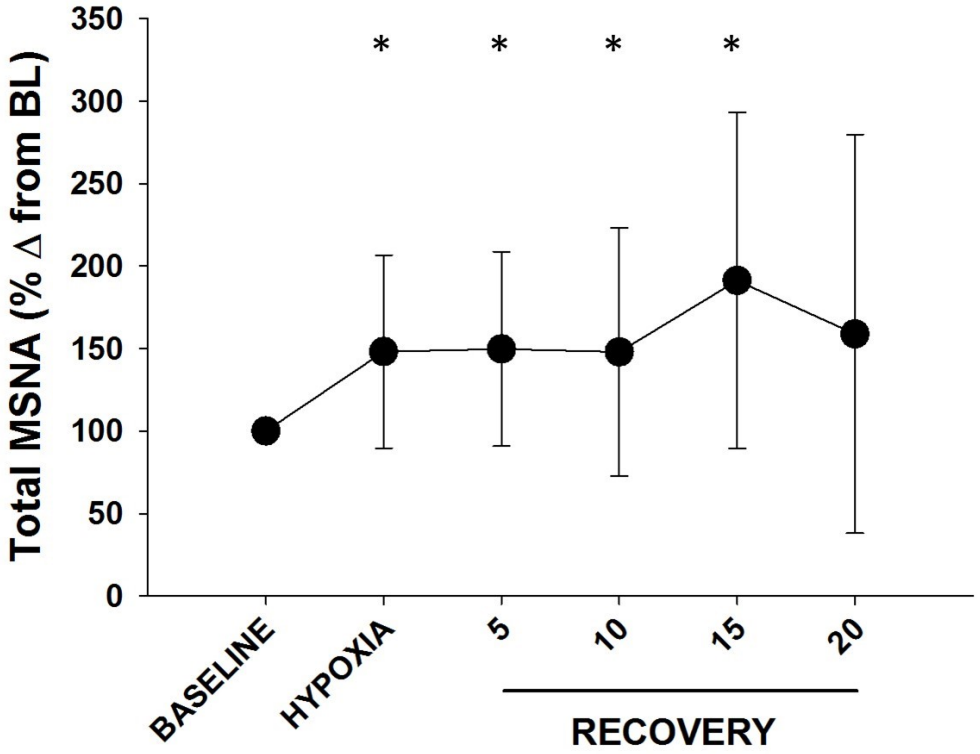

Figure 12. Changes in Total muscle sympathetic nerve activity (MSNA; n=10) during and following a ten-minute poikilocapnic hypoxic (HX) exposure. Total MSNA was increased during and up to 15 minutes following HX. * $p < 0.05$ compared to baseline.

Discussion

This study is the first to comprehensively characterize the SNs and cardiovascular responses following acute hypoxia (ten minutes at SaO_2 ~80%). This study adds to the literature in that it is the first to quantify ABP responses past ten minutes of recovery, the first to measure changes in FVR and PWV and the first to comprehensively analyze the components of the MSNA (i.e. amplitude and frequency).

First, we show that both SBP and MAP are increased after ten minutes of recovery following HX exposure. This follows the work of Steinback and Poulin (2008) who showed a delay in the increase in ABP during twenty minutes of poikilocapnic and not isocapnic HX (ABP was not increased within ten minutes exposure). We also show that Q is increased during acute poikilocapnic HX and returns to baseline following exposure, which parallels the decrease in TPR during HX, returning to baseline following HX. This observation is similar to that previously observed during acute (5 minutes) isocapnic HX (Steinback *et al.*, 2009) where TPR was decreased 20%. Further, both of these studies subject participants to HX that is equivalent (~80% SaO_2 and 45 Torr $P_{ET}O_2$). However, the present study investigated a ten-minute long HX exposure while Steinback et al, (2009) only used a five-minute exposure. A longer duration exposure may increase MSNA to a greater extent and may result in a smaller drop in TPR, the present study observed a decrease in TPR of 11.4%.

We also show, for the first time, the FBF and FVR responses; previous acute poikilocapnic HX studies have only measured vascular changes occurring in the forearm. As described previously, there are regional differences in the response of the peripheral vasculature in that FVR did not change during or following acute isocapnic HX (Morgan *et*

al., 1995, Xie *et al.*, 2001). In the present study we show that FVR is decreased during HX (FBF is increased) and that this response persists at least ten minutes into the recovery (**Figure 5).** This adds to the work of Tamisier *et al,* (2004) who show forearm vascular resistance is decreased during hypocapnic but not hypercapnic HX and increased following hypercapnic but not hypocapnic HX.

Similar to previous publications (Steinback *et al.,* 2012), we have shown that MSNA is increased during HX, and that only burst frequency, and similarly BI, has a persistent increase that lasted 15 minutes into the recovery. However, we did observe an increase in burst amplitude at 15 minutes of recovery (**Figure 10**; this is possibly the artifact of a large inter-individual response in MSNA during and following HX. Lusina *et al*, (2006) did report that both burst frequency and amplitude were increased following 20 minutes of isocapnic HX (SaO_2 75-80%). It is possible that following poikilocapnic HX that chemoreflex gain is altered such that ventilation is increased relative to baseline despite SaO_2 returning to pre-HX levels (see **Table 2**). Ventilation was not measured in the present study, but $P_{ET}CO_2$ remained decreased relative to baseline indicating hyperventilation during the recovery period (while breathing room air; **Table 2**). Continued activation of the peripheral chemoreflex could result in augmented sympathetic burst amplitude that persists into the recovery. A larger cohort of participants with MSNA measurements would be required to determine if burst amplitude is (or is not) increased following poikilocapnic HX.

Lastly, this study is the first to report measures of central and peripheral arterial stiffness (PWV) following acute HX. However, neither central nor peripheral PWV changed during or following HX (**Figure 25**). These results are similar to the study by Boos *et al,* (2012) who measured no change in upper limb arterial stiffness after 180 minutes of

poikilocapnic HX. While acute HX may not have an effect on central and peripheral arterial stiffness, it may be that longer durations of HX or prolonged intermittent HX stimulation may affect PWV. Indeed, Lewis *et al.* (2014) showed that central PWV increased during HX (3-4 days) and continued increasing with chronic exposure (after 12-14 days) although peripheral PWV did not change with exposure to high altitude.

Methodological Considerations

The present study used a ten minute HX (chemoreceptor) stimulus to increase MSNA. This is the same stimulus used in previous studies to elicit a prolonged sympathetic response (Xie *et al,* 2001, Steinback *et al,* 2012). The level of HX achieved in the present study was 78 ± 4.5% SaO_2 and 48 ± 9.6 Torr $P_{ET}O_2$, which is consistent with the work of both Xie *et al,* (2001) and Steinback and Shoemaker (2012). However, we employed a poikilocapnic design as this is more "life-like" stimulus compared to isocapnia.

In the present study we chose to include both males and females for participation as this is more representative of the general population. Although sex has been shown to affect resting MSNA (Matsukawa *et al.*, 1998, Ng *et al.*, 1993) and recent evidence suggests that the MSNA response to chemoreflex, baroreflex and mental stress may differ between sexes and across the phases of the menstrual cycle (Patel *et al.*, 2014, Yang *et al.*, 2013, Usselman *et al.*, 2013, Yang *et al.*, 2012). We did not observe any systematic differences in the responses from female participants.

Potential Mechanisms

The effects of persistent sympathetic activation following acute HX exposure are minimal in a healthy young population. However, the mechanisms responsible for the augmented MSNA response are still unclear. This study provides further evidence that the chemoreflex is involved in the persistent activation of the SNS following HX exposure. Specifically, we observed increases in MSNA burst amplitude following HX which may be indicative of persistent activation (or change in gain) of the PCR reflex. Querido *et al,* 2010 show that the persistent MSNA can be attenuated by giving 100% oxygen indicating the role of the PCR reflex. Secondly, we observed that both SBP and MAP are increasing following HX and MSNA remains increased despite this. This would indicate a re-setting of the baroreflex curve to a higher set-point (dashed line in **Figure 7**). Querido *et al,* 2010 also showed that spontaneous baroreflex set-point but not gain is changed following acute HX. However, in the present study MSNA was not increased at 20 minutes of recovery (when ABP was increased) suggesting that the shift in set-point is only temporary. It may be that the time for the baroreflex function to recover also depends on the severity, duration and CO_2 control during HX.

Additionally, HX is a potent vasodilator, and it may be that there is persistent vasodilation occurring that persists following the removal of the initial stimulus (i.e. return to room air; Dinenno *et al.*, 2003). This could explain why FVR is decreased following HX and lack of change in arterial stiffness in the present study. Determining the exact mechanism by which MSNA is increased in the recovery to acute HX in humans may require further study.

Implications

It is believed that hyperactivity in the SNS may contribute to the pathogenesis of CVD (Mancia and Grassi, 1991); MSNA hyperactivity has been characterized as being a contributing factor to the development of vascular dysfunction and is attributed with several diseases including essential hypertension, obstructive sleep apnea, and others (reviewed in Malpas, 2010). Hypertension has been shown to be associated with high plasma catecholamine concentration; a marker of increased SNA (e.g. DeQuattro *et al.*, 1976) and obstructive sleep apnea patients have elevated resting MSNA compared to weight-matched controls (e.g. Carlson *et al.*, 1993, Coy *et al.*, 1996, Narkiewicz *et al.*, 1998). The potential cardiovascular consequences that may be the result of persistent increases in MSNA following repeated or prolonged HX exposure are of merit to investigate as it may shed light onto mechanisms by which cardiovascular dysfunction develops. It is hypothesized that the peripheral chemoreceptors (PCR) have increased sensitivity in these diseases and that increased PCR sensitivity leads to augmented MSNA (Prabhakar and Peng, 2004). If PCR gain is changed during and following acute poikilocapnic HX, it would follow that it would also be changed following longer durations exposures. We know the ventilatory sensitivity to HX is increased following exposure to altitude. A change in the gain of the PCR reflex during diseases where a person experiences HX could lead to increased MSNA, eventual increases in central PWV (most important clinical determinant) and increase the risk/instances of CVD. Healthy persons purposefully subjecting themselves to HX (repeated visits to altitude, or athletes who use HX tents for training) may also be subjecting themselves to increased CVD risk by these same mechanisms.

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Chapter 3. General Discussion

Main Findings

To our knowledge, this study is the first to comprehensively characterize the neurovascular responses following acute poikilocapnic HX (ten minutes at SaO_2 ~80%). Specifically, we add recovery responses longer than ten minutes in duration to address the time-course of the [persistent] increase in variables following acute HX. Additionally we included complete measures of ABP, measures of femoral blood flow and vascular resistance, multiple measures of arterial stiffness (central and peripheral PWV), and a comprehensive MSNA analysis including burst amplitude responses.

First, we show that MAP is increased after ten minutes of recovery following HX exposure. We also show that Q is increased during acute poikilocapnic HX and returns to baseline following exposure, which parallels the decrease in TPR during HX, returning to baseline following HX. Previous studies investigating the vascular responses to acute poikilocapnic HX have looked at changes in forearm blood flow and vascular resistance; here we show the FBF and FVR responses during and following acute poikilocapnic HX. We show that FVR is decreased during HX (FBF is increased) and that this response persists at least ten minutes into the recovery (**Figure 5).** Additionally, this study report measures of central and peripheral arterial stiffness (PWV) following acute HX. In this study, neither central nor peripheral PWV changed during or following HX (**Figure 6**).Finally, similar to previous publications (Steinback and Shoemaker, 2012), we have shown that MSNA is increased during HX, and that only burst frequency, and similarly BI, has a persistent increase that

lasted 15 minutes into the recovery. However, we did observe a transient increase in burst amplitude at 15 minutes of recovery (**Figure 10**).

Limitations

This study addressed the neurovascular responses during and following a ten minute poikilocapnic HX exposure. The level of HX achieved in the present study was 78 ± 4.5% SaO_2 and 48 ± 9.6 Torr $P_{ET}O_2$, and we used a poikilocapnic design as this is more "life-like" stimulus compared to isocapnia and resulted in decreased $P_{ET}CO_2$ from 41.8 ± 7 Torr during baseline to 36.6 ± 10 during and 39.3 ± 10 following HX. The ventilatory response to HX varies from person to person (Vizek *et al.*, 1987), and in this study some participants remained isocapnic (e.g. ventilation was not increased) while others were hypocapnic. Although this does mimic "real-life" more accurately, it does complicate the results as CO_2 control plays an important role in the sympathetic and cardiovascular responses to HX (see **Appendix I**). With a larger cohort, it may be possible to differentiate between people who have a large ventilatory response (are more hypocapnic) and those who are "non-responders" (remain isocapnic) to determine if the cardiovascular responses are different. Further, we did not control for previous exposure to HX and the ventilatory response to HX is increased with subsequent exposures (Powell *et al.*, 1998). In some of our participants, the data analyzed for this study was a second trial of this protocol and they had experienced at least ten minutes of poikilocapnic HX (SaO_2 ~80%) one month prior to testing.

In the present study we chose to include both males and females for participation as this is more representative of the general population. Although sex has been shown to affect resting MSNA (Matsukawa *et al.*, 1998, Ng *et al.*, 1993) and recent evidence suggests that the

MSNA response to chemoreflex, baroreflex and mental stress may differ between sexes and across the phases of the menstrual cycle (Yang *et al.*, 2012, 2013, Usselman *et al.*, 2013, Patel *et al.*, 2014), we did not observe any systematic differences in the responses from female participants.

The present study investigated peripheral vascular blood flow in the superficial femoral artery. Other work investigating the effects of acute poikilocapnic HX on cardiovascular function have measured forearm blood flow and resistance. We found that FBF was increased during HX (FVR was decreased) and that it remained decreased up to ten minutes following HX. However, we cannot compare these results to previous work to isolate the effects of CO_2 control on FBF and FVR as there are none, nor can we make reliable observations about differences in the response between limbs as we did not collect data in both limbs. Further, we were only able to collect data from the superficial femoral artery in 14 participants (7F).

Future Directions

This study fills in many gaps in the literature, however does not directly address the mechanisms by which the SNS and cardiovascular responses are occurring. Presently, we have collected MSNA data in ten participants (5 F) and observed an increase in burst amplitude following HX; follow up would include testing a larger number of participants with MSNA measurements to accurately determine if the burst amplitude is increased following acute poikilocapnic HX or not. There is inconsistency in the literature respect to the persistence of MSNA burst amplitude following isocapnic HX exposure (Lusina *et al.*, 2006,

Steinback and Shoemaker, 2012), although ventilatory responses in the aforementioned studies would suggest that the chemoreflex is no longer activated following HX (Peng *et al.*, 2003, Cummings and Wilson, 2005, Steinback and Shoemaker, 2012). We observed a decrease in $P_{ET}CO_2$ following HX (**Table 2**), indicating an increase in ventilation. Analysis of the ventilatory responses during and following HX would provide clarification about the potential that the peripheral chemoreflex is still activated following acute poikilocapnic HX. Further recruitment of participants with MSNA recordings will also allow us to be able to more accurately (statistically) determine whether there are sex differences in the neurovascular responses (sympathetic and cardiovascular) to acute poikilocapnic HX.

We collected data sufficient to determine if baroreflex function is altered following acute piokilocapnic HX. Specifically, we could address whether the baroreflex curve is operating at a different set-point, or if the slope (gain) of the curve has changed following HX. Querido et al, (2010) determined that the chemoreflex is involved in the maintenance of increased MSNA in the recovery period. This same research group also determined that the baroreflex curve was reset upward **(Appendix I, Figure 17**) during and following acute HX, (Querido *et al.*, 2011). Together, these suggest the mechanisms regulating MSNA (and resulting cardiovascular responses) are changed following acute isocapnic HX; these studies could be repeated following poikilocapnic HX.

Conclusion

The activation of the sympathetic nervous system (SNS) is a compensatory mechanism promoting adequate blood flow distribution to critical tissues during periods of reduced

oxygen availability. Determining the responses of the SNS and cardiovascular system during in healthy persons without CO_2 control (poikilocapnic), will help to filling in some of the gaps in the literature. Specifically, this study has helped to determine the importance of CO_2 in the vascular responses to HX. Specifically, that higher CO_2 appears to result in higher MSNA and therefore ABP, vascular resistance and arterial stiffness compared to lower CO_2 (which would occur "naturally" in a poikilocapnic HX exposure; see **Appendix I** for more detail). However, longer duration HX exposures are also more likely to result in increased ABP, vascular resistance, and arterial stiffness, which would be important for those persons travelling to altitude for prolonged periods. In summary, vascular function does not appear to be negatively affected during or following acute poikilocapnic HX, despite MSNA being increased.

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Appendices

Appendix I: A Review of the Literature

Activation of the Sympathetic Nervous System by Hypoxia

Peripheral chemoreceptors (PCR) are glomus cells located within the carotid bodies that detect changes in blood gases, primarily O_2. These specialized cells respond to HX by sending afferent signals to the brainstem to increase ventilation and maintain/increase vascular tone (reviewed in Guyenet, 2000). Briefly, decreased PO_2 is detected directly by the PCRs by mechanisms that inhibit voltage-gated potassium ion (BK) channels (Hoshi and Lahiri, 2004). This leads to depolarization of the cell and release of dopamine (**Figure 12**) and subsequent activation of post-synaptic sensory neurons (i.e. carotid sinus nerve, a branch of cranial nerve IX). There have been several proposed mechanisms of O_2 sensing at the glomus cell including activation of a heme-containing protein, increase in intracellular cyclic adenosine monophosphate (CAMP), and inhibition of nitcotinamide adenine dinucleotide phosphate (NADPH) oxidase in the mitochondria, to name a few (shown in **Figure 12;** Richerson, 2009).

Activation of the PCR by HX leads to excitatory signals being transmitted via the carotid sinus nerve (a branch of cranial nerve IX) to the nucleus tractus solitarius (NTS; reviewed in Guyenet, 2000). Signals are integrated in the NTS and relayed to the rostral ventrolateral medulla (RVLM); activation of the RVLM neurons results in increased firing of sympathetic neurons (Guyenet, 2000). Specifically, RVLM neurons receive excitatory inputs from the NTS as the result of PCR activation by HX (indicated by solid lines in **Figure 13**).

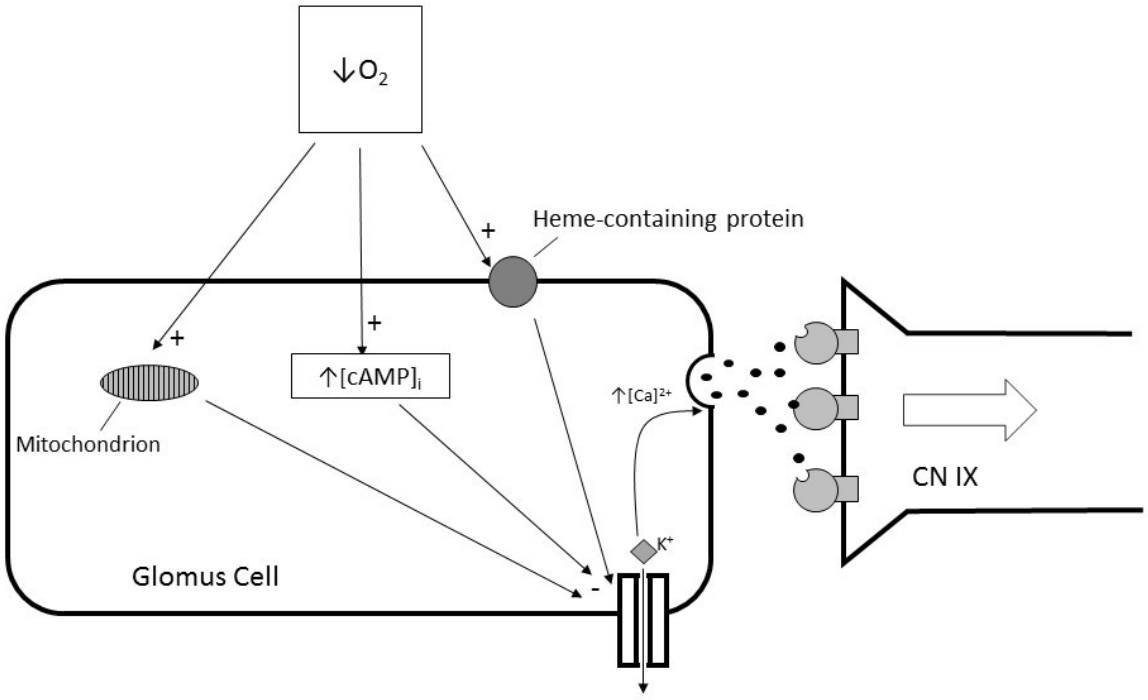

Figure 12. Activation of the peripheral chemoreceptors (PCR) by hypoxia. Reduced oxygen acts through several potential mechanisms to activate the PCR (glomus cells of the carotid bodies). These include: activation of a heme-containing protein, increasing intracellular cyclic adenosine monophosphate (cAMP) concentrations, or inhibition of nitcotinamide adenine dinucleotide phosphate oxidase in the mitochondria. All of these pathways are believed to act to inhibit voltage-gated potassium ion (K^+) channels and result in depolarization of the cell and release of dopamine (black circles). Dopamine activates the receptors on the carotid sinus nerve (a branch of cranial nerve IX).

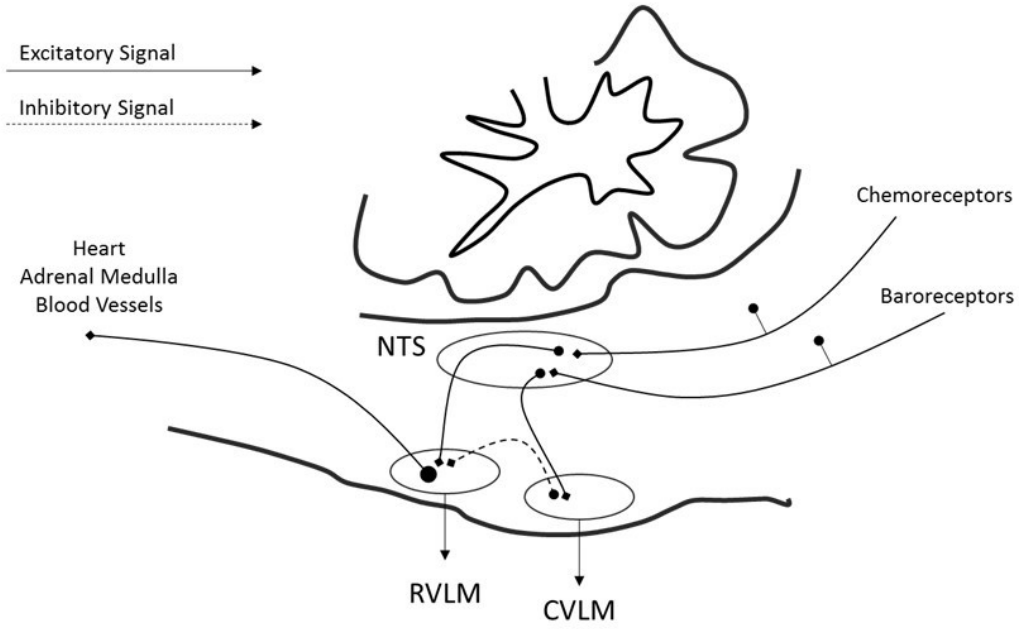

Figure 13. Sagittal view of the brain stem depicting the pathways involved in activation of the sympathetic nervous system by the peripheral chemoreceptors (PCR) and arterial baroreceptors. PCR activation results in excitatory signals (solid lines) from the nucleus tractus solitarius (NTS) being transmitted to the rostral ventrolateral medulla (RVLM). Signals are integrated in the RVLM and result in the activation of the SNS neurons that innervate the heart, adrenal medulla and blood vessels, among other things. Baroreceptor activation is integrated through the NTS and the caudal ventrolateral medulla (CVLM). Activation of CVLM neurons by the baroreceptors results in inhibitory signals (dashed lines) sent to the RVLM. Modified from (Colombari *et al.*, 2001).

Due to the simultaneous activation of the respiratory and sympathetic nuclei in the brainstem, sympathetic nerve activity (SNA) has been shown to be linked closely with the respiratory cycle due to respiratory induced changes in ABP or blood gases, central oscillation theory, alveolar inflation inhibition, or any combination of the aforementioned (Mano *et al.*, 2006). Regardless, voluntary (i.e. apnea, hyperventilation) and involuntary (in the absence of central command) changes in respiration result in changes in SNA (St. Croix *et al.*, 1999).

During spontaneous ventilation, activation of the PCR (in dogs) decreases resistance (vasodilation), however, when ventilation is artificially maintained at a constant rate (CO_2 is maintained), the same stimulus increases vascular resistance (Daly and Scott, 1962). From this, and other similar work by the same research group, it was suggested by Marshall (1998) that the primary response to PCR stimulation (by HX) is vasoconstriction. Therefore, it is possible for the respiratory system to impact the magnitude and direction of the cardiovascular changes observed during and following HX (Bernthal *et al.*, 1951).

Hypercapnia (increased CO_2) has also been shown to increase MSNA, likely via central chemoreceptors (CCR) located in the brainstem (Guyenet, 2000), which are modulated by changes in O_2 (e.g. (Day and Wilson, 2009). The fact that both hypercapnia and HX augment MSNA suggests an important role of the chemoreceptors in modulating and regulating MSNA.

Activation of the Sympathetic Nervous System Results in Vasoconstriction

When the SNS is activated (i.e. by HX), norepinephrine (NE; noradrenaline) is released from post-ganglionic pre-synaptic nerve terminals at the varicosities surrounding the

smooth muscle cells that border blood vessels. NE acts on α_1-adrenergic receptors on the post-synaptic and α_2-adrenergic receptors on the pre-synaptic membrane (vascular smooth muscle cell and sympathetic nerve terminal, respectively). At the smooth muscle cells (α_1-adrenergic receptors), activation of the G-protein coupled receptor $G\alpha_q$ by NE results in the activation of phospholipase C (PL-C) by the α_q subunit, which splits phosphatidylinositol bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 stimulates the release of intracellular calcium (Ca^{2+}) from the sarcoplasmic reticulum (SR), contraction of the smooth muscle and subsequent vasoconstriction. This pathway is shown in **Figure 14**. Simultaneous feedback of NE onto pre-synaptic (α_2-adrenergic) auto-receptors acts to inhibit extracellular Ca^{2+} from entering the cell by the interaction of the $\beta\gamma$ subunit with Ca^{2+} channels. This action also attenuates the release of other co-localized transmitters.

At the neurovascular junction, there is co-release of and neuropeptide Y (NPY) and adenosine triphosphate (ATP), which also play a role in the vascular responses to increased SNA (Macarthur *et al.*, 2011); this is shown in **Figure 15**. Using *in vivo* preparations, high frequency sympathetic nerve stimulation resulted in NPY being co-released from the same large vesicles as NE. NPY acts on post-synaptic Y_1 receptors on the smooth muscle cell membrane to cause vasoconstriction (Pernow and Lundberg, 1989) and negatively feeds back onto pre-synaptic Y_2 receptors to inhibit the release of NE, ATP and NPY (reviewed in Macarthur *et al.*, 2011). NPY has been shown to be a more potent vasoconstrictor than NE, and is responsible for sustained or long duration contractions as it is longer lasting (Lundberg *et al.*, 1982, Macarthur *et al.*, 2011). Smaller vesicles containing ATP and NPY are released at lower frequency nerve stimulation (Macarthur *et al.*, 2011); ATP binds to post-synaptic P_{2X} receptors to induce vasoconstriction and to pre-synaptic P_{2Y} receptors to inhibit NE release.

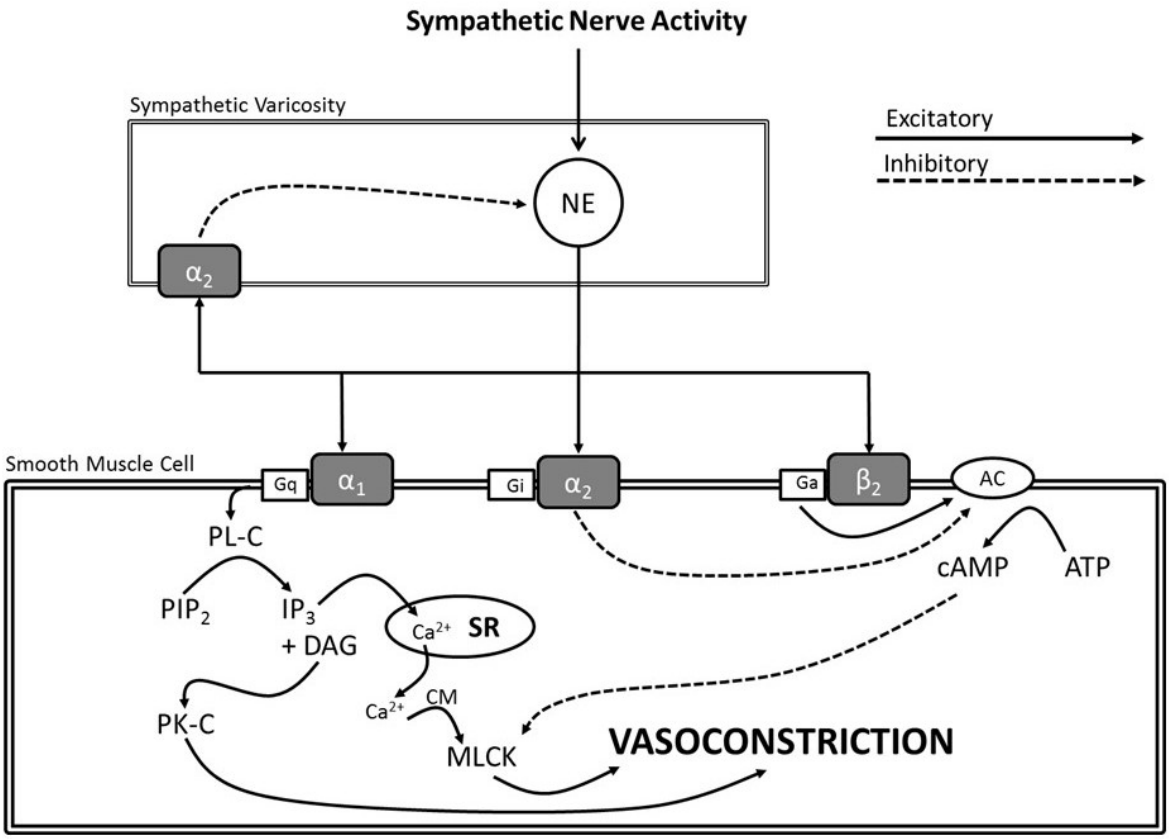

Figure 14. Effects of norepinephrine (NE) on the vascular smooth muscle. NE is released in response to a sympathetic nerve signal arriving at the sympathetic varicosity (end of the sympathetic nerve). NE binds to α_1-adrenergic G-protein coupled receptor G_q and activates phospholipase C (PL-C) which splits phosphatidylinositol bisphosphate (PIP_2) into diacylglycerol (DAG) and IP_3. IP_3 stimulates the release of intracellular calcium ions (Ca^{2+}) from the sarcoplasmic reticulum (SR), while DAG stimulates phosphokinase-C (PKC). Ca^{2+} released from the SR binds to calmodulin (CM) which activates myosin light chain kinase (MLCK) and initiates contraction of the smooth muscle cell. PLK also promotes vasoconstrtiction. Activation of the β_2 receptor (G_a-coupled) causes adenylyl cyclase (AC) to convert adenosine triphosphate (ATP) into cyclic adenine monophostphate (cAMP) which inhibits myosin light chain kinase and promotes relaxation of the smooth muscle cell. Activation of the α_2-adrenergic receptor (G_i-coupled) on the smooth muscle cell membrane inhibits AC and therefore facilitates cell contraction, while activation of the α_2 receptor on the pre-synaptic sympathetic nerve varicosity will inhibit further NE release.

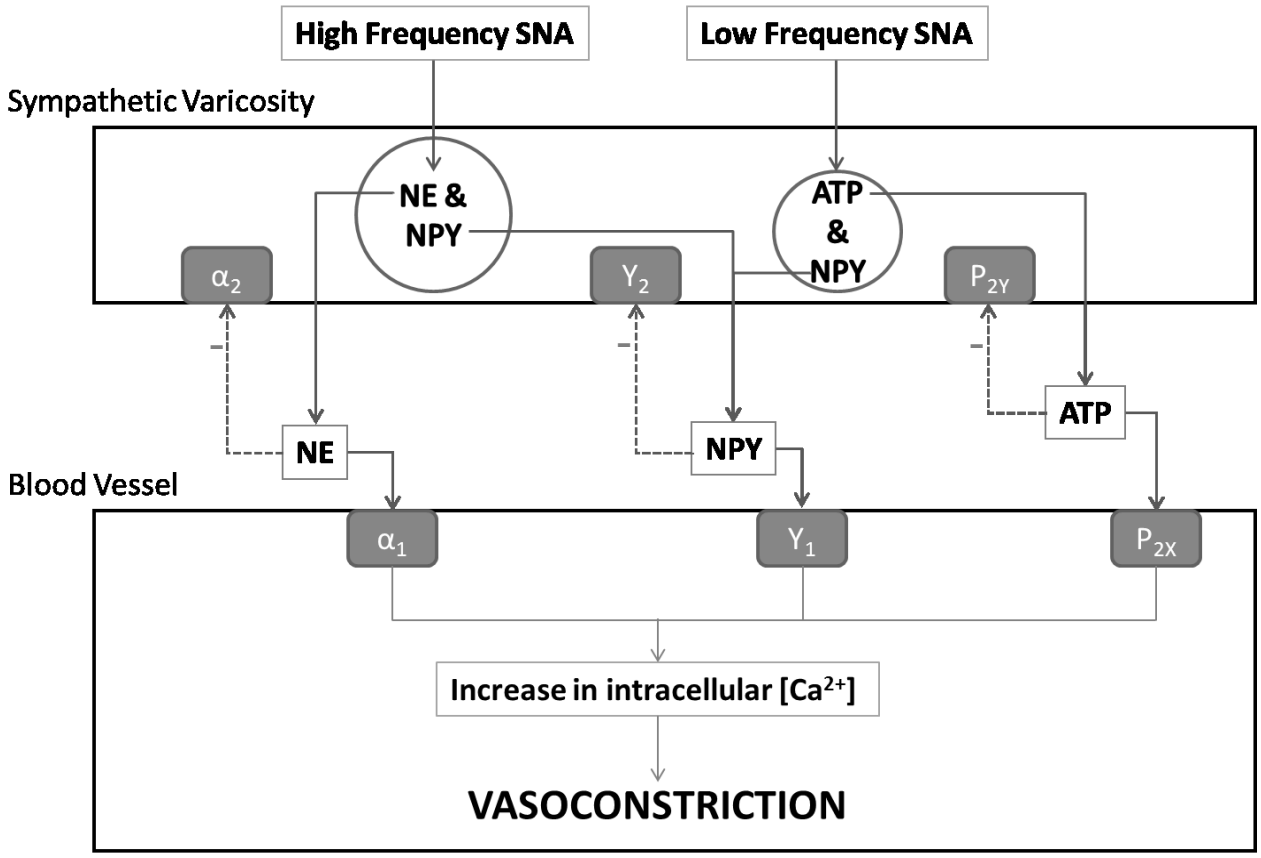

Figure 15. Co-transmission and feedback of neurotransmitters at the sympathetic nerve synaptic varicosity on the smooth muscle cells of the blood vessels. High frequency sympathetic nerve activity (SNA) activates large vesicles containing norepinephrine (NE) and neuropeptide Y (NPY). Low frequency SNA activates smaller vesicles containing adenosine triphosphate (ATP) and NPY. Each neurotransmitter (NE, NPY and ATP) act on their respective G-protein coupled receptor on the blood vessel smooth muscle cell wall (α_1, Y_1, and P_{2X}, respectively; solid lines) and negatively feedback to auto-receptors on the sympathetic nerve varicosity to inhibit further release of neurotransmitters (α_2, Y_2, and P_{2Y}, respectively; dashed lines). Modified from Macarthur et al. (2011).

Activation of the Sympathetic Nervous System by Baroreceptor Activation

Arterial baroreceptors (located at the carotid bifurcation) respond to fluctuations in ABP. **Figure 13** shows the integration of the baroreceptor and chemoreceptor signal in the brainstem. The response to increased ABP (increased stretch of the baroreceptor cell wall) is increased firing of the baroreceptor afferents; baroreceptor neurons project into the NTS. The baroreceptor signal is an inhibitory signal (high ABP inhibits the SNS), and activate NTS neurons by releasing glutamate which stimulates inhibitory interneurons (indicated by dashed lines in **Figure 13**) that project from the caudal ventrolateral medulla (CVLM) to the RVLM. Conversely, as ABP decreases (even within a single cardiac cycle), sympathetic neurons increase the rate at which they transmit action potentials (Chapleau and Abboud, 1987); shown in **Figure 16**). An increase in total MSNA (frequency of neuron firing, or the number of neurons firing), results in greater amounts of vasoactive neurotransmitter (primarily NE) being released onto the smooth muscle cells surrounding the arteries and leads to increased vasoconstriction. It is important, however, to remember that baroreflex control of blood pressure is driven by changes in blood pressure that result in adjustments in MSNA (see **Figure 17**, solid line).

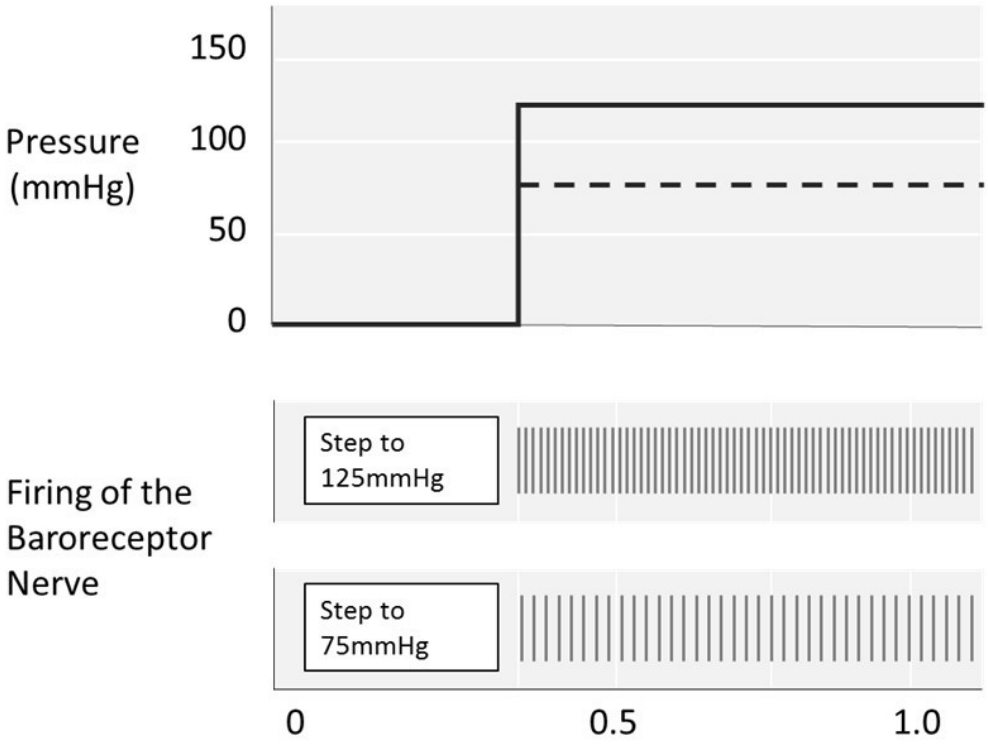

Figure 16. Responses of the baroreceptors to changes in arterial blood pressure (ABP). Increased ABP to 125mmHg (solid line) results in increased firing rate of the baroreceptors while decreasing ABP to 75mmHg (dashed lines) slows baroreceptor firing. Data from Chapleau and Abboud, 1987.

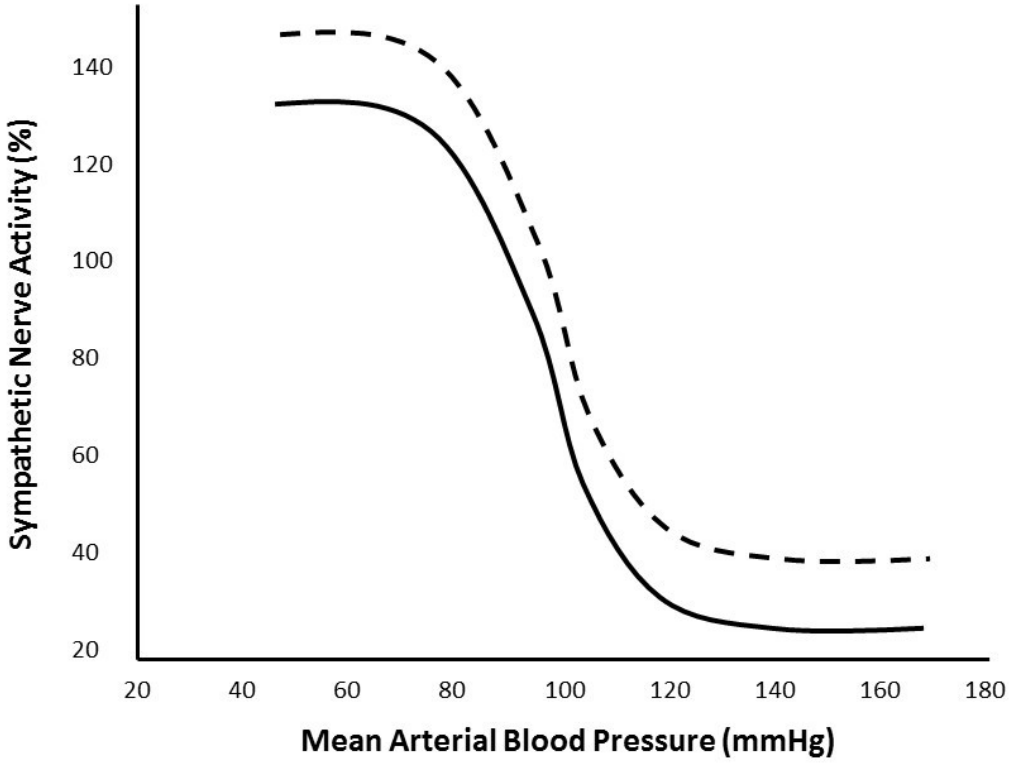

Figure 17. Baroreflex curve. A typical baroreflex curve (solid lines) such that any decreases in mean arterial blood pressure (normal is ~90mmHg) increases sympathetic nerve activity and increases in mean arterial blood pressure reduces sympathetic nerve activity. Re-setting of the curve upward (dashed lines) occurs during hypoxia and results in higher sympathetic nerve activity for any given blood pressure.

MSNA is under baroreflex control, such that periods of low BP result in higher MSNA and vice-a-versa (Delius *et al.*, 1972, Sundlof and Wallin, 1978). It has been shown that diastolic blood pressure (DBP) most accurately reflects the burst frequency of MSNA observed (Sundlof and Wallin, 1978) but not burst strength (amplitude or combined measurement; (Kienbaum *et al.*, 2001). That is to say, the baroreflex is "responsible" for increasing the frequency (Macefield and Wallin, 1999), while the chemoreflex may play a more active role in increasing the amplitude (neuronal recruitment) of the sympathetic signal during HX (Kienbaum *et al.*, 2001, Lusina *et al.*, 2006, Steinback *et al.*, 2010, Steinback and Shoemaker, 2012). Despite the fact that both burst frequency and amplitude are increased during HX (see **Figure 18**), burst amplitude has been shown to correlate most strongly with changes in ventilation during and following HX compared to burst frequency (Steinback and Shoemaker, 2012). The fact that burst frequency is increased following isocapnic HX implements the baroreflex regulation of MSNA to be changed (Querido *et al.,* 2011, Steinback and Shoemaker, 2012). Specifically, the curve is shifted upward (dashed lines in **Figure 17**) to a new set-point such that for a given ABP there is more SNA. Little is known about the changes in baroreflex function following HX, specifically, no study has addressed these changes following acute poikilocapnic HX.

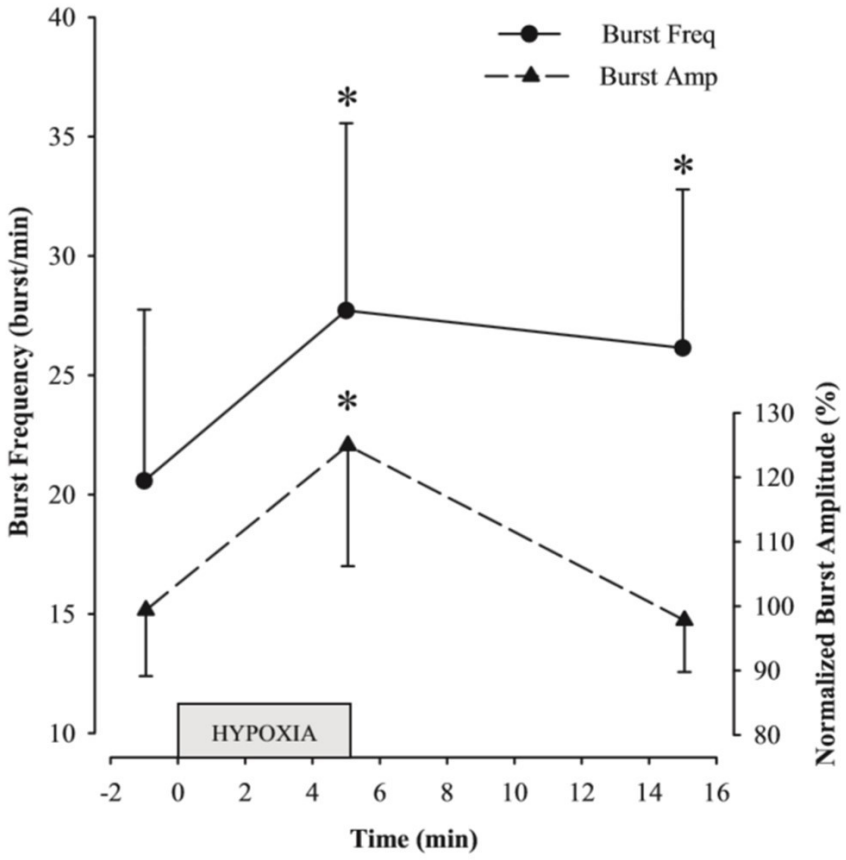

Figure 18. Sympathetic burst frequency and normalized amplitude preceding, during and after hypoxia. Burst frequency remains elevated poststimulus, whereas amplitude returns to prehypoxic values. Values are means ± SD. *significantly different from baseline, $p < 0.05$. From Steinback and Shoemaker, 2012.

Local Vasodilation during Exposure to Hypoxia

Although this review focuses on neurovascular effects and outcomes, it is important to highlight the direct dilatory effects of HX on the peripheral vasculature. During HX, there are competing effects of both vasodilation (direct effect) and vasoconstriction (SNS effect) on the peripheral vasculature. The endothelium releases factors that induce vasodilation to maintain oxygen delivery during HX. HX mediated vasodilation is primarily regulated by nitric oxide (NO) and prostaglandin (PG) pathways (Markwald *et al.*, 2011). Briefly, Adenosine acts on A_1 receptors on the endothelial cell membrane to activate PG (Ray *et al,* 2002) and increase vasodilation (Busse *et al.*, 1984, Messina *et al.*, 1992, Goodwill *et al.*, 2008, Markwald *et al.*, 2011). Further, both the endothelium and red bloods cells release ATP in response to HX (Sprague *et al.*, 1996, Ellsworth *et al.*, 2009) and this stimulates the NO pathway to induce vasodilation. The red blood cells may also respond by directly releasing NO in the form of S-ntiroshemoglobin (SNO-Hb; reviewed in Allen *et al.*, 2009). Additionally, the endothelium mediated vasodilation has been suggested to be modulated by acetylcholine, bradykinin and substance P, to name a few (reviewed in Busse *et al.*, 1985). Simultaneously, circulating EPI activates β_2-adrenergic receptors on endothelial cells (Blauw *et al.*, 1995, Weisbrod *et al.*, 2001) and activates the NO pathway to stimulate vasodilation (Sun and Reis, 1992, Blitzer *et al.*, 1996, Casey *et al.*, 2010, Markwald *et al.*, 2011).

The two primary vasodilatory pathways (NO and PG) interact and both can modulate the other's response to HX (Ray *et al.*, 2002, Nicholson *et al.*, 2009). Specifically, increase in PG synthesis by adenosine receptor activation has been shown to increase CAMP and stimulate NO synthesis in the endothelium (Ray *et al,* 2002) and blockade of only one of NO

or PG synthesis does not abolish the vasodilatory response to HX; blockade of both NO and PG synthesis is required to abolish the vasodilatory response to HX (Markwald *et al.*, 2011).

The body's ability to maintain vascular tone despite vasodilatory stress relies on the SNS. However, vasodilation can occur by NE binding to β-adrenergic receptors on the vascular smooth muscle cells (Richardson *et al.*, 1967, Blauw *et al.*, 1995). It has been suggested that HX may impair the vasoconstriction (SNS) response of the vasculature by a reduction in the post-junctional α_1-adrenergic receptors (respond to norepinephrine; NE) responsiveness (Heistad and Wheeler, 1970). This hypothesis has been supported by work in animals (Skinner Jr. and Costin, 1969, Marriott and Marshall, 1990). However, further studies investigating the responsiveness of the SNS to elicit vasoconstriction during HX in humans have shown that this response is well-preserved through a range of SaO_2 (down to 75%; (Rowell and Blackmon, 1986, Weisbrod *et al.*, 2001, Dinenno *et al.*, 2003, Hansen *et al.*, 2000). Specifically, Dinenno and colleagues (2003) showed that α-adrenergic responsiveness is maintained to 75% SaO_2. Thus, they postulated that more severe HX exposure may be needed to blunt the HX vasoconstriction response.

Effects of Hypoxia on the Sympathetic Nervous System

Chemoreceptor activation during HX results in an increase in MSNA that correlates strongly with the level of HX (or simulated altitude), increasing as arterial O_2 saturation (SaO_2) drops (Saito *et al.*, 1988, Somers *et al.*, 1989b). Hypoxia increases MSNA in healthy individuals after as little as 30 seconds (Smith *et al.*, 1996) and remains increased during prolonged (4 wk) exposure (Hansen and Sander, 2003). This increase can be measured using

the gold-standard microneurography technique which directly assesses the post-ganglionic nerve traffic (reviewed in Esler *et al.*, 1985, Mano *et al.*, 2006). Briefly, a small tungsten electrode is inserted into a peripheral sympathetic nerve (i.e. the peroneal nerve), and electrical activity of the nerve can be recorded. MSNA specifically refers to the sympathetic nerve activity of those post-ganglionic fibers innervating the smooth muscle cells of the blood vessels (arteries) of the skeletal muscle.

Acute HX exposure results in increased MSNA. This increase has been shown to occur after as little as 30 seconds after the start of the exposure (Smith *et al,* 1996) and that the response was graded to the level of HX (percent O_2) breathed (Saito *et al.*, 1988, Rowell *et al.*, 1989). However, an increase in MSNA is not observed until a "threshold" SaO_2 is reached, which is reported to occur at approximately 80% in healthy humans (Smith and Muenter, 2000). For example, Somers *et al,* (1989b) reported no changes in total MSNA at 14% inspired O_2 (91% SaO_2) but did see changes at 10% inspired O_2 (82% SaO_2).

Studies observing changes in MSNA over short durations (less than one hour) often report increases occurring after 5 minutes that plateau (do not continue to increase) for the remainder or the HX duration (Morgan *et al.*, 1995, Tamisier *et al.*, 2004a, Querido *et al.*, 2010, 2011). Thus it would appear that for a short duration stimulus, a plateau is reached quickly and MSNA remains at a constantly increased level for the remainder of the exposure. Intermittent duration (8hr) HX exposure, however, may result in decreased MSNA (Gilmartin *et al.*, 2006, Tamisier *et al.*, 2007). Tamisier *et al,* (2007) report initial increases in MSNA occurring after 1 hour HX exposure, but that MSNA burst occurrence is decreased significantly by 7 hours of HX; in this study, burst frequency at 7 hours was shown to not be different from baseline and not significantly decreased compared to 1 hour HX. The

observation of decreased burst occurrence is related to the increase in HR at 7 hours relative to 1 hour HX exposure (Tamisier *et al,* 2007). However, Gilmartin *et al,* (2006) also observed no increase in MSNA following 8 hours of HX exposure, but studies showing longer duration exposures (4 weeks) do show increased MSNA (Hansen and Sander, 2003). This is likely the result of the variation in respiratory pattern (**Figure 19.B**) occurring with acclimatization to altitude where the initial increase in ventilation results in decreased CO_2 and subsequent decrease in MSNA occurring between 1-8 hours after the start of the stimulus (HX).

The increase in MSNA during HX is influenced by CO_2, in that CO_2 acts as a SNS stimulant in addition to HX. Tamisier *et al,* (2004a) showed that MSNA was increased +70% in hypercapnic HX compare to +40% in hypocapnic HX. However, Somers *et al,* (1989b) showed that poikilocapnic (hypocapnic) HX resulted in a greater increase in total MSNA compared to isocapnic HX. While there is a lot of variability in the increase in MSNA during HX (ranges from +25% to +67%; Xie *et al,* 2001, Querido *et al,* 2010, 2011, Tan *et al,* 2013), it appears as though increased CO_2 levels result in increased MSNA within a specific time frame. Additionally, it may be that CO_2 modulates increases in MSNA by increasing amplitude (by neuronal recruitment) of sympathetic bursts (Steinback *et al.*, 2009). Further work investigating the role of CO_2 in the magnitude of the MSNA response to HX is needed to make further conclusions.

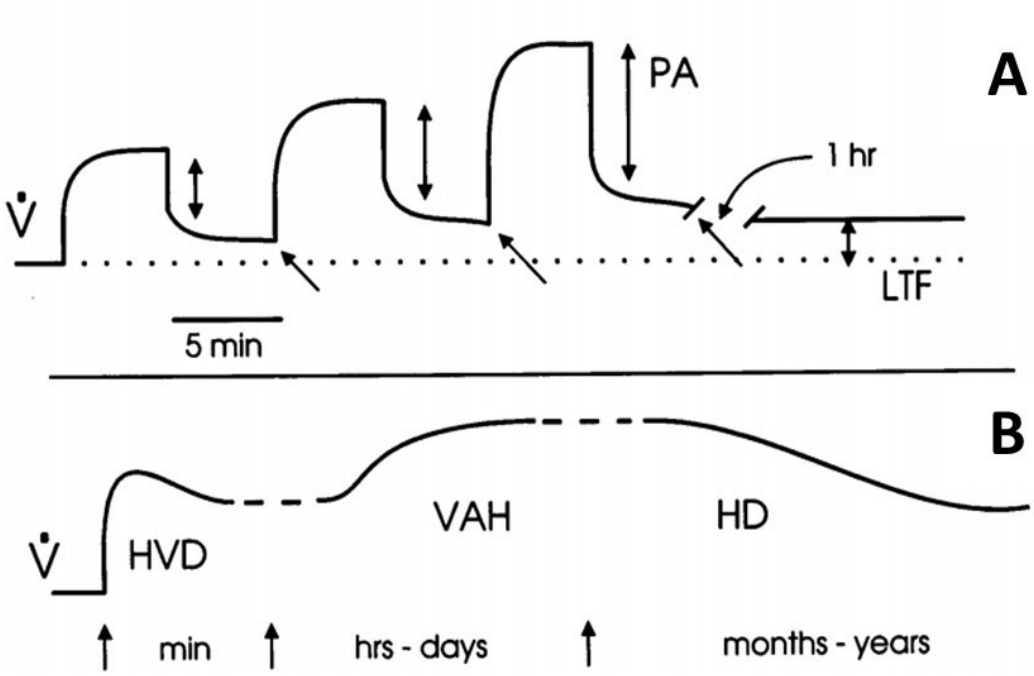

Figure 19. A) Ventilatory responses during and after episodic hypoxic exposures include progressive augmentation (PA) and long term facilitation (LTF). **B)** Ventilatory responses during and after prolonged exposures include hypoxic ventilatory decline (HVD), ventilatory acclimitization to hypoxia (VAH) and hypoxic desensitization (VDH).

Effect of Hypoxia on MSNA Post-Hypoxia

HX is the only sympathetic stimulus that has been shown to result in an augmentation of MSNA in the period following exposure (Morgan *et al.*, 1995, Steinback and Shoemaker, 2012, Steinback *et al.*, 2009) with the increase in MSNA persisting for a period at least that of the stimuli time (Morgan *et al.*, 1995, Xie *et al.*, 2001, Steinback and Shoemaker, 2012). This is true of stimuli ranging from as little as five minutes (Steinback and Shoemaker, 2012) up to 4 weeks (Hansen and Sander, 2003). Specifically, neither lower body negative pressure nor exercise will cause a persistent increase in MSNA (Mark *et al.*, 1985, Vissing *et al.*, 1989). Hypercapnia does activate the SNS to the same extent as HX, but MSNA does not remain activated in the recovery period (Somers *et al.*, 1989a, Xie *et al.*, 2001, Steinback and Shoemaker, 2012). Additionally, hyperoxic hypercapnia activates the SNS to a lesser extent than hypercapnic HX (Somers *et al.,* 1989a, Morgan *et al.,* 1995) indicating a role of oxygen sensing in mediating and maintaining the sympathetic response. Further, hyperoxia given in the recovery period will cause attenuation of the persistent MSNA (Querido *et al.,* 2010) demonstrating that there is something specific regarding HX and the PCR that changes the regulation of MSNA.

A threshold decrease to below ~80% SaO2 is required to increase MSNA during HX (Smith and Muenter, 2000). However, there appears to be no clear conclusion about whether or not the severity of the HX exposure affects the possibility that MSNA will remain increased in the recovery period. MSNA is shown to be increased following HX exposures that range from 85% down to 75% SaO_2 (Morgan *et al,* 1995, Xie *et al,* 2001, Tamisier *et al.,* 2004a, Lusina *et al,* 2006, Querido *et al,* 2010, 2011, Steinback and Shoemaker, 2012). Tamisier *et al,* (2004) was the only other study that did not observe persistence in the MSNA response

following 20 minutes of hypocapnic HX exposure, however they only targeted 85% SaO_2; they did, however observe persistence in their hypercapnic HX group at 85% SaO_2 indicating a role of CO_2 control in this response. Saito *et al,* (1988) subjected participants to up to 6000m simulated altitude (90 minutes total exposure, 30 minutes at three different simulated altitudes) and observed that MSNA burst frequency returned to baseline values in the recovery period; they are the only research group to show no persistence in MSNA following acute HX exposure to 80% SaO_2 or less (the proposed threshold).

Not all studies have shown that MSNA persists into the recovery (Saito *et al,* 1988, Gilmartin *et al,* 2006, Tamisier *et al,* 2007). These studies all investigated the effects of intermittent duration (1-8 hours) HX on MSNA. As described previously, these studies did not show increases in MSNA by the end of the HX exposure due to the fact that ventilation is decreased within this time-frame (relative to shorter or longer duration exposures; **Figure 19.B**; (Powell *et al.*, 1998), and this is likely why there is no carry-over effect.

The persistent augmentation of MSNA following HX also appears to be CO_2 dependent in that poikilocapnic (hypocapnic) exposures are less likely to result in an augmentation in this response (Tamisier *et al.*, 2004a, Gilmartin *et al.*, 2006, Tamisier *et al.*, 2007). All of the isocapnic HX studies looking at MSNA in the recovery period have shown a persistent MSNA response (Lusina *et al.,* 2006, Steinback *et al.,* 2009, Querido *et al.,* 2010, 2011, Steinback and Shoemaker, 2012, Tan *et al.,* 2013); the same goes for hypercapnic HX exposure (Morgan *et al.,* 1995, Tamisier *et al.,* 2004a). However, long duration poikilocapnic exposures (i.e. altitude studies) have shown MSNA to be increased up to three days after ascent to sea level (Hansen and Sander, 2003).

There have been several suggestions as to the mechanisms involved in maintaining the augmented SNA post-HX including sensitization of the peripheral chemoreceptors (Querido *et al.*, 2010, Steinback and Shoemaker, 2012), baroreceptor resetting (Querido *et al.,* 2011), and sustained vasodilation (Dinenno *et al.*, 2003, Tamisier *et al.*, 2004b). Querido *et al,* (2010) showed that the persistence of MSNA post-HX can be attenuated by hyperoxia, suggesting that it may be mediated in part by increased PCR reactivity following HX (Querido *et al.,* 2010, 2011). However, activation of the PCR leads to increased ventilation and ventilation is shown to return to baseline following exposure to acute HX (Steinback and Shoemaker, 2012). This is also demonstrated in that the firing of the carotid sinus nerve decreases (Peng *et al.*, 2003, Cummings and Wilson, 2005) following HX.

Steinback and Shoemaker (2012) found differences in the regulation of this persistence in MSNA, determining that burst frequency but not burst amplitude is increased in the period following HX stimulus. This suggests differential regulation of gating (frequency) and neuronal recruitment (individual burst amplitude) during and after HX, perhaps by different mechanisms. Steinback and Shoemaker (2012) suggest that the PCR is necessary to increase MSNA during HX and that activation of the PCR modulates the baroreceptor reflex during and following HX.

The PCR reflex has also been suggested to play a role in increasing baroreceptor gain in cardiovascular and respiratory diseases (Despas *et al.*, 2012). The baroreflex curve (sensitivity of the SNS to changes in ABP) has been shown to be re-set during and following HX in humans (Monahan *et al.*, 2006, Querido *et al.*, 2011) to higher MSNA for a given DBP with no change in the gain (reactivity) of the system (dashed line in **Figure 17**; Halliwill and Minson, 2002). Querido *et al,* (2011) showed that baroreflex re-setting can been attributed to

the persistence of the MSNA following acute HX whereby the sensitivity of the baroreflex entrainment of MSNA is changed by HX such that there are higher levels of MSNA for any given MAP. There may also be a baroreceptor resetting in hypertension. Wallin et al. (1973) showed in normotensive participants, reductions in ABP below normal levels (120-150/70-90 mmHg) blunted the MSNA, whereas in hypertensive participants this inhibition occurred at much higher ABP (150-200/100-120 mmHg). Further investigation is required to determine the interactions between these potential mechanisms leading to the persistent augmentation of MSNA post-HX and whether cardiovascular function is impaired [acutely] by this.

Effects of Hypoxia on Arterial Blood Pressure

During HX, the peripheral cardiovascular system is influenced by competing vasoactive factors. The reduction in PaO_2 induces local vasodilation by the interaction of autonomic and endothelial influences, while vasoconstriction is regulated primarily through sympathetic neural pathways. The net effect of these competing pathways is determined by varying factors including, but not limited to, strength of the HX stimulus, duration of HX, previous exposure to HX, ventilatory response, and CO_2 control.

Of the acute isocapnic HX studies (less than 1 hour) in which participants were exposed to similar severities of HX seven studies, show increases in ABP (Xie *et al.*, 2001, Lusina *et al.*, 2006, Steinback and Poulin, 2008, Querido *et al.*, 2010, 2011, Steinback and Shoemaker, 2012); and three show no increase in ABP (Dinenno *et al,* 2003, Thompson *et al,* 2006, Casey *et al,* 2014, Tan *et al,* 2013)0; these studies all varied in duration of exposure (5-20 minutes). Further, all acute poikilocapnic HX studies showed no persistent increase in ABP following HX (Heistad and Wheeler, 1970, Rowell and Blackmon, 1986, Saito *et al.*, 1991,

Cooper *et al.*, 2005), however recovery was only measured for a maximum of ten minutes following cessation of HX. Of the poikilocapnic HX studies reviewed here, only one study showed increased ABP with a duration less than 3 hours (Steinback and Poulin, 2008). In this study the target $P_{ET}O_2$ was 45 Torr which may have reduced SaO_2 below 80% (threshold; Smith and Meunter, 2000). Studies directly comparing poikilocapnic and isocapnic HX exposures have found increases in ABP during isocapnic and not poikilocapnic exposures (Somers *et al,* 1989; Ainslie and Poulin, 2004), with the exception of Steinback and Poulin (2008) who showed that the increase in ABP during HX may be delayed during poikilocapnic exposure.

From the two studies investigating the effects of different degrees of HX severity on ABP, it is difficult to make any conclusions about the effects of severity on changes in ABP. Specifically, Saito *et al,* (1988) exposed participants to simulated altitudes ranging from 4000m to 6000m; they observed decreased DBP and MAP at 4000m and 6000m which appeared to decrease with intensity (decreasing PO2). Rowell and Blackmon (1986) also looked at two different FiO_2 (10.4 and 7.6%) and also observed a decrease in MAP that increased in magnitude with decreasing FIO_2%. However, this protocol tests the two different severities of HX in sequence (without a break between) and the duration of the protocol may affect the outcomes as there is inconsistency in the ABP responses reported by those studies looking at poikilocapnic HX exposures ranging from 1-8 hours. Specifically, of the four studies reviewed here two showed increased MAP (Boos *et al.*, 2012, Parati *et al.*, 2013) and two showed no change in MAP (Tamisier *et al,* 2007, Lewis *et al,* 2014); there was no pattern with respect to duration of exposure for these studies although the observation that ABP is not increased at 8 hours follows that MSNA is also not increased at this time point.

Longer duration HX exposure may be accompanied by an increase in ABP, from as early as one day (Kanstrup *et al.*, 1999) to 4 weeks exposure (Hansen and Sander, 20003), although following acclimatization it is likely that ABP returns to sea level values (Dhar *et al.*, 2014). Dhar and colleagues (2014) collected ABP measures from 104 healthy male participants after 6 and 18 months at 4500-4800m altitude and reported no increase in ABP. Lewis *et al,* (2014) did not report an increase in any measure of ABP (DBP, SBP or MAP) after either 3 days or 12 days exposure at 5050m altitude (81-83% SaO_2), however, it may be that there are large inter-individual differences in the acclimatization time between people and the response of ABP to HX exposure has a different time course for each person.

The Effect of Hypoxic Exposure on Arterial Blood Pressure Post-Hypoxia

Following acute HX, it is accepted that MSNA is still increased, despite the removal of the initial stimulus and this may or may not result in increased ABP in the recovery period. The chances of having increased ABP following HX exposure largely depend on the conditions of the HX exposure including the severity and duration of the exposure, and CO_2 control during the exposure and whether ABP is increased during HX.

ABP is increased following both acute poikilocapnic (Rowell and Blackmon, 1986, Steinback and Poulin, 2008) and isocapnic (Xie *et al,* 2001, Lusina *et al,* 2006; Querido *et al,* 2010, 2011, Steinback and Shoemaker 2012) HX exposure. Many studies do not report the recovery data for ABP, specifically, of the acute HX studies reviewed here, only 8/13 isocapnic and 3/10 poikilocapnic HX report ABP in the recovery; these reports will need to be validated by future work in this area.

Few studies have reported ABP following different severities of HX exposure (Rowell and Blackmon, 1986, Saito *et al.,* 1988). ABP was shown to return to baseline following both of these exposures, although ABP and MSNA were not increased by the end of the exposure in either study (Rowell and Blaockmon, 1986, Saito *et al.,* 1988). Further, both of these studies looked at the effects of differing levels of HX in sequence (without a break) such that conclusions regarding the effects of ABP in the recovery cannot be made for different severities of HX. Thus, it appears that progressively more severe HX results in larger amount of vasodilation such that the reflex increase in MSNA (which is progressively increased) cannot compensate and a drop in ABP is observed.

Following intermittent HX exposure (1-24 hours), three out of four studies observed no change in ABP (Saito *et al,* 1988, Tamisier *et al,* 2007, Lewis *et al,* 2014); all of these studies observed no change or decreased ABP during HX. Gilmartin *et al,* (2006) observed increased SBP following 8 hours hypobaric poikilocapnic HX exposure (in a chamber, SaO_2 80%), however, they did not measure ABP during the exposure. Following 4 weeks at extreme altitude (5250m), MAP is shown to still be increased three days after return to sea level (Hansen and Sander, 2003). Additionally, ABP is returned to baseline by 18 months at high altitude, it remains the same (i.e. does not increase) upon decent (Dhar *et al,* 2014).

Vedam *et al,* (2009) investigated the effect of nitric oxide synthase (NOS) inhibition on the cardiovascular response to a twenty minute isocapnic HX exposure; NOS inhibition resulted in increased MAP during HX that did persist into the recovery. This implicates the vasodilation by nitric oxide during HX as a factor in observed ABP following HX. If there is a carry-over or exaggerated vasodilatory response occurring during HX, it may be that systemic ABP is not increased following HX (when HR and CO return to baseline). However,

following poikilocapnic HX exposure, increased ABP persisting into the recovery (Steinback and Poulin, 2008). This suggests that the persistent increase in MSNA lasting post-stimulus causing vasoconstriction that increases ABP. The differences in CO_2 control are likely responsible for the different ABP responses.

Effects of Hypoxia on Vascular Resistance

There is evidence that ABP is increased in conjunction with increased MSNA during acute isocapnic and long term HX exposure in humans. There is less evidence, however, in other vascular effects of HX occurring both during HX in the recovery period following HX exposure (while MSNA is still increased), including potential changes in vascular resistance to blood flow. This includes changes in both TPR and systemic vascular resitance (i.e. limbs).

TPR is based on Ohm's law that pressure and resistance are related such that:

$$\mathbf{MAP = Q \times TPR}$$

Where Q is the product of HR and SV. If HR, and thus Q, is increased without a change in MAP, TPR would be decreased and if MAP is increased, TPR would be decreased to a greater extent. It is entirely possibly for TPR to be decreased and for vascular resistance in one system (i.e. limbs) to be increased. The differential regulation of vascular resistance across the systems is regulated in part by the SNS whereby increases in MSNA would increase resistance in the periphery in the absence of vasodilation.

Effect of Hypoxic Exposure on Total Peripheral Resistance

TPR is shown to be decreased after acute HX exposure (Steinback *et al,* 2009). However, after exposure to hypobaric HX simulating ascent to 6100m (SaO_2 = 76%) TPR is increased by 10% with respect to sea level (Reeves *et al.*, 1987). Further increases in simulated altitude (7620m and 8840m; SaO_2 68 and 59%, respectively) results in increased TPR to up to 15% compared to sea level (Reeves *et al.,* 1987). This suggests that there are time-dependent changes in other cardiovascular variables that are changing during the HX exposure that affect TPR (e.g ventilation, changes in MSNA). Further, TPR refers to the whole body and it may be that there is differential regulation at the systemic level (i.e. peripheral vs central) and that this change is regulated by the severity of HX, duration of HX and by CO_2 regulation in a manner similar to MSNA and ABP.

During acute isocapnic HX, ABP and cardiac output (Q) are increased, and TPR has been shown to be decreased during acute HX exposure (Steinback *et al,* 2009); longer duration exposures (greater than 24 hours), however, appear to result in increased TPR (Kanstrup *et al,* 1999). Specifically, Kanstrup *et al,* (1999) observed a 23% increase after 1 day and a 33% increase in TPR by 5 days exposure to 4559m altitude despite SaO_2 being higher on day 5 (85%) compared to day 1 (79%). This suggests that the increase in resistance is independent of SaO_2 and is likely the result of persistent MSNA.

Stroke volume (SV) is decreased during 10 days at an altitude of 3100m (Alexander and Grover, 1983), and would result in an increase in TPR if ABP is unchanged (i.e. Reeves *et al.,* 1987). Indeed, TPR has been shown to decrease during acute HX exposure (Steinback *et al,* 2009). This speaks to the differential regulation of vasoconstriction by the SNS during HX, favoring blood flow redistribution to the central organs and brain while maintaining muscle

blood flow. CO_2 does not appear to play a role in the decrease in TPR during HX, in fact, all studies reporting TPR report at least a 20% decrease in TPR during HX (Steinback *et al,* 2009, Boos *et al,* 2012). Although the aforementioned studies all report decreased TPR during HX they all vary in duration of HX exposure (from 5 minutes to 5 days) and it may be that for the same duration of exposure CO_2 will affect the decrease in TPR in that the greater the $P_{ET}CO_2$, the smaller the magnitude of decreased TPR. Indeed, Thompson *et al,* (2006) reported that systemic vascular resistance index was only decreased 5% during 1 hour of isocapnic HX while 180 minutes of poikilocapnic HX results in a 20% decrease in TPR (Boos *et al.,* 2012). This implies that the increase in MSNA from the extra CO_2 may help maintain vascular resistance. This implies that a person travelling to altitude (whom is hyperventilating and experiencing hypocapnia) will experience a greater drop in TPR compared to someone who is being supplemented with CO_2 (in the lab) and may help explain why people feel light headed at altitude. However, during the acclimatization process, ventilation is slowly restored (over days) and the persistent MSNA may result in increased TPR (Kanstrup *et al.,* 1999).

Effect of Hypoxic Exposure on Peripheral Vascular Resistance

Although changes in TPR may not be directly related to the intensity of the HX stimulus, vascular resistance at the systemic level changes during acute HX exposure do appear to depend on the severity of the stimulus (total drop in O_2). Specifically, Heistad and Wheeler (1970) investigated the effect of breathing 12% and 10% FiO_2 for 16 minutes on forearm vascular resistance and found that resistance was decreased at 10% FiO_2 (decreased 5.9% from baseline) with no change at 12% FiO_2. The authors did not report the SaO_2 during each exposure, however it may be that 12% FiO_2 was not be sufficient to reach the threshold

(SaO_2 ~ 80%) for observing HX related responses (Smith and Meunter, 2000). Further work illustrating that a more severe HX stimulus will result in a greater magnitude drop in vascular resistance comes from Rowell and Blackmon (1986) who observed that splanchnic resistance was decreased by 16% at 10.4% O_2 and by 26% at 7.6% O_2.

Peripheral (limb) vascular resistance responses also appear to be time-dependent with short and intermediate duration (up to 8 hours) exposures to HX resulting in either no change in systemic vascular resistance (i.e. forearm or leg; Heistad and Wheeler, 1970, Morgan *et al.,* 1995, Xie *et al.,* 2001, Tamisier *et al,* 2004, Cooper *et al.*, 2005, Tamisier *et al.,* 2007) or decreased resistance (vasodilatory effect; Leuenberger *et al.*, 1991, Dinenno *et al.,* 2003, Tamisier *et al.,* 2004, Casey *et al.,* 2014, Lewis *et al.,* 2014). Longer duration exposures (greater than 24 hours), however, appear to result in increased forearm (Cruz *et al.,* 1976), leg (Hansen and Sander, 2003) vascular resistance.

Further data suggesting a decrease in resistance during longer HX exposures comes from work by Lewis *et al,* (2014) that shows an increase in carotid compliance at 3-4 days of HX exposure that normalizes with acclimatization (by 12-14 days). The carotid artery is much more prone to arterial stiffening (which would increase resistance) due to the fact that it is an elastic artery (Avolio *et al.*, 1985, Stewart *et al.*, 2003). If resistance in an elastic artery is increasing, it would suggest that HX affects vascular function negatively; it appears as though the increase in MSNA and blood pressure are observed as a "counter-balance" to maintain blood flow during a period of vasodilatory stress and that the decrease in resistance is observed due to an increase in blood flow in these regions.

Hypercapnic HX results in a greater increase in MSNA and this is likely the reason why blood flow resistance is maintained despite the large vasodilatory stress (Tamisier *et al,*

2004b). For example, hypocapnic but not hypercapnic HX results in decreased forearm vascular resistance during HX (Tamisier *et al,* 2004a). While blood flow resistance is decreased in the forearm during acute HX exposure (Leuenberger *et al,* 1991, Dinenno *et al,* 2003, Tamisier *et al,* 2004, Casey *et al,* 2014, Gilmartin *et al,* 2006), studies have shown no changes in leg vascular resistance during 20 minutes of isocapnic (Xie *et al.,* 2001, Tan *et al.,* 2013) or hypercapnic (Morgan *et al.,* 1995) HX exposure.

Effect of Hypoxic Exposure on Vascular Resistance Post-Hypoxia

Similar to the changes in vascular resistance occurring during acute HX exposure, there are regional differences in the vascular resistance responses occurring post-HX. These responses are also likely dependent on the severity or duration of the HX exposure, and CO_2 control during the exposure. In this review, only one study was shown to report TPR, four studies report forearm vascular resistance, and three studies report leg vascular resistance in the period of time following HX. For this reason, the effects of severity, duration and CO_2 control will be reviewed together for each region.

Effect of Hypoxic Exposure on Total Peripheral Resistance post-Hypoxia

Thompson and colleagues (2006) showed that the systemic vascular resistance index (similar to TPR) is returned to baseline following one hour of isocapnic HX (83% SaO_2). It should be noted that in this study the smallest magnitude decrease of any HX study reporting changes in TPR. It maybe that following more severe HX exposures (where TPR is decreased more than 20%), there is a delay in the return to baseline. Further, following poikilocapnic HX

exposures where TPR is not reduced to the same extent, but MSNA remains increased in the recovery, it may be that TPR increases in the recovery (as observed with the long term studies; i.e. Kanstrup *et al*, 1999). This would have implications for short duration poikilocapnic HX exposure (i.e. a day tour to altitude) where resistance would be increased in the period of time following descent. Increased resistance could impair oxygen delivery and result in altitude-related illnesses at sea level, where they might be misdiagnosed or mistreated.

Effect of Hypoxic Exposure on Peripheral Vascular Resistance post-Hypoxia

Forearm vascular resistance is increased in the post-HX period only when resistance is not decreased during HX (Tamisier *et al.,* 2004b, Cooper *et al.,* 2005). Specifically, Tamisier *et al,* (2004b) observed that forearm vascular resistance was increased following hypercapnic HX and not hypocapnic HX (sustained at -5mmHg below nomocapnic levels). Hypercapnic and isocapnic HX are shown to result in smaller decreases in forearm vascular resistance compared to poikilocapnic and hypocapnic HX because of the augmented MSNA response. However, Cooper *et al,* (2005) also showed an increase in forearm vascular resistance post-HX where no change was observed during HX; this is likely due to the less severe (12% FiO_2) HX exposure.

Duration of exposure may also play a role in the forearm vascular response following HX. Following 8 hours of poikilocapnic HX, forearm vascular resistance is decreased by at least 27% (Gilmartin *et al.,* 2006, Tamisier *et al.,* 2007). In both of these studies, no measures of vascular resistance were taken during the HX exposure, but it may be that the persistent vasodilation that follows a similar pattern in that longer duration HX exposures result in larger

vasodilatory stimuli. Additionally, Cruz *et al,* (1976) measured forearm vascular resistance following 72 hours of poikilocapnic HX and reported no significant changes during HX.

It has been shown that there is no significant change in FVR following acute HX, however, in these studies, there was no change observed during the HX exposure either (Morgan *et al.,* 1995; Xie *et al.,* 2001). Longer duration (4 wk) HX exposures resulting in increased leg vascular resistance during the exposure also report a return to baseline (by three days after; Hansen and Sander, 2003). It may be that although the MSNA responses are not different between limbs, there is greater vascular responsiveness in the leg (Carter *et al.*, 2005, Monahan and Ray, 2002) such that resistance is not changed following HX.

Effects of Hypoxia on Arterial Stiffness

Arterial stiffness varies at different regions of the body such that central regions (i.e. aorta) are less stiff (due to the elastic properties of the arteries) and peripheral locations are stiffer (reviewed in Laurent *et al,* 2006). Very few studies have investigated the effects of HX exposure on the stiffening in the arteries.

Effect of Hypoxic Exposure on Central (Aortic) Stiffness

Carotid-femoral PWV is the gold standard non-invasive measure for clinical arterial stiffness (Laurent *et al.*, 2006) and gives an index of changes in aortic stiffening. Many studies report augmentation index (AIx), and although these measures are not the same (central PWV vs AIx), they do represent aortic stiffening and have been validated (Yasmin and Brown, 1999, Laurent *et al.*, 2006).

All studies investigating the effects of HX on aortic stiffness (central PWV or AIx) investigate SaO_2 between 80-85%. An increase in central stiffness (AIx) is observed during acute HX if the measure is corrected for increases in heart rate (AIx75; the AIx if the heart rate is 75bpm; Parati *et al,* 2013) or if the duration of the exposure is greater than 1 day (Rhodes et al, 2011, Lewis *et al,* 2014; measured central PWV). Interestingly, Parati et al, (2013) did not observe an increase in central PWV, but only AIx75 at both 6 hours and 2 days.

It may also be that central stiffness is influenced by CO_2 in that poikilocapnic HX is more likely to result in decreased aortic time to reflection (measure of central PWV; Wilkinson *et al.*, 2002, Sharman *et al.*, 2005) compared to isocapnic studies (Vedam *et al,* 2009); a decreased time to reflection is correlated with an increased pulse wave velocity and increased stiffness of the aorta. Poikilocapnic HX results in greater decreases in resistance in TPR and the increased stretch (vasodilation) may lead to increased stiffness measured during HX.

Effect of Hypoxic Exposure on Peripheral Arterial Stiffness

Peripheral PWV is a measure of the stiffness of the non-central vasculature (i.e. arms or legs). Using applanation tonometry, ultrasound or other pulse transducers to detect the pulse at a peripheral location (i.e. finger or toe), the velocity of the pulse wave can be determined. Similar to central PWV, an increase in velocity is indicative of stiffer arteries. Peripheral PWV, however is directly regulated by the SNS such that increases in MSNA can lead to increased velocity in the peripheral vasculature (Failla *et al.*, 1999, Giannattasio *et al.*, 2005, Parati *et al.*, 2013, Parati and Salvi, 2015).

Boos *et al,* (2012) report no change in upper limb PWV but a decreased reflection time (increase in arterial stiffness) during 180 minute poikilocapnic HX exposure (72% SaO_2). Investigations of peripheral PWV (measured to the finger or toe) have shown no change in peripheral PWV up to 14 days duration (Boos *et al.*, 2012, Lewis *et al.*, 2014) despite MSNA being increased; these were measured at 180 minutes in Boos et al, (2012) and days 3-4 and 12-14 in Lewis et al, (2014). However, Rhodes *et al,* (2011) measured arterial stiffness daily and showed that the stiffness index decreased upon ascent to 3450m (days 1-2), increased above baseline values (+14%) by day 4, and that it decreases again when ascending to higher altitudes (4750m). This suggests that the initial response to HX is a decrease in peripheral arterial stiffness, but that acclimatization (if the level of HX does not change) results in return of stiffness measures to sea level values. However, once acclimatized, if the severity of the HX stimulus changes it will decrease stiffness again. Therefore, it is possible that the study by Lewis et al, (2014) showed no change in PWV due to the timing of their measurements.

The pattern of changes in peripheral arterial stiffness is likely a direct result of increasing vasodilation at the peripheral arteries with increasing HX severity. Vedam *et al,* (2009) determined that this initial decrease in arterial stiffness (after 20 minutes HX) is in part mediated by NO by using L-NMMA to block NOS and attenuating the reduction in AIx during HX. While no studies have specifically investigated the role of CO_2 in the peripheral PWV response to HX, isocapnic HX may increase the stiffness of the peripheral arteries to a greater extent than poikilocapnic HX by increasing MSNA to a greater extent.

Effects of Hypoxic Exposure on Arterial Stiffness Post-Hypoxia

All (two) studies investigating the effects of HX on arterial stiffness following HX exposure have found no difference in central (aortic) or peripheral stiffness (PWV or AIx) compared to baseline (Vedam *et al,* 2009; Boos *et al,* 2012). This suggests that short duration (less than 1 day) HX exposure does not persistently alter vascular stiffness despite a persistent vasoconstrictor signal from augmented MSNA post-HX. This also provides evidence of possible persistent vasodilation. However, no studies have investigated the effects of long-term exposure (i.e. sojourn to altitude) on the stiffness of the arteries in the weeks following decent to sea level.

Summary

Exposure to HX results in increases in MSNA that are immediate and reflective of the strength of the stimulus (level of O_2), the duration of the stimulus and the control of CO_2. Longer duration exposures may result in larger increases in MSNA, and higher CO_2 levels increase MSNA, likely by increasing the amplitude (strength) of the bursts of sympathetic activity. This increase in MSNA has been shown to persist following exposure for a time at least as long as the original exposure (for acute exposure) and for days after high altitude travel.

The increase in MSNA appears to be the result of a response to the vasodilation occurring during HX. As such, blood flow resistance is decreased and ABP is only increased during acute exposures with high CO_2 or during long term exposures. That is to say, ABP is

increased when MSNA is increased to a greater extent. Further, ABP may only become increased after some delay and may occur during the time following HX exposure.

Despite an increase in MSNA and ABP, vasodilation results in a decrease (or no change) in vascular resistance. There may be a regional response in changes in vascular resistance in that peripheral resistance is maintained while total (systemic) resistance in decreased. This would suggest a re-distribution of blood flow during HX to favor oxygen delivery to central organs/brain. The decrease in total vascular resistance has been shown to persist into the recovery (or have a delayed response becoming apparent in the recovery) during short term exposures, while long term exposures are more likely to result in increased resistance following exposure.

Arterial stiffness may be decreased peripherally during acute HX exposure, but appear to return to baseline during long term exposure. However, central arterial stiffness may be increased following long term HX exposure. These changes may be the result of vascular dysfunction occurring as the result of HX exposure. Understanding the differences in the results will help clarify for future studies the impact of exposure severity, duration and CO_2 control on the vascular outcomes. Particularly, it appears as though more severe, long duration, and hypercapnic HX exposure result in the most negative vascular outcomes (increased ABP, vascular resistance and artery stiffness). These conditions are of particular significance as they mimic real-life situations whereby persons would be exposed to HX. For example, a person might be exposed to HX for a long duration (and severe Hx for a short duration) if they are climbing Mt. Everest. Furthermore, persons with OSA experience repeated acute hypercapnic HX exposures (apneas) throughout the night. This review points

out the need for future research investigating the period of time following HX exposure when MSNA is still increased but (for the most part) vascular variables have returned to normal.

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Appendix II: A Review of the Methodology

Measuring Sympathetic Nerve Activity

Direct recording of SNA using microneurography

Direct recording of SNA by microneurography was first introduced by Hagbarth and Vallbo (1968), and is often used as a direct measure of activity of SNS output (reviewed in Mano *et al.*, 2006). This is a process that involves placing a small (35mm) tungsten microelectrode (needle) into a peripheral nerve (Hagbarth and Vallbo, 1968). The tip of the microelectrode is un-insulated (3-5μm) to allow for the electrical activity from the efferent muscle sympathetic nerve to be recorded. A similarly sized reference needle is placed just deep to the epidermis near-by and allows for background noise to be subtracted (blue flag in **Figure 20**). This process is widely used, to record MSNA, skin SNA and afferent nerve impulses, in both humans and animals.

Specifically, the electrode measures electrical signals from nerve traffic within the insonated nerve (i.e. the peroneal nerve). When an action potential travels down the axon of a muscle sympathetic nerve fibre (stained brown in **Figure 21**) within the proximity of the electrode it will be detected. The spatial relation to the electrode and the specific nerve fascicle it is located within will affect the absolute strength of the MSNA signal; the number of postganglionic muscle sympathetic nerves located surrounding the electrode will also affect the signal strength (Wallin *et al.*, 1973). So long as the position of the electrode is not changed throughout the recording, multiunit MSNA recordings are credited with showing excellent,

within subject, spontaneous temporal and reflex mediated variations in sympathetic outflow (Wallin *et al.*, 1973).

MSNA is often used as a direct measure of the influence of the sympathetic nervous system on the peripheral vasculature as increases in MSNA result in vasoconstriction (reviewed inMcAllen and Malpas, 1997) and increased vascular resistance. It is also important to note that a measurement of MSNA is only a measurement of sympathetic outflow (the specific frequency and integrated amplitude of multiple action potentials within the nerve) and does not necessarily denote catecholamine release or effector activity. The vascular responses depend not only on release of NE, but also α-adrenergic receptor density and sensitivity (Mancia and Grassi, 1991). For these reasons, ABP, HR, and other outcome variables are often measured in conjunction with MSNA.

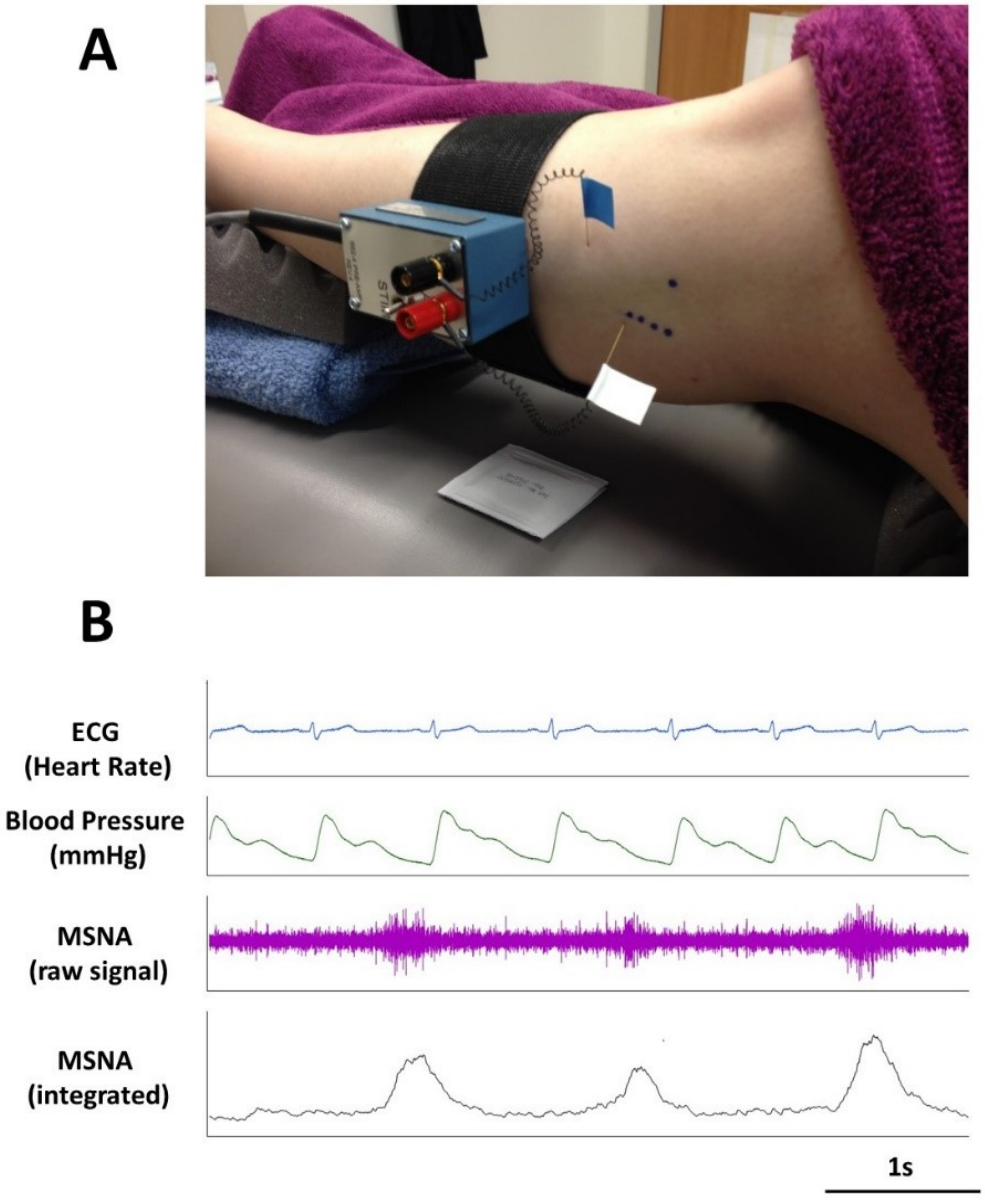

Figure 10. Microneurography. A) Image of the reference electrode (blue flag) inserted just under the epidermis and the sampling electrode (white flag) inserted into the peroneal (Fibular) nerve in a participant. The blue dots on the leg represent the head of the fibula (top dot) and the path of the nerve (row of four dots). B) Trace of five seconds of muscle sympathetic nerve activity showing the band-pass filtered signal (top) and the integrated signal (bottom) for five consecutive heart beats.

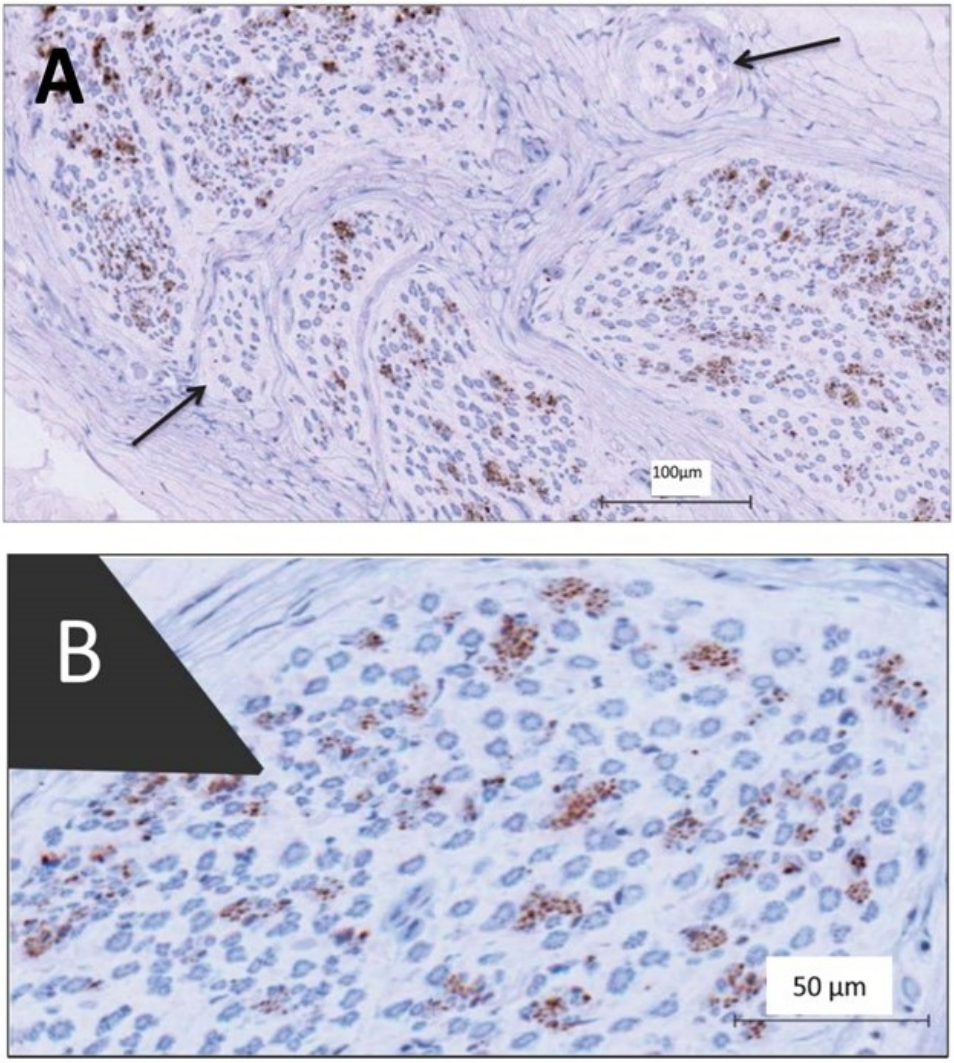

Figure 11. Muscle sympathetic nerve fasicles. A) View of how sympathetic fibres (positively stained for tyrosine hyrdroxylase) vary within each fascicle in a representative section of the common peroneal nerve. The amount of staining within the fascicle varied between fascicles of the same nerve. Some fascicles displayed no staining, while others showed a large abundance. Black arrows indicate fascicles with no sympathetic neurons. B) Schematic of microneurographic needle on a common peroneal nerve slice (enlarged image) to illustrate the relationship between microelectrode and sympathetic axon bundles in this nerve. From Tomkins *et al,* 2013.

Measuring SNA using plasma catecholamine concentrations

Blood plasma samples can be used to determine the levels of circulating catecholamines (sympathetic neurotransmitters). Activation of the SNS has been shown to increase plasma NE in humans (Rosenthal *et al.*, 1978, Watson *et al.*, 1979, Rowell *et al.*, 1989, Leuenberger *et al.*, 1991). MSNA has been shown to be positively correlated with plasma NE concentration at rest and during SNS stress (e.g. HX) in humans (Wallin *et al.*, 1981, Leuenberger *et al.*, 1991; see **Figure 22**). Hypertension (characterized by overactivity of the SNS) has also been shown to be associated with high plasma catecholamine concentration (e.g. DeQuattro *et al.*, 1976).

However, plasma NE concentration depends on multiple factors including release at the nerve endings and clearance. Furthermore, increases in blood volume, blood flow redistribution and differences across the vascular tree (arterial vs. venous) can alter the measured plasma concentrations. Therefore, circulating NE is estimated to represent only 5-10% of secreted NE (reviewed in Mancia and Grassi, 1991). For these reasons, plasma NE measurements are less sensitive/ reproducible measure compared to MSNA (Grassi *et al.*, 1997). Grassi and colleagues (2009) provide evidence that multiple (at least 3) plasma NE samples need to be taken and averaged to produce reproducible results that are comparable to MSNA in resting humans.

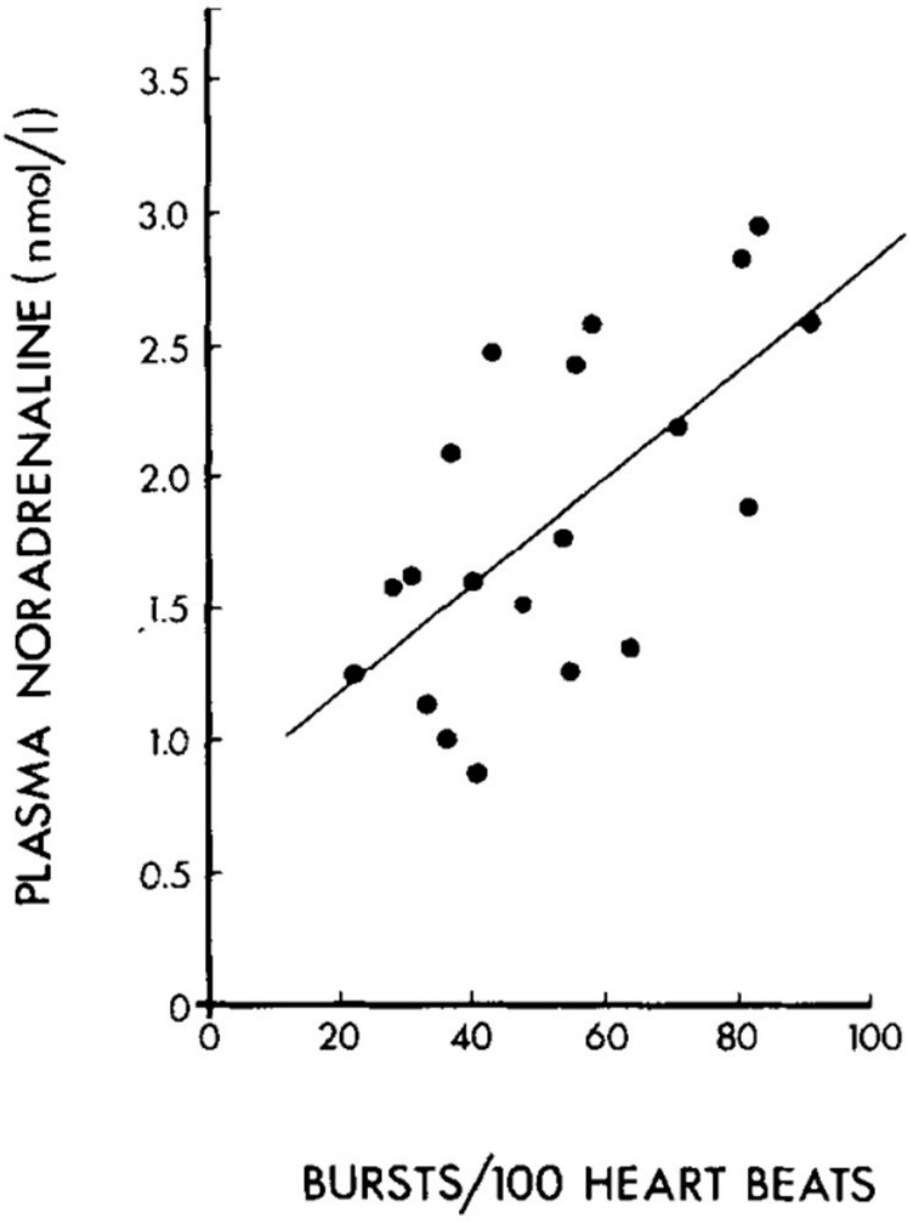

Figure 12. Relationship between mean level of MSA (expressed as bursts per 100 heart beats) and plasma concentration of noradrenaline. Each point represents one subject. $r = 0.65$, $p < 0.01$. (Figure from Wallin *et al,* 1981).

During acute HX, when MSNA is increased, many studies have not shown similar increases in plasma NE (reviewed in Rostrup, 1998). Indeed, only Leuenberger *et al,* (1991) showed increased plasma NE in response to 30 minutes of HX. They reported that although NE release is increased during HX, NE clearance is also increased (Leuenberger *et al.*, 1991). Leuenberger *et al,* 1991 most likely observed changes in NE during HX due to the fact that they were measuring from the arterial circulation, however not all studies measuring arterial NE have reported increased NE during HX (Dinenno *et al.,* 2003); this is likely because the aforementioned study only subjected participants to 5 minutes HX compared to 25-30 minute exposure in Leuenberger *et al,* (1991). Further, studies looking at chronic (greater than 1 week) HX exposure consistently show increased plasma NE (reviewed in Rostrup *et al.,* 1998). However, following acclimatization at altitude (after 1 week), blood plasma volume is decreased relative to whole blood volume (hematocrit is increased) and concentration may be increased for this reason.

Pattern of MSNA

MSNA occurs, in humans, in bursts (McAllen and Malpas, 1997). Human burst patterns have been shown to occur at a lesser frequency compared to animals, at a rate approximately equal to once every three or four heart beats at rest (McAllen and Malpas, 1997). MSNA is also strongly time-locked with the cardiac cycle (Delius *et al.*, 1972, Fagius, 1988, Juan *et al.*, 1987) , such that bursts only occur during the diastolic (filling) phase, and that the occurrence of bursts is inversely related to variations in diastolic pressure (Juan et al., 1985 in Fagius, 1988). The synchronicity of MSNA bursting pattern with the ECG tracing is a key characteristic of MSNA used in identifying microelectrode location within a nerve fascicle containing muscle sympathetic traffic (see **Figure 21**). Other criteria used for

establishing location include that MSNA (1) is modulated by respiration, (2) responds to changes in blood pressure, and (3) can be increased by increasing intra-thoracic pressure (Mano *et al.*, 2006).

Furthermore, it has been shown that DBP is most strongly associated with the burst frequency of MSNA observed (Sundlof and Wallin, 1978) but not burst strength (amplitude or combined measurement; (Kienbaum *et al.*, 2001). This differential relationship in frequency and strength has been supported with animal work cats (Malpas and Ninomiya, 1992), and rats (DiBona and Jones, 1998) and in humans following acute HX (Steinback and Shoemaker, 2012; see **Figure 18**). A model describing the convergence of two pathways has been described (Kienbaum *et al.*, 2001), and evidence supporting this model have been obtained (Keller *et al.*, 2006).

MSNA has also been shown to be linked closely with the respiratory cycle (Eckberg *et al.*, 1985). This may be due to respiratory induced changes in blood pressure or blood gases, central oscillation theory, alveolar inflation inhibition, or any combination of the aforementioned (Mano *et al.*, 2006). Regardless, voluntary (i.e. apnea, hyperventilation) and involuntary (in the absence of central command) changes in respiration result in changes in MSNA (St. Croix *et al.*, 1999).

Quantification of MSNA

MSNA occurs in bursts of activity that need to be processed in order to quantify. The raw MSNA signal is amplified, band-pass filtered (700-2000Hz), rectified and integrated (0.1s time constant) to obtain a mean voltage neurogram where bursts of MSNA are easily

identified. Both **Figures 20 and 23** show representative tracings from the filtered and integrated MSNA signals.

MSNA is quantified in terms of frequency (number of bursts and burst probability or occurrence) and amplitude (see **Figure 24**). Frequency is basically counting the number of sympathetic bursts that occur within a specific time frame (i.e. five minutes). A burst of sympathetic activity is characterized as occurring during the diastolic phase of the heart beat and there is never more than one burst occurring for any given heartbeat. Further, most persons quantifying MSNA use criteria that the burst must have a signal to noise ratio of 3:1, that is to say the burst is at least three times as large as background noise. Burst frequency can be assessed in both absolute (bursts/minute) and relative (bursts/100 heart beats) terms.

MSNA amplitude varies with electrode position and as such, is not able to be quantified in absolute terms. For studies investigating changes in MSNA, baseline amplitude can be determined as the average amplitude measured during a specific period of time or the amplitude of any given burst can be assessed as a percentage of the largest burst occurring during quiet rest (baseline; largest burst would be equal to 100% and all other bursts scaled accordingly). Lastly, total MSNA is expressed as the absolute frequency multiplied by amplitude and is a measurement that talks both frequency and amplitude into account when quantifying MSNA.

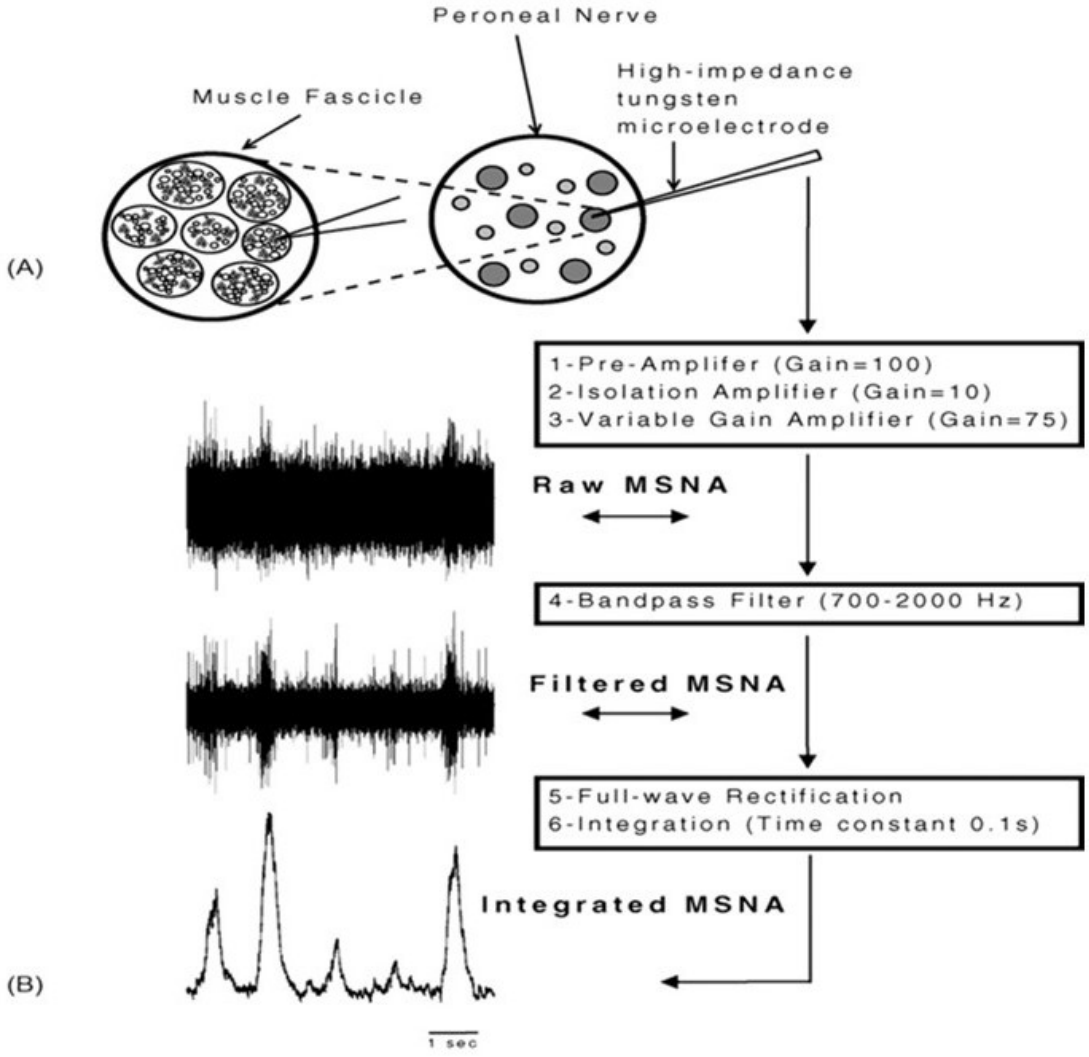

Figure 13. (A) Schematic representation of a microelectrode inserted into a human peripheral nerve for sympathetic recordings. The nerve contains bundles of sympathetic nerves that target blood vessels in muscle or skin, Adapted from (Wallin, 2004, Macefield et al., 2002). (B) Representative examples of the raw, filtered and integrated muscle sympathetic nerve activity. From (Salmanpour *et al.*, 2010).

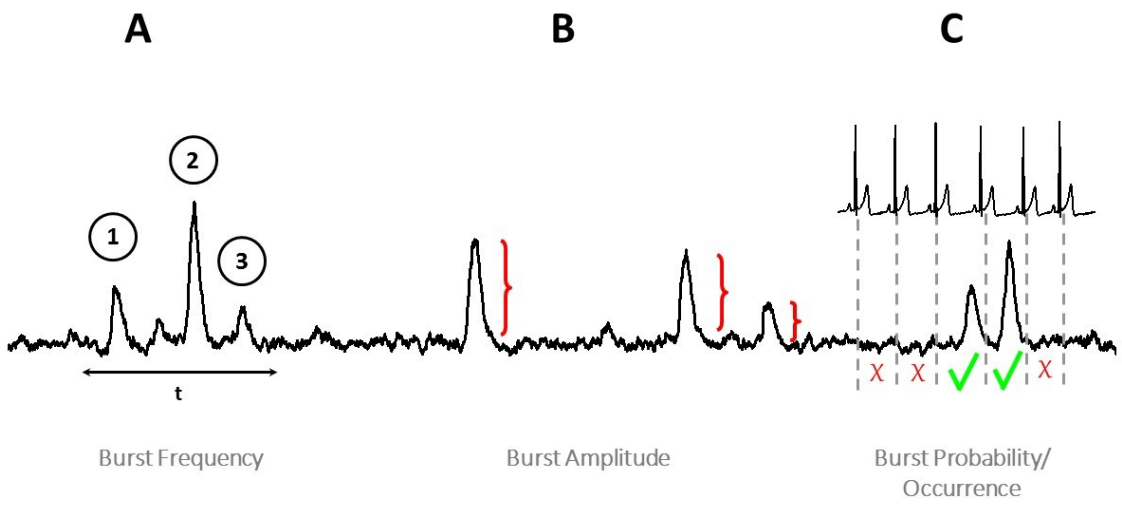

Figure 14. Quantification of muscle sympathetic nerve activity (MSNA). **A)** Burst frequency refers to the number of bursts for a given amount of time (usually one minute). **B)** MSNA burst amplitude refers to the given amplitude (height) of a specific burst of MSNA. Burst amplitude is usually quantified as a percent change from baseline. **C)** MSNA burst occurrence refers to the probability of a specific heart beat having a burst of MSNA associated with it. Burst occurrence is sometimes referred to as burst occurrence and is typically measured as the number of bursts occurring for every 100 heartbeats. Figure created by Dr. Steinback, used with permission.

Factors Affecting Resting MSNA Values

Many factors contribute to resting MSNA including age, sex, body mass index (BMI) and others (reviewed in Wallin, 2007). Sundlof and Wallin (1978) first characterized MSNA as increasing with age; this has been confirmed by others (Sundlof and Wallin, 1978, Fagius and Wallin, 1993). Although sex has been shown to affect resting MSNA (Matsukawa *et al.*, 1998, Ng *et al.*, 1993) initially, responses to sympathetic stressors did not appear to be different between sexes (Jones *et al.*, 1996). However, recent evidence suggests that the MSNA response to both chemoreflex and baroreflex stress does differ between sexes and across the phases of the menstrual cycle (Usselman *et al.*, 2013, Patel *et al.*, 2014).

Increased BMI and body fat percentage have been shown to be correlated with MSNA (Scherrer *et al.*, 1994). It was also hypothesized that resting MSNA may correlate strongly to MAP such that persons with high levels of MSNA also had higher resting MAP. This is not the case, and in fact there is little to no correlation between the two variables (Sundlof and Wallin, 1978, Skarphedinsson *et al.*, 1997, Charkoudian *et al.*, 2005). Furthermore, although there are large inter-individual variability/differences (reviewed in Wallin, 2007), it is likely that genetics plays a role (Wallin, 1993).

Reliability and Validity of MSNA Measurement

Long term (> 10 years) reproducibility of MSNA burst frequency in the same group of individuals has been shown (Fagius and Wallin, 1993). However, as previously mentioned, MSNA burst frequency has been described as increasing over time (Fagius and Wallin, 1993, Sundlof and Wallin, 1978). Intra-observer variability in MSNA analysis has been shown to be less than 5% and inter-observer variability is less than 10% (Fagius and Wallin, 1993).

Advances in technology have made the detection of MSNA bursts easier and most likely decreased both the intra and inter-observer variability's (Lambert *et al.*, 2012)

The decision to record from peroneal nerve or other lower extremity nerve (i.e. tibial nerve) is dependent on the research group and training environment; in North America, the peroneal (fibular) nerve is most often used to measure MSNA, with the microelectrode inserted near the fibular head (Mano *et al.*, 2006). Regardless of nerve choice, Mano et al. (1996) showed that the discharge patterns of both nerves are almost identical in pattern and respond synchronously (Wallin *et al.*, 1973, Vallbo *et al.*, 1979). This observation suggests that post-ganglionic sympathetic neurons directed at the skeletal muscle are homogeneous in their activity; that is to say, they all receive similar directions from the spinal/ganglionic level (Vallbo et al., 1979).

Safety of MSNA Measurement

Due to the nature of the microneurography recording, it is possible that adverse events may occur. These may include, but are not limited to: infection, hyperesthesia, partial paresis, pain and tenderness, and numbness (reviewed in Mano *et al.*, 2006). In order to avoid infection, microelectrodes should always be sterilized, and used at a 1:1 ratio for electrodes to participants (Mano *et al.,* 2006). In a large study conducted by Eckberg and colleagues (1989), questionnaires were sent to over 1000 persons who had participated in microneurography studies. Conclusions from this report indicate that the occurrence of neuropathy following microneurography is extremely rare (occurring in 0.3% of participants), and none were permanent (resolved by six months; Eckberg *et al.,* 1989). Further, symptoms of tingling, prickling, pain, and numbness lasting less than two weeks only occurred in 9% or persons

(Eckberg *et al.*, 1989). Animal work has shown that any damage caused by prolonged probing or recording is transient and no marked axonal degeneration was reported (Takahashi *et al.*, 1982) with half of the nerves healing completely by 4 months and the other half under the process of repair (Fried *et al.*, 1989).

Measuring the Cardiovascular Variables Associated with Changes in Sympathetic Nerve Activity

Measuring Heart Rate and Arterial Blood Pressure

Heart rate (HR) can be determined using three-lead electrocardiography (ECG) using a lead-II configuration. This involves placing the negative (white) electrode on the right side just below the clavicle, positive (red) electrode on the left side on the lower ribs and the ground electrode on the left shoulder. This configuration provides an ECG tracing similar to **Figure 25**, which allows for instantaneous HR to be determined from the ECG waveform as the duration of the R-R interval divided by sixty (quantified as beats per minute; BPM).

Using finger photoplethysmography (Finometer Pro®, Finapres, Netherlands), beat-by beat ABP can be determined and changes in ABP across time determined. The blood pressure waveform, and derived cardiac output (model flow algorithm from Finometer) are exported from the Finometer and MAP, SBP, and DBP can be calculated from the pressure waveform using Lab Chart peak analysis software. This measure is calibrated using a return to flow measurement from the Finometer and corrected with three manual blood pressure measurements taken prior to the start of the protocol.

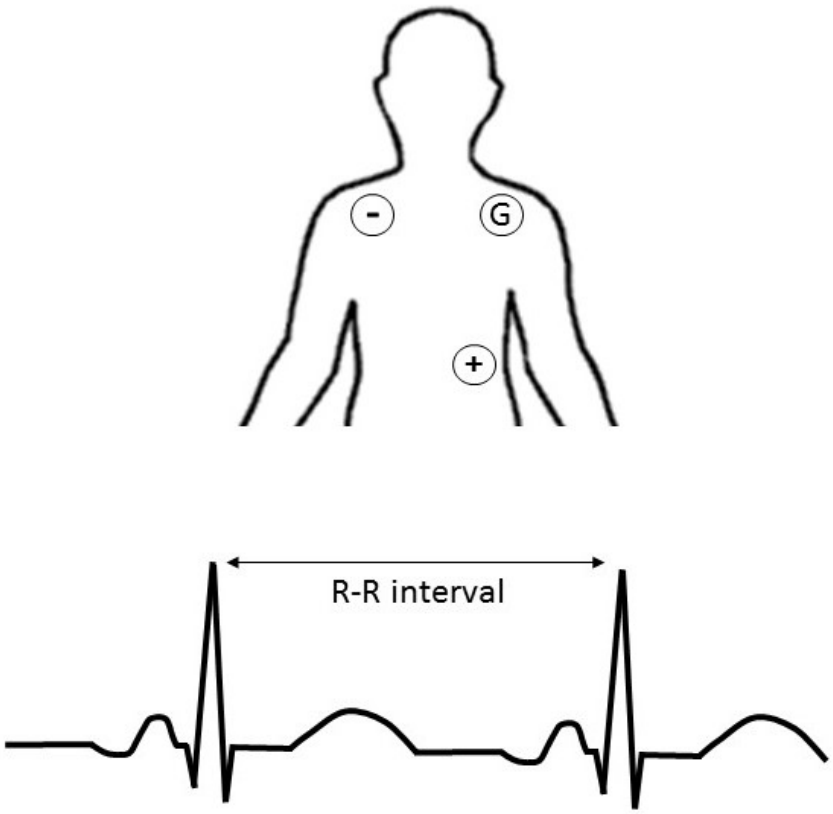

Figure 15. Using electrocardiography (ECG) to determine instantaneous heart rate. Three ECG electrodes are placed on the participant in a lead-II fashion, with the negative electrode on the right shoulder just under the clavicle, the positive electrode placed on the left side on the ribs and the ground placed on the left shoulder. This configurations (shown on top) gives an electrical recording similar to the bottom panel. From this, the time between two heart beats (R-R interval) can be determined and instantaneous heart rate (beats per minute) is calculated as R-R divided by 60.

Measuring Femoral Blood Flow and Femoral Vascular Resistance

Femoral artery blood flow (FBF; L/min) can be easily determined using peripheral ultrasound. Briefly, high frequency ultrasound waves (sound waves) are produced by vibration of piezoelectric crystals (by electrical current) that can be transmitted through a probe into the body in bursts (see **Figure 26**). Different tissues in the body will reflect the waves back from their respective distances from the probe and these are received back at the probe and transmitted to the machine. The internal processing performed by the ultrasound machine use the speed of sound travelling through tissue (1570 m/s) and the time for the return of the sound wave to display each sound wave reflected as part of an image on a screen (**Figure 26**).

Using the mean (area under the curve) from the peak velocity signal to calculating FBF is not the same as blood flow calculated directly on the ultrasound machine. The weighted mean (from the ultrasound) takes into account the proportion of blood travelling at each speed and displays this on the ultrasound machine as a more intense signal for a greater proportion of blood travelling at that speed. Assuming the proportion of blood travelling at each velocity does not change throughout a protocol, and if the same method for determining FBF, FVR and FVC is used across the protocol, it is possible to make comparisons between time points.

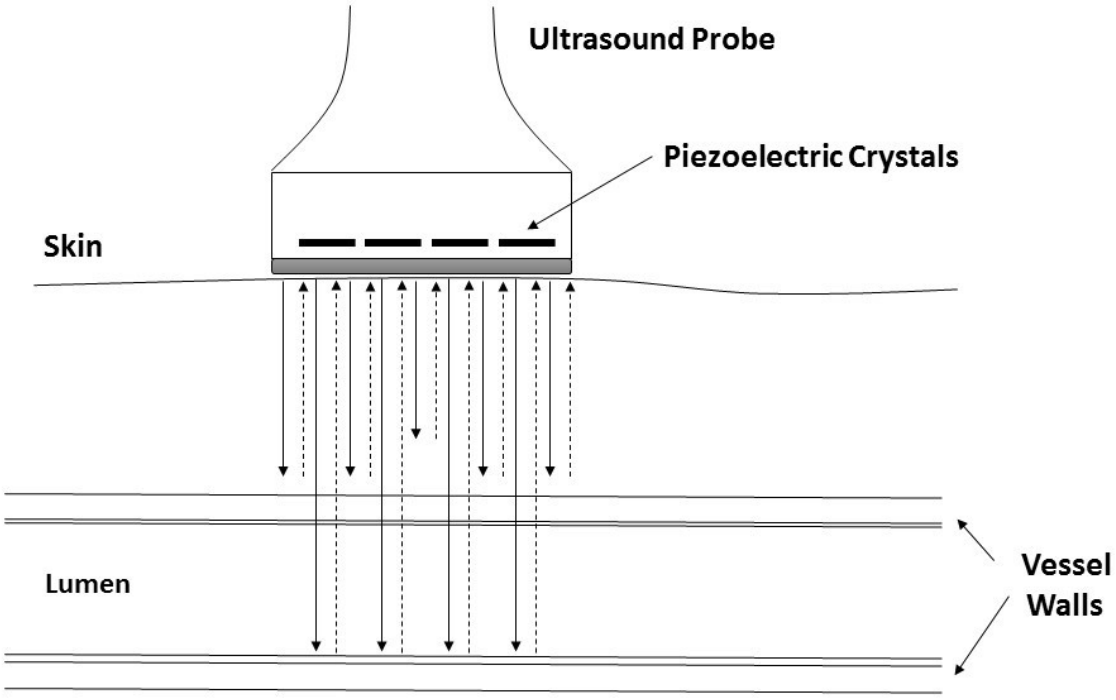

Figure 16. Measurement of blood flow using ultrasound. Electrical current is applied to piezoelectric crystals located in the ultrasound probe which transmit ultrasound waves into the tissue. These waves are reflected off of surfaces of organs (i.e. blood vessel walls) and can be combined to form an image (see **Figure 27**).

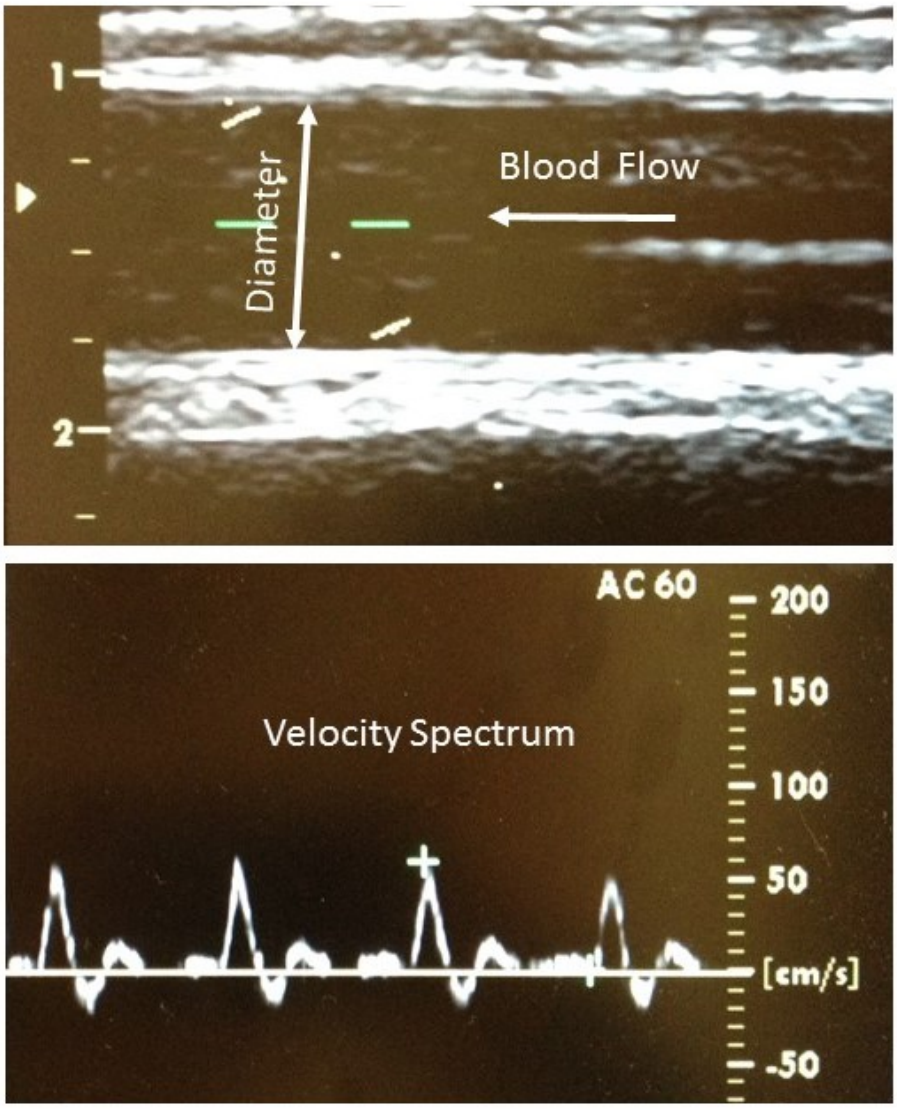

Figure 17. Femoral blood flow signal from the peripheral ultrasound. **Top:** Image of blood flow through the femoral artery. The diameter is determined as the distance from the upper wall of the blood vessel to the lower wall of the blood vessel. **Bottom:** The blood velocity signal for each heart beat indicating the speed of the red blood cells through the femoral artery.

The superficial femoral artery is a large artery running down the medial surface of the upper leg in humans. It can be insonated using ultrasound at a position just distal to the bifurcation of the common femoral artery (**Figure 28**). The peak femoral blood velocity signal can be used in conjunction with ultrasound images to determine FBF. Briefly, the blood vessel diameter is determined during systole and diastole and the cross sectional area of the blood vessel can be determined from blood vessel diameter according to equation:

$$\textbf{Cross Sectional Area} = \prod \textbf{x (radius)}^2$$

Where the radius is equal to half of the diameter. Using a mean cross sectional area (1/3 systolic +2/3 diastolic), mean FBF determined according to the following equation:

$$\textbf{Mean FBF = mean FBV * mean cross sectional area}$$

Using MAP, femoral vascular resistance (FVR; mmHg/L/min) can be calculated as:

$$\textbf{FVR (mmHg/L min}^{-1}\textbf{) = MAP (mmHg) / FBF (L/min)}$$

And femoral vascular conductance (FVC) can be calculated as the inverse of FVR.

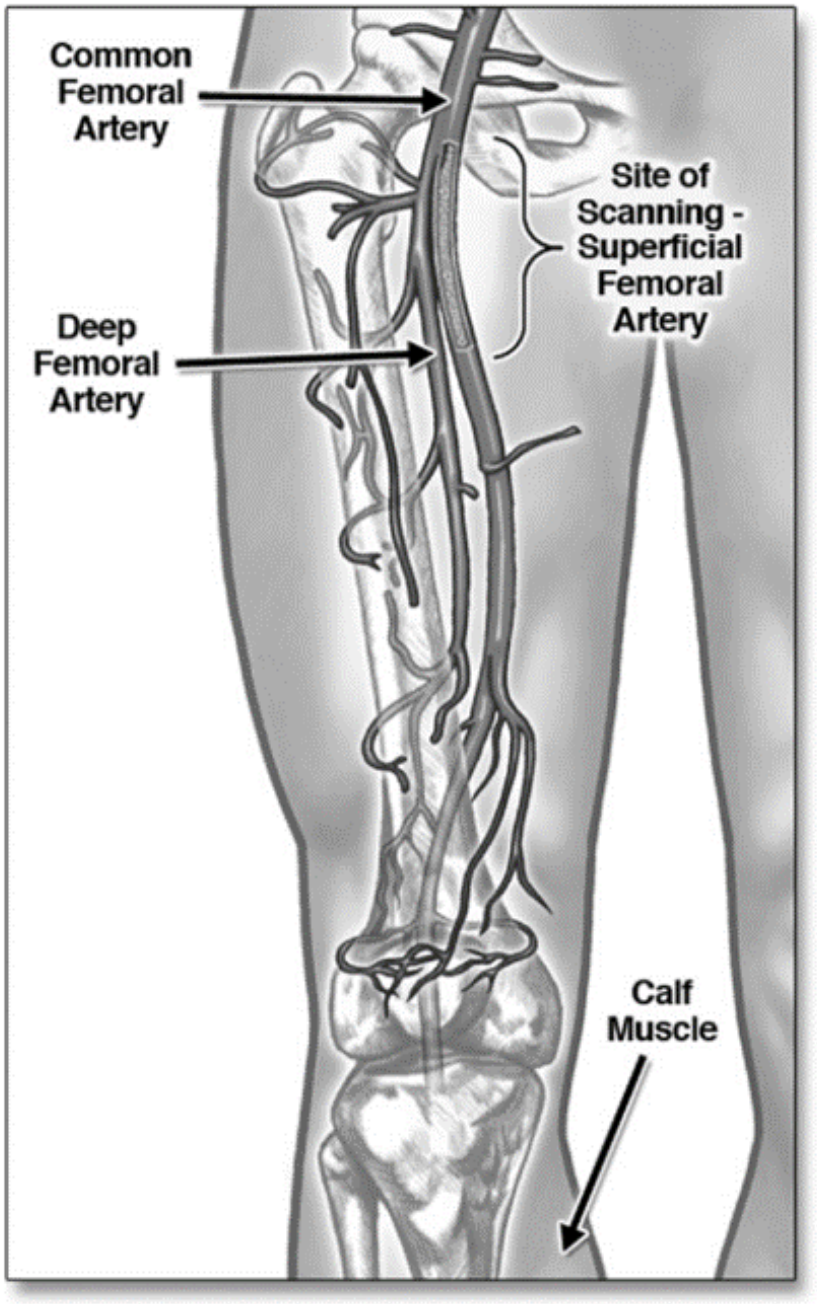

Figure 18. Location of superficial femoral artery imaging. The superficial femoral artery is insonated just distal to the bifurcation of the common femoral artery in all participants. From (McDermott *et al.*, 2011).

Measuring Pulse Wave Velocity to Determine Arterial Stiffness

Pulse wave velocity (PWV) is a non-invasive measurement of arterial stiffness and is determined by measuring the pulse transit time at multiple locations in the body to determine the velocity at which the pulse wave is travelling (**Figure 27**). For a given heartbeat, there is a pressure waveform which travels through the vascular tree. The time for the wave to travel to a given location is dictated by the initial pressure (systolic blood pressure) and by the stiffness of the vessels. A stiffer blood vessel will allow blood to travel faster, where a more compliant vessel will result in slower blood flow. Arterial stiffness varies at different regions of the body such that central regions are less stiff (due to the elastic properties of the arteries) and peripheral locations are stiffer (reviewed in Laurent *et al.,* 2006).

Central PWV is a measurement of aortic stiffness and is the most clinically relevant (Laurent *et al,* 2006). The gold standard for determining central PWV is simultaneous pulse measurements at the carotid and femoral arteries (Laurent *et al.,* 2006). Peripheral pulse wave velocity (i.e. using the radial artery) can also be determined using this technique, where the PWV measured at the carotid is used to account for any changes in heart contractility (see **Figure 29**).

There are a variety of devices designed to assist in the determination of PWV, many of which use applanation tonometry to record pulse waves. However, methods using mechanotranducers or Doppler ultrasound are well accepted for determining PWV (reviewed in Laurent *et al,* 2006).

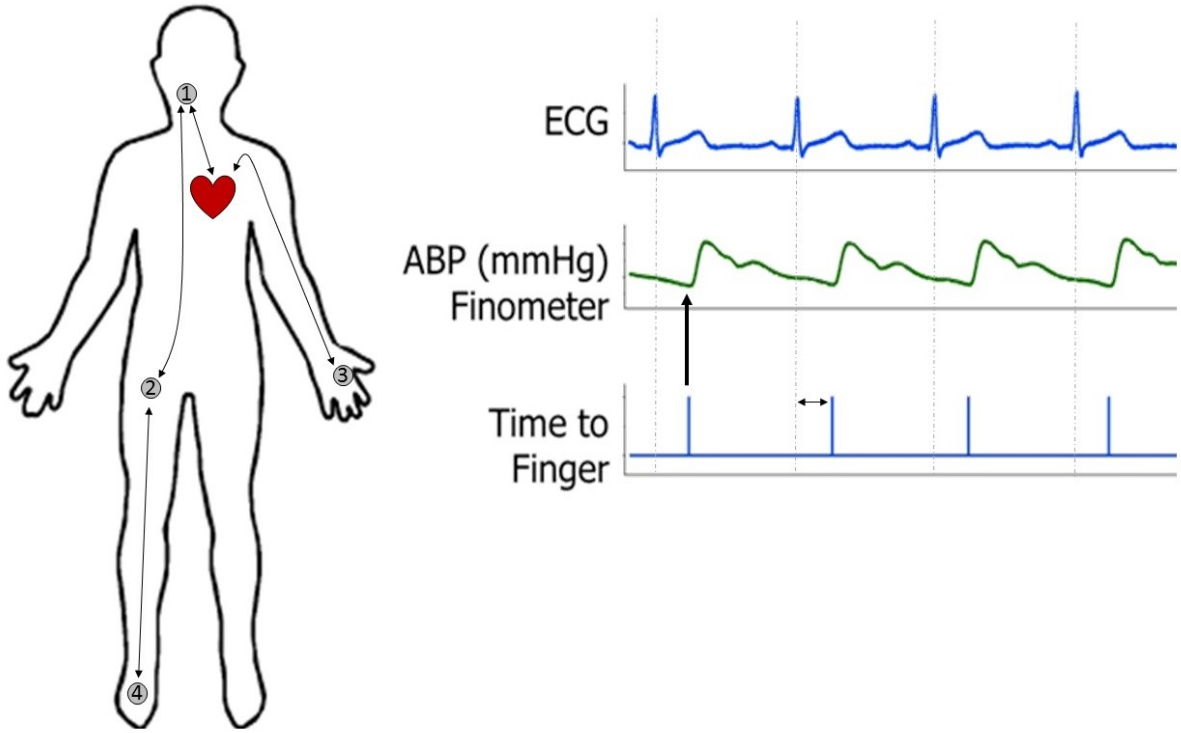

Figure 19. Measuring pulse wave velocity (PWV). Pulse wave transit time is the time from the QRS wave in electrocardiogram (ECG) signal (indicated by dashed lines) to the foot of the pulse wave (shown by the arrow and measured by the spikes in the bottom row of the trace). Pulse transit time was determined at four locations 1) carotid artery, 2) femoral artery, 3) finger, and 4) toe. Using the distances between the heart (measured at the sternum) and each peripheral location, PWV was determined. ABP, arterial blood pressure.

PWV is calculated according to

$$\textbf{Velocity} = D / T$$

Where T is the time from heart contraction (QRS wave) and D is the distance from the heart (sternum) to measurement site. The PWV at the carotid artery is used to correct for changes in heart contractility. Specifically, using the numbered references for location from **Figure 29**:

$$\textbf{Central (Femoral – Carotid) PWV} = D_2 - D_1 / T_2 - T_1$$

$$\textbf{Upper Limb (Finger – Carotid) PWV} = D_3 - D_1 / T_3 - T_1$$

$$\textbf{Lower Limb (Toe – Femoral) PWV} = D_4 - D_2 / T_4 - T_2$$

Measuring the Respiratory Responses to Hypoxia

Measuring Expired Oxygen and Carbon Dioxide Content

The partial pressure of the end-tidal O_2 ($P_{ET}O_2$) and CO_2 ($P_{ET}CO_2$) approximate arterial blood gas concentrations (PaO_2 and $PaCO_2$, respectively). However, it is established

the arterial blood gases are often different (increased) compared to end-tidal measures due to dead space, ventilation-perfusion mismatch, diffusion limitations and shunting (Lindinger and Heigenhauser, 2012, Stickland *et al.*, 2013, Elliott *et al.*, 0000); specifically, $P_{ET}CO_2$ and $P_{ET}O_2$ may underestimate $PaCO_2$ and PaO_2. This gradient between end-tidal and arterial blood gases, however, may change in different conditions (e.g. HX). Tymko *et al,* (2015) showed that the $P_{ET}CO_2$-$PaCO_2$ gradient remains the same (6.9mmHg) while $P_{ET}O_2$-PaO_2 increases from -2.0mmHg to 0.2mmHg between room air and HX (ten minute isocapnic HX; $P_{ET}O_2$ = 47Torr). Thus, $P_{ET}O_2$ more closely represents PaO_2 while $P_{ET}CO_2$ is underestimated by almost 7mmHg in both conditions.

When V_E is increased, $P_{ET}CO_2$ levels decrease as more CO_2 is removed from the body. As demonstrated previously, CO_2 plays a role on the sympathetic and cardiovascular response to HX and must be measured. This can be done by taking gas samples at the mouth and passing them through a gas analyzer. The gas analyzer houses an infrared to detect CO_2 and an optical O_2 sensor, and has a 200ml/min sampling rate (ADInstruments ML206). It can detect CO2 in the range of 0-10% (up to 76 Torr at sea level) which is far outside of the physiological range ($P_{ET}CO_2$ is approximately 40Torr in a healthy human at sea level).

Appendix III: Sample Trace from Lab Chart