INTERMITTENT VERSUS CONTINUOUS HYPOXIA: IMPACT ON HEMODYNAMIC VARIABLES AND GENE EXPRESSION

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The physiologic response to hypoxia may be determined by variables such as exposure pattern and inducible nitric oxide synthase (iNOS) expression. Blood pressure (BP) and heart rate (HR) responses to daily exposure (X 3 days) of intermittent hypoxia (IH) vs. continuous hypoxia (CH) (equivalent total exposure time) were compared. The relation between BP and HR responses and iNOS expression under IH and CH conditions was also examined. On 3 consecutive days, 10 normal males had six 10-min. hypoxic exposures (oxyhemoglobin saturation, SpO2: 80-90%), with each exposure separated by 10 min. of normoxia. Subjects also had 3 consecutive days of CH (60 min/day; SpO2: 80-90%). IH and CH exposure blocks were separated by a $\geq$7 days. BP, HR, and SpO2 were recorded during the 5 min. prior to and the last 5 min. of each daily IH and CH exposure. Venous blood for iNOS mRNA was obtained before exposure on day 1, and 2 hrs. after the last exposure on day 3. HR, systolic and diastolic BP were significantly ($p<0.05$) increased from baseline- to end-exposure on each day, regardless of IH or CH. There was a significant negative correlation ($p<0.01$) between both diastolic and mean BP with iNOS at the end of the day 3 IH session. Hypoxic stress reflected by IH and CH is associated with significant, but comparable changes in BP. Negative correlation between BP and iNOS mRNA in conjunction with IH, but not CH, exposure suggests that the hemodynamic response to IH may be modulated by iNOS.

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PREFACE

I would like to acknowledge and sincerely thank the following people for their support during the many years of pursuit of this research and degree:

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My family and friends, and in particular my wife Nancy and daughter Kelsey, for their love, support, and especially tolerance as I navigated through a process that at times seemed a Himalayan-sized challenge

I see this as an endpoint only from the perspective of it perhaps being the end of the beginning. I can only hope that future scholarly projects will be as full of adventure as this was.

1. INTRODUCTION

It is estimated that 12-20 million people in the United States have obstructive sleep apnea (OSA), a disorder typified by nightly exposure to intermittent hypoxia (IH) secondary to upper airway obstruction during sleep (Sieck, 2001). Epidemiologic, retrospective, and cross-sectional studies have identified associations between OSA and increased risk for cardiovascular disease, e.g., stroke, ischemic heart disease, and systemic hypertension (Leung & Bradley, 2001; Neubauer, 2001; Prabhakar, 2002). There is particularly strong evidence of an association between OSA and systemic hypertension, independent of age, obesity or other confounding factors (Leung & Bradley, 2001; Nieto et al., 2000; Peppard, Young, Palta, & Skatrud, 2000; Young et al., 1997). Consequently, there is an emerging consensus that OSA has serious physiologic consequences beyond those associated with sleep disruption (Brooks, Horner, Kozar, Render-Teixeir, & Phillipson, 1997; Fletcher, Lesske, Qian, Miller, & Unger, 1992; Neubauer, 2001).

During apneic episodes, mechanical and neurochemical responses produce dramatic fluctuations in blood pressure (BP) and heart rate (HR) in conjunction with changes in respiration (Lam & Ip, 2002). Current thinking implicates the pathophysiologic consequences of these episodic surges as the mechanism responsible for cardiovascular disease, including systemic arterial hypertension and myocardial infarcts in patients with OSA (Leung & Bradley, 2001). Fragmented sleep is also characteristic of OSA, but experimental studies in dogs (Brooks et al., 1997) and rats (Fletcher et al., 1992) support the hypothesis that IH, not sleep fragmentation, causes persistent hypertension (Neubauer, 2001).

There is also emerging evidence that the pattern of hypoxia (repetitive or continuous) is a

critical factor in determining physiologic response, with IH being a more potent stimulus than continuous hypoxia (CH). For instance, the reduction in ventilation following initial augmentation during CH has been shown to be attenuated during IH in adult humans (Nieuwenhuijs, Sarton, Teppema, & Dahan, 2000) and animals (Gozal & Gozal, 1999). This ventilatory enhancement persists during normoxia following IH exposure (Cao, Zwillich, Berthon-Jones, & Sullivan, 1992). Similarly, animal studies have shown that IH produces marked increases in BP and sympathetic activity which were not seen following exposure to a comparable duration of CH (Prabhakar, 2002; Young et al., 1997).

The major difference between IH and CH relates to episodic reoxygenation in the former, but not in the latter (Prabhakar, 2001, 2002). In this context, IH seems to resemble ischemia-reperfusion (Prabhakar, 2002). Prior work indicates that the episodic re-oxygenation that occurs during IH represents an oxidative stress that results in cellular generation of reactive oxygen species (Prabhakar, 2001, 2002). There are a number of ways that oxidative stress could mediate effects of IH (Prabhakar, 2001). Regardless of the mechanism, there is increasing recognition that oxidative stress is likely influential in the transcriptional activation of the specific genes through which hypoxia influences adaptive (or maladaptive) responses (Prabhakar, 2001). Hypoxia-inducible factor-1 alpha (HIF-1α), a transcription factor, has been the focus of much recent interest as the possible factor responsible for signaling specific gene expression and the resultant physiologic response to IH. HIF-1α regulates expression of a number of genes associated with hypoxic adaptation, including those involved in vasomotor control.

However, there are major gaps in knowledge regarding the linkage of biologic and molecular responses to IH with physiological responses. The proposed study in humans

represents an initial step in increasing understanding of the physiological importance of episodic periods of IH and CH on BP and HR, as well as the potential modulator roles of oxidative stress and HIF-1α transcription activation. Normal subjects will be recruited, rather than patients with OSA, to eliminate potentially confounding effects of the underlying disease process.

1.1. Purpose

The purpose of this study was to define and compare the impact of short-term exposure to IH and CH with regard to selected cardiovascular variables as well as circulating and exhaled biomarkers of oxidative stress and HIF-1α (at the level of messenger ribonucleic acid [mRNA]). Results were assessed to determine if a potential relationship exists between changes in these biologic markers and changes in the cardiovascular variables.

1.2. Specific Aims

1) To assess and compare the cardiovascular effects of short-term IH versus short-term CH exposure to a fraction of inspired oxygen (F_IO_2) that results in an arterial oxygen saturation (SpO2) between 80 and 90%;

2) To assess and compare the effect of IH and CH on circulating and exhaled biomarkers of oxidative stress and HIF-1α;

3) To explore the relationship between the cardiovascular impact of IH / CH and oxidative stress and HIF-1α. .

1.3. Research Questions and Hypotheses

RQ 1 - Is there a difference in the impact of short-term exposure to IH versus CH on selected cardiovascular variables (systolic and diastolic blood pressure [SBP, DBP]) and heart rate (HR)?

H1.1 - SBP, DBP and HR will increase to a greater extent following short-term exposure to IH compared to short-term exposure to CH.

H1.2 - The increase in SBP, DBP and HR will be greater following 3 consecutive days of IH exposure compared to 3 consecutive days of CH exposure.

RQ 2 - What is the effect of IH and CH on circulating and exhaled biomarkers of oxidative stress and HIF-1α activation (mRNA)?

H2.1 - Circulating and exhaled biomarkers of oxidative stress and HIF-1α activation (mRNA) will increase to a greater extent following IH compared to CH.

H2.2 - The increase in circulating and exhaled biomarkers of oxidative stress and HIF-1α activation (mRNA) will be greater following 3 consecutive days of IH exposure compared to 3 consecutive days of CH exposure.

RQ3 - Is there a relationship between the increase in circulating and exhaled biomarkers of oxidative stress and HIF-1α activation (mRNA) and the change in SBP, DBP or HR?

H3.1 - Participants with the greatest increase in circulating and exhaled markers of oxidative stress and HIF-1α (mRNA) will demonstrate the greatest change in SBP, DBP and HR during IH exposure on a given day and across 3 consecutive days of exposure

H3.2 - Participants with the greatest increase in circulating and exhaled markers of oxidative stress and HIF-1α (mRNA) will demonstrate the greatest change in SBP, DBP and HR during CH exposure on a given day and across 3 consecutive days of exposure.

1.4. Definition of Terms

IH: Exposure to a fraction of inspired oxygen (F_IO_2) that results in a peripheral arterial oxygen saturation (SpO_2) between 80 and 90% for six 10 minutes periods (interrupted by 10 minute intervals of normoxia).

CH: Exposure to a F_IO_2 that results in a SpO_2 between 80 and 90% for 60 consecutive minutes.

Cardiovascular variables: Systolic blood pressure, diastolic blood pressure and heart rate.

Circulating and exhaled biomarkers of oxidative stress: Any oxygen species capable of independent existence that contains one or more unpaired electrons, e.g., nitric oxide (NO), superoxide, or hydroperoxyl, is known as a free radical, also called reactive oxygen (or nitrogen) species, capable of inducing oxidative stress. Exhaled NO will be the primary exhaled biomarker of interest in this study, and is measured using exhaled breath. There are many known circulating biomarkers of oxidative stress. Inducible nitric oxide synthase (iNOS), one of the target genes of HIF-1α and a precursor of NO, will be the primary circulating biomarker of interest in this study and will be measured using venous blood.

HIF-1α activation (mRNA): HIF-1 is a transcriptional activator that mediates changes in gene expression (e.g. iNOS) in response to changes in cellular oxygen concentrations. HIF-1 is a heterodimer consisting of, in part, an oxygen-regulated HIF-1α subunit. The regulation of HIF-1α expression and activity in vivo occurs at multiple levels, including messenger ribonucleic acid (mRNA) expression, which carries the genetic code to the cytoplasm for controlling the formations of the proteins that are responsible for phenotypic adaptations (or maladaptations).

1.5. Conceptual Framework

The conceptual framework shown in Figure 1 depicts the proposed relationships between IH and CH and the variables to be examined in this study. As conceptualized, IH and CH influence SNA and HIF-1α activity, with IH having a more marked effect (darker lines) and CH a more minor effect (lighter lines).

With the onset of IH or CH, 1) HIF-1α transcriptional activity increases, mediated by NO, emanating from cell mitochondria; 2.) this in turn increases expression of hypoxia-inducible genes (target genes); 3.) which in turn leads to increased generation of NO in cell mitochondria, and 4.) inhibition of HIF-1α transcriptional activity and subsequent inhibited expression of target genes influenced by HIF-1α (e.g the hypoxia-inducible genes downstream from HIF-1α) due to interference with HIF-1 DNA binding. The extent of inhibition in HIF-1α is dependent on NO levels, i.e., the higher the level of generation of NO, the greater the inhibition of HIF-1α activity. This action and reaction can be viewed as analogous to a negative feedback loop. The NO is generated by the hypoxia-inducible transcription of the gene encoding iNOS.

IH and CH also increase SNA and hence sympathetic vasoconstrictor tone via peripheral chemoreceptor stimulation manifested as changes in HR and BP. HIF-1α regulates expression of genes, e.g., iNOS, involved in regulation of neurotransmitter properties also affecting SNA and vasomotor control.

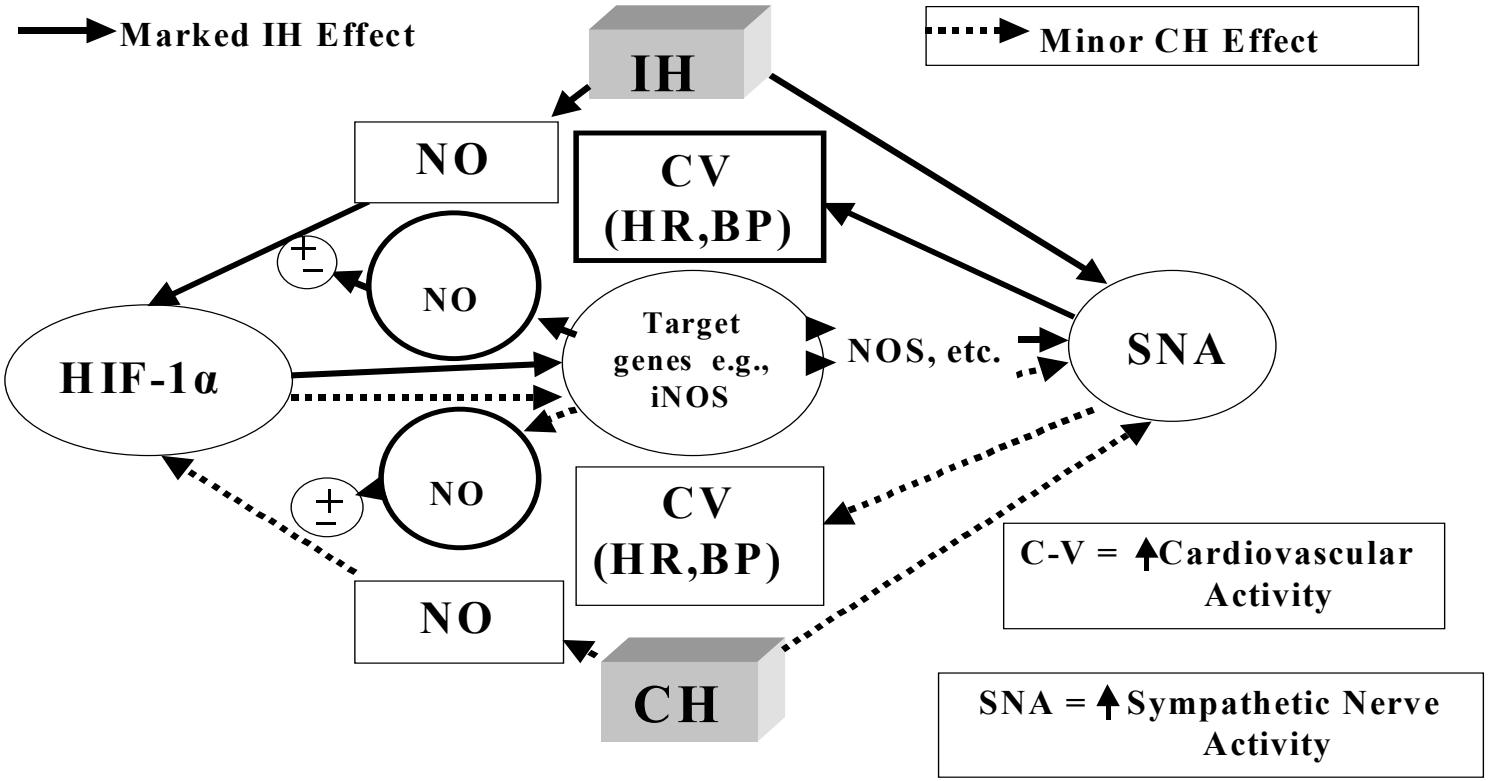

Figure 1 Conceptual framework: The conceptual relationship of study variables.

2. BACKGROUND AND SIGNIFICANCE

2.1. Cardiovascular Effects of Normal Sleep

During normal sleep, there is a progressive reduction in central ventilatory drive accompanied by a reduction in ventilation and SpO_2. In deeper stages of sleep, respiration is predominately under metabolic control with a very regular pattern. Cardiovascular autonomic regulation mirrors the change in respiratory control. Parasympathetic nervous system tone increases and sympathetic nervous system activity (SNA), BP and HR decrease, and the cardiovascular system reaches a state of hemodynamic and autonomic quiescence during which myocardial workload is decreased (Leung & Bradley, 2001).

2.2. Cardiovascular Effects of OSA

Patients with OSA experience repetitive episodes of apnea, from several to as many as 100 times per sleep hour, as a result of upper airway collapse and obstruction at the level of the pharynx.(Lam & Ip, 2002). The shift from apnea to normal breathing causes abrupt surges as cardiovascular autonomic nervous system activity and hemodynamic variables (BP, HR) oscillate between apneic and ventilatory phases. Surges in HR and BP typically occur 5-7 seconds after apnea termination,(Ringler et al., 1990; Tilkian et al., 1976) coincident with arousal from sleep, peak ventilation, and the nadir of SpO2. The usual fall in HR and BP that accompanies normal sleep is counteracted by these repetitive surges (Leung & Bradley, 2001). Hypoxia stimulates peripheral chemoreceptors which, in turn, increases sympathetic outflow and BP (Lam & Ip, 2002). During obstructive apneas, the sympathoexcitatory effect of hypoxia is amplified by carbon dioxide retention associated with cessation of breathing (Leung & Bradley,

2001). This results in increased sympathetic vasoconstrictor tone (Somers, Mark, Zavala, & Abboud, 1989). Hence, it follows that OSA is associated with chronic abnormalities of cardiovascular autonomic nervous system regulation during sleep including increased SNA, reduced baroreflex sensitivity and HR variability, and increased BP variability (Carlson et al., 1993; Hedner, Ejnell, Sellgren, Hedner, & Wallin, 1988; K Narkiewicz et al., 1998; K. Narkiewicz, van de Borne, Cooley, Dyken, & Somers, 1998; Somers, Dyken, Clary, & Abboud, 1995). Further, several studies have shown that individuals with OSA have higher SNA during sleep and wakefulness than control subjects (Carlson et al., 1993; Hedner et al., 1988; Somers et al., 1995).

2.3. Physiologic Effects of IH

The ventilatory response to IH is believed to be related to long-term facilitation of ventilatory activity by a mechanism that may be serotonergic-dependent (Turner & Mitchell, 1997). Neubauer (Neubauer, 2001) has suggested the sympatho-excitatory region of the rostral ventrolateral medulla as a potential site of ventilatory adaptation to IH, since neurons in this region are sensitive to the direct effects of hypoxia (Mazza, Edelman, & Neubauer, 2000). In contrast to CH, in which there may be a biphasic ventilatory response characterized by a reduction in ventilation following initial augmentation, IH appears to be associated with attenuation of this decline in adult humans (Nieuwenhuijs et al., 2000) and animals (Gozal & Gozal, 1999).

Emerging evidence suggests that IH has the potential to alter ventilatory neuronal activity. (Gozal & Gozal, 1999) tested the hypothesis that repeated short exposures to hypoxia modifies hypoxic ventilatory response in an animal model. The study involved 30-minute hypoxic challenges in 2 to 3-day old rat pups before and 6 hours after completing a series of 8 IH

cycles consisting of 5 minutes hypoxia and 10 minutes normoxia, or normoxia throughout. Periods of IH were associated with ventilatory enhancement that persisted during normoxia following the IH. Cao et al.(Cao et al., 1992) examined the normoxic ventilatory response following successive episodes of 2-minute eucapnic hypoxic challenges in awake tracheotomized dogs and reported similar findings. In contrast, a similar facilitation of ventilatory response was not demonstrated when awake goats were exposed to either 4 hours (n=10) or 30 minutes (n=7) of CH (Dwinell, Janssen, & Bisgard, 1997). In the latter study, minute ventilation, tidal volume, and frequency were measured before, during, and after the CH exposure.

Additional studies suggest that IH may augment SNA in animals and healthy humans. Bao et al.(Bao, Metreveli, Li, Taylor, & Fletcher, 1997) studied the role of renal artery sympathetic nerves and the adrenal medulla in BP increase during IH in rats. Adult male rats had either an adrenal medullectomy (n=22), bilateral renal artery denervation (n=19), or sham surgery (n=36). Rats from each group were subjected to IH (FiO_2 lowered twice per minute for 6-8 hours) for 35 consecutive days. Results confirmed that the IH-mediated increase in diurnal BP requires both intact renal artery nerves, as well as an intact adrenal medulla. Xie et al.(Xie et al., 2000) reported that even short-term IH exposure (20 seconds per minute of isocapnic hypoxia to SpO_2 = 82 ± 1% for 20 minutes) augmented SNA in 7 healthy humans. SNA remained elevated for at least 20 minutes after return to normoxic baseline SpO_2. Arabi et al.(Arabi et al., 1999) reported that even 2 continuous 8-hour nocturnal exposures (successive nights) to hypobaric hypoxia (SpO_2 = 73±4%) in 10 healthy normotensive subjects was associated with elevated DBP that persisted after termination of hypoxic exposure.

There is also evidence from studies utilizing adult rats exposed to periods of IH for 8 hours per day for 30 (Greenberg, Sica, Batson, & Scharf, 1999) and 70 (Kraiczi et al., 1999) days

suggesting that IH exerts a greater influence on sympathetic activity and BP than CH. Recent research with a rat model provides preliminary evidence that the link between IH and hypertension may be mediated by decreased activity of NO, an exhaled biomarker of oxidative stress (Fletcher, 2001). In addition, two recent studies in humans support the theory of increased resting basal vascular tone through lack of NO activity in the OSA patient. Carlson et al. (Carlson, Rangemark, & Hedner, 1996) showed that forearm vascular relaxation to acetylcholine (which is NO dependent) decreased in apneic subjects compared with nonapneic controls. Duchna et al. (Duchna et al., 2000) found that endothelium-dependent NO (bradykinin) vascular relaxation was depressed in OSA patients compared with controls, whereas NO independent (nitroglycerine) vasorelaxation was unaffected. In summary, the suggestion that specifically implicates the repetitive nature of hypoxic exposure as being pivotal in the development of cardiovascular (e.g. BP) alterations (Prabhakar, 2001) is gaining ever-increasing support from the investigational community.

2.4. Response to IH may be related to Oxidative Stress and HIF-1α Gene Expression

Since episodic hypoxia/re-oxygenation and sympathetic activation are associated with oxidative stress (Leung & Bradley, 2001), a relationship between oxidative stress and changes in BP during IH is plausible. Inter-individual variability in cardiovascular responses to IH may be due to differences in genetic expression (Kraiczi et al., 1999). Kraiczi et al (Kraiczi et al., 1999) reported that increased left ventricular (LV) weight-to-body weight ratio and enhanced LV expression of atrial natriuretic peptide mRNA was present in 6 spontaneously hypertensive rats, but not in the 6 normotensive rats after 70 days of IH (8 hours per day). This preliminary evidence indicates that at least some long-term consequences of IH may be modified by

preexisting constitutional (genetic) traits. Further testing, as proposed in this study, is necessary to confirm this potential.

HIF-1α regulates expression of a number of genes associated with hypoxic adaptation, including those involved in vasomotor control such as endothelin-1, adrenomedullin, iNOS; iron metabolism, and HO-1 products, e.g., CO, bilirubin, biliverdin, and ferritin (G. Semenza, 2000). HO-1 and associated substances have vasodilator, anti-inflammatory and neurotransmitter properties that are important in the response to oxidative stress (Ameredes et al., 2002; Prabhakar, 2001; Sethi & Choi, 2001). HIF-1α also plays a role in the development of pulmonary artery hypertension and pulmonary artery medial hypertrophy with hypoxic exposure (G. L. Semenza, 2001). Mice experienced delayed development of pulmonary artery hypertension and right ventricular (RV) hypertrophy during CH exposure when the gene encoding for HIF-1α was inactivated (G. Semenza, 2000; G. L. Semenza, 2001; Yu et al., 1999). Analysis of knockout mice has also demonstrated that HIF-1α is required for embryonic development and survival. Conversely, mice that are heterozygous for the knockout allele and thus partially HIF-1α deficient develop normally (G. L. Semenza, 2001). Yu et al. (Yu et al., 1999) compared the physiological response of partially HIF-1α deficient heterozygous mice to the response of their normal (wild-type) littermates after breathing 10% oxygen for 1 to 6 weeks. The partially HIF-1α deficient heterozygous mice demonstrated significantly delayed development of polycythemia, RV hypertrophy, pulmonary hypertension, and pulmonary vascular remodeling compared with their normal littermates. These findings provide suggestive evidence that partial inhibition of HIF-1α activity may in the future provide a means to prevent pulmonary vascular remodeling in at-risk patients with diseases due to or exacerbated by IH or CH.

2.5. Significance and Innovation

No prior studies have explored the potential role of HIF-1α or oxidative stress in producing individual variability in response to IH and CH. Notable cardiovascular sequelae such as stroke, ischemic heart disease, and systemic hypertension do not develop in all persons who experience IH. Conceptually, oxidative stress arising from IH may play a pathophysiologic role in these consequences (Neubauer, 2001). Clinical research utilizing awake, normal subjects may provide beginning support for the physiological importance of episodic periods of IH on clinically important physiological parameters, the mediating effects of biomarkers of oxidative stress and HIF-1α transcription activation.

It is intriguing to speculate that inter-individual variability of HIF-1α activation and oxidative stress explains at least some of the variability in response to IH between individuals. Since episodic hypoxia/re-oxygenation and sympathetic activation are associated with oxidative stress (Leung & Bradley, 2001), a relationship between oxidative stress and changes in cardiovascular response to IH is logical. Current thinking suggests that HIF-1α regulates expression of a number of genes associated with hypoxic adaptation, including those involved in vasomotor control such as endothelin-1, NO synthase, and HO-1 products such as CO (G. Semenza, 2000). HO-1 and associated substances have vasodilator, anti-inflammatory and neurotransmitter properties that are important in the response to oxidative stress (Ameredes et al., 2002; Prabhakar, 2001; Sethi & Choi, 2001). HIF-1α also plays a role in the development of pulmonary artery hypertension and pulmonary artery medial hypertrophy with hypoxic exposure (G. L. Semenza, 2001).

Inter-individual variability in cardiovascular responses to IH may be due to differences in genetic expression. Kraiczi et al (Kraiczi et al., 1999) reported that increased left ventricular

(LV) weight-to-body weight ratio and enhanced LV expression of atrial natriuretic peptide mRNA was present in 6 spontaneously hypertensive rats, but not in the 6 normotensive rats after 70 days of IH (8 hours per day). This preliminary evidence indicates that at least some long-term consequences of IH may be modified by preexisting constitutional (genetic) traits. Further testing, as proposed in this study, is necessary to confirm this potential.

If in fact inter-individual variability in cardiovascular responses to IH is due at least in part to differences in genetic expression, assessment of oxidative stress and HIF-1α expression may facilitate risk stratification to identify those individuals most susceptible to adverse cardiovascular outcomes. Better understanding of the consequences of IH and those factors that influence expression of these consequences may translate into improved development of effective therapeutic paradigms. Successful risk stratification and aggressive long-term management of patients most susceptible to adverse cardiovascular outcomes may eventually allow acute-care health resources to be optimally directed to patients other than those suffering the sequelae of long-term IH.

Translational research of this sort, meant to bridge the gap between the bench and the bedside, will not have immediate bedside nursing implications. The ability to identify and thus aggressively manage, in a practical clinical fashion, those individuals most susceptible to the adverse cardiovascular outcomes of IH will be a goal for the near future. Convenient and cost-effective testing for presence of transcription factor and target gene activation will be needed before clinicians are able to provide useful and effective traditional therapy in a timely, aggressive manner.

Manipulation of genetic factors, already successful in animal models, may also become an important aspect of therapeutic paradigms for those individuals most susceptible to the

adverse cardiovascular outcomes of IH. An example of the future possibilities of gene manipulation is illustrated by mice that experience delayed development of pulmonary artery hypertension and right ventricular hypertrophy during exposure to hypoxia when the gene encoding for HIF-1α is inactivated (G. Semenza, 2000; G. L. Semenza, 2001; Yu et al., 1999).

3. METHODOLOGY

3.1. Research Design

The study used a within-subjects repeated measures design. Each subject participated in 3 blocks (acclimatization, 3 consecutive days of IH, 3 consecutive days of CH) as shown below. The order of the IH and CH blocks was determined by subject availability.

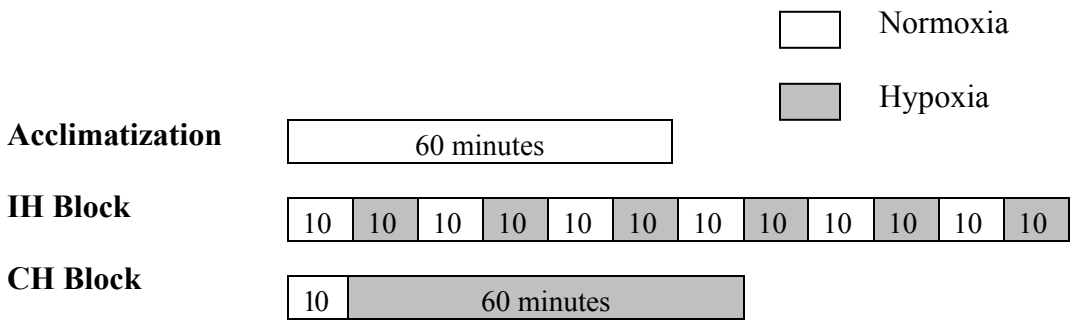

Figure 2 Scheme of normoxic (acclimatization) and hypoxic (IH and CH) exposures

3.2. Research Methods

3.2.1. IH Block

On each of 3 consecutive days, participants experienced 6 exposures to hypoxia as follows: 1) Subjects initially breathed air (fraction of inspired oxygen, F_IO_2=21%, the percentage that is in room air) through a commercially available facemask for 10 minutes; 2) A F_IO_2 ~13.5% was administered via the facemask with subsequent adjustment of the F_IO_2 to achieve and maintain an arterial oxyhemoglobin saturation by pulse oximetry (S_pO_2) = 80-90% for 10 minutes; 3) IH ended and room air was administered for 10 minutes. Steps #1-3 were repeated

for a total of 6 IH exposures. Each day required approximately 3-4 hours of the participant's time, including calibration, application and removal of the monitoring devices. During data collection, BP, HR, S_pO_2, end-tidal carbon dioxide tension ($P_{et}CO_2$), and minute ventilation were recorded continuously.

3.2.2. CH Block

On each of 3 consecutive days, participants experienced one 60-minute exposure to hypoxia as follows: 1) Subjects initially breathed F_IO_2=21% (room air) for 10 minutes to achieve a steady state (as reflected by stable S_pO_2, $P_{et}CO_2$ and breathing pattern) and to collect baseline data. The subject then began to breathe F_IO_2~13.5% with subsequent adjustment in the same manner as during the IH block to achieve and maintain S_pO_2 at 80-90% for 60 minutes. Each day required approximately 3-4 hours of the participant's time, including calibration, application and removal of the monitoring devices. BP, HR, S_pO_2, $P_{et}CO_2$ and minute ventilation were recorded continuously.

3.2.3. Acclimatization Session

Participants breathed F_IO_2=21% (equivalent to room air) through the same facemask for 60 minutes on one day. BP, HR, S_pO_2, $P_{et}CO_2$, and minute ventilation were continuously recorded. This day required approximately 3-4 hours of the participant's time, including calibration, application and removal of the monitoring sensors.

3.2.4. Washout

There was $\geq$ 1-week between the IH and CH blocks. The acclimatization session served to acclimatize subjects to the research procedure and was the first session in the series.

3.3. Research Procedures

3.3.1. Subject Instructions

Subjects were instructed not to eat or drink for at least 2 hours and to avoid food and beverages containing caffeine for 24 hours prior to arriving for each study block. They were asked to avoid meat, cured or smoked foods (e.g. ham, bacon, smoked fish) and leafy greens/vegetables (e.g. broccoli, kale, spinach) which may contain degraded red blood cells and or nitrates which may impact measured variables, for at least 48 hours before and during each study block. Participants were also asked avoid taking vitamin pills which may contain anti-oxidants, for at least 3 weeks. Compliance with these requests was by self-report. Subjects were not permitted to eat or drink during the recording periods. However, they were permitted to drink water during the 2 hour interval between the end of the recording and the final blood sampling on day-3 of the IH and CH Blocks.

3.3.2. Monitored Variables

S_pO_2 was recorded via finger pulse oximetry, HR and rhythm was recorded utilizing a modified EKG chest lead, BP was recorded utilizing commercially available device (Portapres^R and Modelflow^R software or Finapres®) using a cuff that was placed on a finger of the subject's non-dominant hand and inflated. The hand from which BP was recorded was supported at approximately the level of the left atrium during data collection. Breathing rate and pattern were recorded using respiratory inductance plethysmography (RIP) (Respitrace®). RIP is an established, non-invasive means to obtain semi-quantitative measures by recording chest wall movement via elastic bands that fit snugly around the rib cage and abdomen. The Respitrace® is calibrated by asking participants to breathe with a normal, stable rate and tidal volume (Vt) for 5 minutes. A baseline average for breathing was established (in order to calculate subsequent tidal

volume expressed as % baseline Vt). $P_{et}CO_2$ was measured utilizing a commercial infrared capnograph connected by or to a thin catheter placed within the facemask. The capnograph was calibrated daily before each session with gas containing a known amount of CO_2.

3.3.3. Gas Delivery

Subjects were semi-recumbent for 20 minutes prior to baseline data collection and during data collection periods. During each hypoxic exposure, subjects wore a commercially available partial rebreather or non-rebreather facemask (the same that is clinically used to deliver oxygen to patients) connected to a commercially available generator (Hypoxico, Inc.) which delivered gas at an adjustable F_IO_2. Guided by a published table (Hultgren, 1997), the generator was initially set to deliver an F_IO_2 = 13.5% and subsequently adjusted such that the subject's S_pO_2=80-90%. Thereafter, the F_IO_2 was titrated as needed to maintain this S_pO_2 level by a dedicated member of the research team whose role was to constantly monitor participant status. During *acclimatization* sessions, subjects wore a facemask delivering room air.

3.3.4. Blood Collection

At each visit, measures of blood pressure (BP), heart rate (HR) and rhythm, arterial blood oxygen saturation (S_pO_2), end-tidal carbon dioxide tension ($P_{et}CO_2$), and breathing pattern data were obtained. A subtotal of 90 cc of venous blood was obtained over each of the 3 days of Study Block 1 and Study Block 2. 20cc of blood were also obtained during the Acclimatization Block. The grand total for all blood draws across all sessions was 200cc (see table below). Exhaled breath was sampled before and after each daily test session and analyzed for NO. The timing of blood draws was as follows: 10 cc of venous blood will be obtained by venipuncture for analysis of biomarkers of oxidative stress immediately prior to and after the last hypoxic exposure each day during the IH Block and CH Blocks. In addition, 15 cc of blood was drawn

before the test run on day 1 and 2 hours after the test run on day 3 of the IH Block and CH Block to assess HIF-1α and iNOS mRNA. All blood draws were done by an individual experienced in phlebotomy using standard venipuncture techniques.

Table 1 Timing of blood collection

	Control	CH			IH			Total
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	
Blood draws in cc	20	35	20	35	35	20	35	**200**

3.3.5. Exhaled NO

Samples were obtained by having participants exhale from maximum to minimum lung volume at a constant flow rate into a gas analyzer (Logan Research, Kent, UK). Participants then breathed tidally for 4 minutes into a mixing chamber. A one-way valve was used to prevent rebreathing. Subjects wore wear a nose-clip during exhaled breath collection.

3.3.6. Subject Distraction

To discourage participants from concentration on breathing which could influence the study variables, distraction was provided via television viewing or music.

3.4. Setting and Sample

All data collection occurred in the Pulmonary Sleep Evaluation Lab at the University of Pittsburgh Medical Center.

3.4.1. Entry Criteria

Healthy men between 18 and 45 years of age; able to speak and understand English; nonsmoker for at least three months; not on prescription medications.

3.4.2. Exclusion Criteria

A history of cardiovascular disease (including but not limited to arrhythmia, syncope or near-syncope, systemic or pulmonary hypertension, angina pectoris, hyperlipidemia, congenital heart disease, primary family member with coronary artery disease).

A history of significant medical illness including thyroid abnormality, diabetes mellitus, pulmonary disease, or sleep-disordered breathing, neurologic disorder, anemia, sickle cell disease; 3) athlete in training; 4) obesity (body mass index >30); 5) history of high altitude illness, 6) resting SpO_2 < 95%; 7) EKG consistent with coronary artery disease, right or left ventricular hypertrophy, or any dysrhythmia other than sinus arrhythmia; 8) $P_{ET}CO_2 \geq 45$ mm Hg at rest on room air; 9) spirometry indicating abnormal lung function indicated by % predicted values for forced vital capacity (FVC), forced expiratory volume at 1 second (FEV_1), and/or FEV_1/FVC ratio outside normal ranges for height, weight and sex.

3.5. Limitations

Critical study variables (target SpO_2, timing of IH and CH) were based on an extensive literature search and discussions with experts. The proposed design had not been previously used and, while modeled on prior work, might have been insufficient stimulus to create the changes of interest. Notable cardiovascular sequelae do not develop in all who experience IH and it is possible that participants may not have been responsive to IH. However, prior studies investigating the effect of IH on ventilatory responsiveness (Garcia, Hopkins, & Powell, 2000; Katayama et al., 2001) and erythropoiesis (Garcia et al., 2000) in humans have demonstrated significance with < 9 subjects, suggesting this is unlikely. Subjects were healthy young males with no comorbidity, thereby excluding generalizability of results to a great number of individuals. However, it is hoped that study findings will be immensely valuable for the purpose

of evaluating the study design and methodology in order to determine if a larger future study is justifiable.

3.5.1. Sample Size Justification

Power analysis was performed using data from two studies using a similar design. Arabi et al. (Arabi et al., 1999) examined HR and daytime BP elevation after nocturnal hypoxia in 10 humans (DBP & SBP presented graphically, numeric data not provided). Xie et al.(Xie et al., 2000) examined neurocirculatory consequences of intermittent asphyxia in 7 humans. Both studies are similar to the proposed study in the cardiovascular variables examined and the interval of IH exposure, but not in regard to the timing of hypoxic exposure, as well as its multi-day design. Published literature provided no examples of IH research using an identical design and methodology to the proposed study, including biomarker examination. Given the nature of the proposed study, results from Arabi et al. (Arabi et al., 1999) and Xie et al.(Xie et al., 2000) were judged most similar and used to determine a sample size that would result in a power of $\geq$ 0.80, with alpha set at 0.05 (See below). Based on this analysis, 12 subjects would appear adequate. Sample size was set at 20 participants to allow for attrition and missing data.

Table 2 Power analysis

Study	HR	Est. Sample	Effect Size	Power
Xie et al.	Baseline = 61 $\pm$ 4 Asphyxia = 64 $\pm$ 4	20	0.53	0.97
Arabi et al.	Normoxia = 61 $\pm$ 9 Hypoxia = 74 $\pm$ 13	10	0.83	0.96

3.5.2. Data Analysis

Monitored variables were recorded continuously and downloaded to a computer. Mean values for SBP, DBP, HR, and SpO_2 were analyzed for the first and last 10 minutes for control and CH blocks and for the first and last 2 minutes for the IH block. Changes in breathing pattern and $P_{ET}CO_2$ were analyzed descriptively and used as covariates. Preliminary analysis was conducted to: 1) compare measures of central tendency and dispersion; 2) examine distribution; and 3) plot and analyze residuals to detect nonlinear patterns and assure that statistical assumptions were met.

3.5.2.1. Research Question 1

Is there a difference in the impact of short-term exposure to IH versus CH on selected cardiovascular variables (SBP, DBP, and HR)?

The last measurement of cardiovascular variables from each day's IH session was compared with the same respective measurement of cardiovascular variables from the end of each day's CH session by using repeated measures ANOVA to assess for changes over time. Differences in cardiovascular variables across measurement times for each day of IH exposure were examined using repeated measures ANOVA as well.

3.5.2.2. Research Question 2

What is the effect of IH and CH on circulating and exhaled biomarkers of oxidative stress and HIF-1α activation (mRNA)?

Values for exhaled biomarkers of oxidative stress obtained prior to and after each day's IH session were compared with the same respective measurements of exhaled biomarkers of oxidative stress obtained prior to and after each day's CH session by using repeated measures

ANOVA to assess for changes over time. The circulating marker for HIF-1α obtained before IH and CH session one, and after IH and CH session three, were also be examined using repeated measures ANOVA to assess for changes over time.

3.5.2.3. Research Question 3

Is there a relationship between the increase in exhaled biomarkers of oxidative stress and HIF-1α activation (mRNA) and the change in SBP, DBP or HR?

Linear regression analysis was utilized to examine the relationship between biomarkers (iNOS, HIF-1α, and oxidative stress) and cardiovascular parameters (SBP, DBP, and HR) measured at the end of the same respective IH and CH sessions. Pearson correlation coefficients between biomarkers and HR, SBP, and DBP changes were examined in each subject each day (in those measured each day) and over the course of each 3-day session.

The results of this study will be presented in the format of two manuscripts entitled *IH versus CH: Impact on Hemodynamic* Variables (Chapter 4) and *IH versus CH: Relationship of Hemodynamic Response to Molecular Response* (Chapter 5).

4. IH VS. CH: IMPACT ON HEMODYNAMIC VARIABLES

4.1. Introduction

Intermittent exposure to hypoxia (intermittent hypoxia, IH) is common in the population. Obstructive Sleep Apnea (OSA), representing one temporal profile of IH secondary to upper airway obstruction during sleep, has been estimated to be present in 12-20 million people in the United States (Sieck, 2001). Acute and chronic cardiovascular alterations (Amin et al., 2004; Leung & Bradley, 2001; Neubauer, 2001; Prabhakar, 2002; Shahar et al., 2001; Silvestrini et al., 2002), including systemic arterial hypertension and endothelial cell dysfunction (Fletcher, 2003; Lavie, 2003) have been associated with OSA. OSA, however, reflects a constellation of physiologic exposures including oxyhemoglobin desaturation-resaturation (IH), intermittent hypercapnia and central nervous system arousal. However, recent studies in animals suggest that the pattern of hypoxia (intermittent versus continuous) is important in determining the nature and magnitude of the physiologic response, with IH being a more potent stimulus than CH. Exposure of rats to IH is associated with augmented sympathetic nervous system activity and increases in blood pressure (BP) that are not seen following exposure to a comparable duration of CH in separate studies (Prabhakar, 2002; Young et al., 1997). Evidence from studies utilizing adult rats exposed to periods of IH for 8 hours per day for 30 (Greenberg et al., 1999) and 70 (Kraiczi et al., 1999) days also suggests that IH produces sustained BP increases and enhanced responsiveness of BP to short term hypoxia after long-term IH, respectively.

Studies in healthy humans (Cutler, Swift, Keller, Wasmund, & Smith, 2004; Xie et al., 2000) have indicated that IH may augment sympathetic nerve activity (SNA) and BP. Xie et al.(Xie et al., 2000) reported that even short-term IH exposure (20 seconds per minute of

isocapnic hypoxia resulting in arterial blood oxygen saturation [SpO_2] = 82 ± 1%, over 20 minutes) augmented SNA in 7 healthy humans with SNA remaining elevated for at least 20 minutes after return to baseline SpO_2. In another IH study, Cutler et al. (Cutler et al., 2004) similarly observed that muscle sympathetic nerve activity (MSNA) and BP were increased during 20 minutes of intermittent voluntary "hypoxic apneas" (intermittent breath holding combined with breathing of mildly hypoxic air) in 7 healthy humans with MSNA, but not BP, remaining elevated during 180 minutes of recovery. However, numerous recent studies have hypothesized that alterations in the chemoreflex control of MSNA activity can influence the development of hypertension in patients with OSA (K Narkiewicz et al., 1999; Trzebski, 1992; Trzebski & Smietanowski, 1996).

Exploring a different profile of IH exposure, Arabi et al. (Arabi et al., 1999) indicated that exposure of 10 healthy normotensive subjects to 8-hours of hypobaric hypoxia (SpO_2 = 73±4%) on each of 2 consecutive nights with intervening normoxia was associated with diastolic BP (DBP) elevation that persisted after termination of hypoxic exposure. A recent study by Tamisier et al. (Tamisier, Anand, Nieto, Cunnington, & Weiss, 2005) is of particular relevance to the present study, as muscle SNA and hemodynamic response to IH and CH in normal humans was directly compared. This the only other instance that we are aware of where hemodynamic response to both IH and CH has been examined with the same subjects within the same study. Interestingly, Tamisier and co-workers found significant increases in BP and muscle SNA after 2 hours of CH but not after 2 hours of IH.

The purpose of this study was to compare cardiovascular response to IH exposure on each of 3 consecutive days versus CH exposure on each of 3 consecutive days (with comparable daily hypoxic exposure time regardless of the exposure profile) in healthy subjects. We

hypothesized that IH exposure would be associated with a greater increase in BP and/or HR from baseline to end-exposure on each day. We also hypothesized that augmentation of HR and/or BP would be greater with each IH exposure day compared with each CH exposure day.

4.2. Methods

4.2.1. Sample

Subjects were 10 healthy non-obese males aged 25.3 ± 5.7 years [mean ± SD, range 20-35 years, BMI < 30]. During screening, all subjects had resting $SpO_2 \geq 95\%$, resting end tidal carbon dioxide ($P_{ET}CO_2$) $\leq$ 45 mm Hg, and spirometry indicating normal lung function. The subjects were abstinent from tobacco smoking for at least 3 months, had no history of pulmonary or cardiovascular disease and were not on any prescription medications. All had normal electrocadiograms and none were in athletic training. Females were not eligible to participate due to the potential confounding effects of varying hormonal environments across the study interval. The study received approval from the University of Pittsburgh Institutional Review Board for Biomedical Research and all subjects provided written, informed consent.

4.2.2. Procedure

Each subject participated in 3 exposure protocols: 1) normoxic acclimatization; 2) 3 consecutive days of IH exposure; and 3) 3 consecutive days of CH exposure (Figure 3). The normoxic session acclimatized subjects to the procedure and was always the first in the series. Subjects were assigned to the IH or CH session based on their availability (in a non-randomized fashion); 4 subjects performed the IH protocol first and 6 performed the CH protocol first. To reduce the possibility of an interaction between the protocols, IH and CH sessions were separated by a washout period (≥ 7 days).

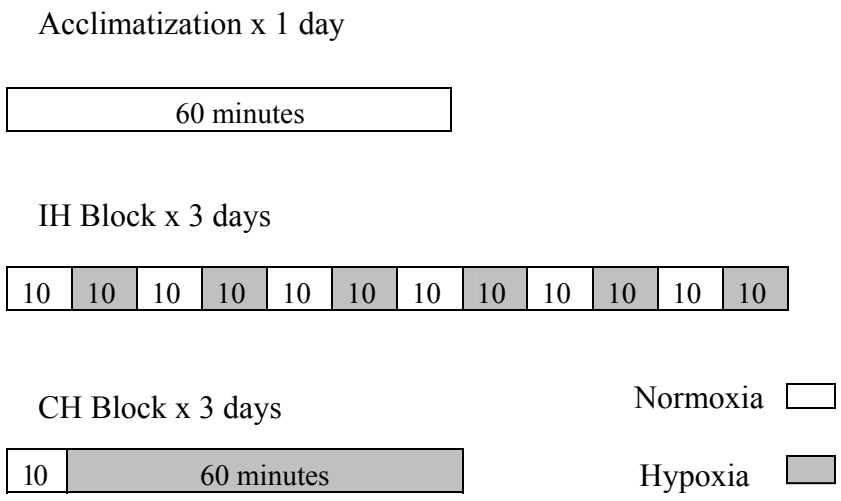

Figure 3 Scheme of normoxic and hypoxic exposures for the acclimatization session (60 minutes room air) IH session (6 hypoxic [FIO_2 0.13.5] exposures for 10 minutes each) and CH session (one 60 minute hypoxic [FIO_2 0.13.5] exposure).

Subjects were semi-recumbent in a hospital bed for 15 minutes prior to and during data collection for the control, IH, and CH sessions. Distraction (television or music) was provided during all test sessions to discourage subjects from concentrating on their breathing, since this could influence study variables. Subjects could not be blinded to the nature of the exposure protocol, but were blinded to the hypothesis, as none of the subjects had advanced knowledge of hypoxia/cardiovascular physiology.

4.2.2.1. Normoxic Acclimatization Protocol

Subjects breathed a fraction of inspired oxygen (F_IO_2) equal to 0.21 using a source of medical grade air [MGA]) through a commercially available non-rebreather facemask (also used for IH and CH sessions) for 60 minutes. The MGA was fed from a wall-mounted tap into a 15-liter Douglas bag (also used during IH and CH sessions) which was connected to the mask worn

by the subject via a one-way valve. Systolic, diastolic, and mean blood pressure (SBP, DBP, MBP), heart rate (HR), S_pO_2, and $P_{et}CO_2$ were continuously recorded for the first and last 5-minute periods of the 60-minute session.

4.2.2.2. IH Session

On each of 3 consecutive days, subjects were provided with 6 ten minute exposures to IH. Subjects initially breathed MGA through the non-rebreather facemask for 10 minutes to achieve a stable S_pO_2, $P_{et}CO_2$ and breathing pattern, after which baseline data were collected during 5 minutes of continuous recording. A F_IO_2 ~0.135 was then administered from a commercially available hypoxic gas generator (Hypoxico, Inc., New York, N.Y.) to the Douglas bag and facemask until the S_pO_2 was 80-90%. The hypoxic gas generator used in this study utilizes “pressure swing adsorption”, wherein zeolite crystals are used as a “molecular sieve”. The generator passes room air under pressure through a vessel containing an adsorbent bed (with zeolite crystals) that attracts oxygen more strongly than nitrogen such that gas coming out of the vessel is relatively enriched in nitrogen. SpO2 was maintained in this range with a target of 85% by bleeding in MGA as needed. After 10 minutes, the hypoxic exposure was ended and MGA was administered for 10 minutes. The same procedure was followed for a total of 6 IH exposures. Continuous recordings of SBP, DBP, MBP, HR, S_pO_2, and $P_{et}CO_2$ were obtained during the last 5-minutes of each day’s 6th IH exposure.

4.2.2.3. CH Session

On each of 3 consecutive days, subjects had one 60-minute exposure to hypoxia. After initially breathing MGA through the non-rebreathing facemask for 10 minutes to achieve stable breathing, and then collecting baseline data for 5 minutes prior to the CH exposure interval, the F_IO_2 was adjusted to 0.135 in the same manner as during the IH session to achieve and maintain

S_pO_2 at 80-90% for 60 minutes. Continuous recordings of SBP, DBP MBP, HR, S_pO_2, and $P_{et}CO_2$ were obtained during the last 5-minutes of each day's CH exposure.

4.2.2.4. Instrumentation

The S_pO_2 was monitored via finger pulse oximetry (CO_2SMO *Plus*!® respiratory profile monitor, Novametrix Medical Systems, Inc., Wallingford, CT, USA). Heart rate and rhythm were monitored utilizing a standard 3-lead electrocardiogram (in lead 2)**.** $P_{et}CO_2$ was obtained utilizing an infrared capnograph with a solid state mainstream flow sensor (CO_2SMO *Plus*!® respiratory profile monitor) connected to the facemask. The CO_2 sensor was calibrated daily before each session using internally generated values of 0 and 38 mmHg. SBP, DBP, and MBP were recorded using a Portapres® (Finapres Medical Systems**,** Amsterdam, Netherlands) via an inflated cuff placed on one or two fingers of the subject's non-dominant hand. The Portapres® was calibrated before each session with known pressures (0, 50, 100, and 150 mmHg) generated internally by the device and verified using a standard sphygmomanometer. Data were acquired and recorded using Labtech Notebook Pro software, version 10.1 (Andover, MA, USA). The recording channels for blood pressure, S_pO_2, and $P_{et}CO_2$ were calibrated with known values from the Portapres® and CO_2SMO *Plus*!®.

4.2.3. Data Analysis

Data are reported as the mean $\pm$ standard deviation (SD). Statistical significance was set at $p \leq 0.05$ *a priori*. Cardiopulmonary parameters were analyzed by a 2 (IH vs. CH) x 3 (day 1, 2, and 3) x 2 (baseline vs. end hypoxia) ANOVA. SPSS statistical package version 11.5 was used for analysis.

4.3. Results

Values of all measured variables for baseline and end hypoxia during IH and CH sessions, reported as mean ± SD, are reported in Table 3.

Table 3 Hemodynamic values

	S_pO_2 (Percent)	$P_{et}CO_2$ (mmHg)	HR (BPM)	SBP (mmHg)	DBP (mmHg)	MBP (mmHg)
IH 1						
Baseline	96.8±0.8	41.0±3.1	65.5±8.7	126.8±14.0	66.6±4.2	84.4±4.6
End	85.0±2.3	39.3±2.6	77.6±8.6	134.2±17.0	71.3±11.8	87.5±11.8
IH 2						
Baseline	97.0±1.3	41.7±2.4	65.0±5.6	128.7±14.5	67.6±9.2	85.5±10.3
End	83.9±2.2	39.0±1.9	73.6±9.0	141.6±18.3	75.9±9.1	93.0±10.6
IH 3						
Baseline	96.8±0.9	39.6±1.7	64.8±6.7	124.2±12.5	68.7±10.5	84.9±10.1
End	84.3±1.8	38.1±1.5	73.4±5.9	136.3±14.5	75.0±9.4	90.7±10.0
CH 1						
Baseline	97.0±1.0	39.5±4.2	64.0±9.6	131.5±16.3	74.4±8.0	91.6±9.0
End	86.0±2.3	38.7±4.5	72.0±10.6	139.1±16.8	77.0±7.7	93.1±8.8
CH 2						
Baseline	97.0±1.5	40.5±2.5	64.1±14.7	124.9±17.8	66.0±11.7	84.4±10.4
End	86.3±2.8	39.3±2.3	74.2±9.2	131.2±14.3	69.2±8.7	87.1±6.7
CH 3						
Baseline	96.4±3.4	38.2±3.2	68.3±8.5	123.8±13.0	69.9±11.3	87.3±8.4
End	86.9±5.0	39.0±3.3	76.8±5.8	129.0±13.0	70.6±7.6	87.6±6.7

4.3.1. Arterial Oxygen Saturation

The baseline S_pO_2 prior to all IH exposures was 96.9 $\pm$ 0.1% and 84.4 $\pm$ 0.5% during the last 5 minutes of all IH exposures. The baseline S_pO_2 prior to all CH exposures was 96.8 $\pm$ 0.3% and 86.4 $\pm$ 0.6% during the last 5 minutes of all CH exposures. For the group, the S_pO_2 decreased more from baseline during IH than during CH exposure ($p = 0.01$).

4.3.2. End Tidal CO_2

$P_{et}CO_2$ values are reported for 8 of the 10 subjects, due to technical problems. The $P_{et}CO_2$ prior to and during the last 5 minutes of IH exposure were 40.8 $\pm$ 1.1 mmHg and, 38.8 $\pm$ 0.6 mmHg, respectively. The $P_{et}CO_2$ prior to and during the last 5 minutes of CH exposure were 39.4 $\pm$ 1.2 mmHg and 38.9 $\pm$ 0.3 mmHg, respectively. The $P_{et}CO_2$ mean decreased more during IH than during CH from baseline to end exposure ($p = 0.02$, Figure 4).

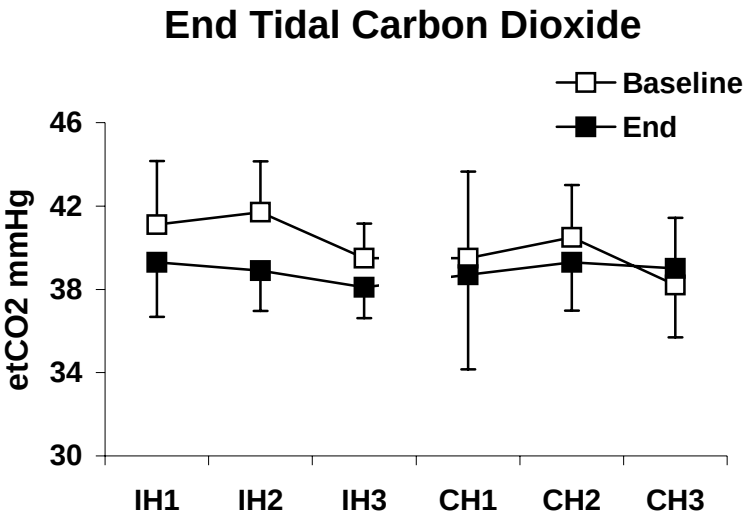

Figure 4 End tidal carbon dioxide

4.3.3. Heart Rate

When comparisons were made between data obtained at baseline and end exposure on each study day (e.g., IH-day 1, IH-day 2, IH-day 3), and each CH exposure (e.g., CH-day 1, CH-day 2, and CH-day 3), there was a statistically significant ($p < 0.001$) increase in HR, irrespective of exposure type (IH vs. CH) on day 1, day 2, and day 3 (Figure 5). The magnitude of the HR increase was not significantly different between the two exposure protocols. Thus, to summarize, HR increased from baseline to end exposure during both IH and CH, and the magnitude of the change was statistically similar in both exposure protocols.

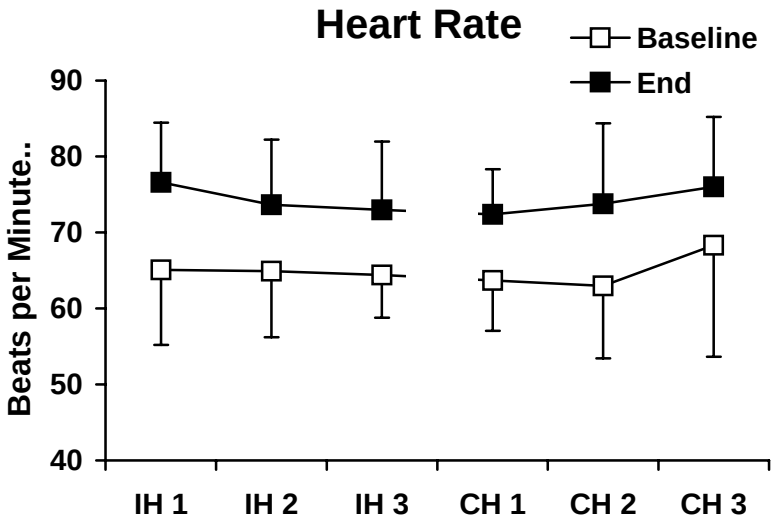

Figure 5 Heart rate

4.3.4. Systolic Blood Pressure

When comparisons were made between data obtained at baseline and end exposure to IH-day 1, IH-day 2, IH-day 3, and CH-day 1, CH-day 2, and CH-day 3, there was a statistically significant ($p=0.009$) increase in SBP, irrespective of exposure type (IH vs. CH) on day 1, day 2, and day 3 (Figure 6). The magnitude of the SBP increase was not significantly different between

the two exposure protocols. Thus, SBP increased from baseline to end exposure during both IH and CH, and, as in the case of HR, the magnitude of the change was statistically similar in both exposure protocols.

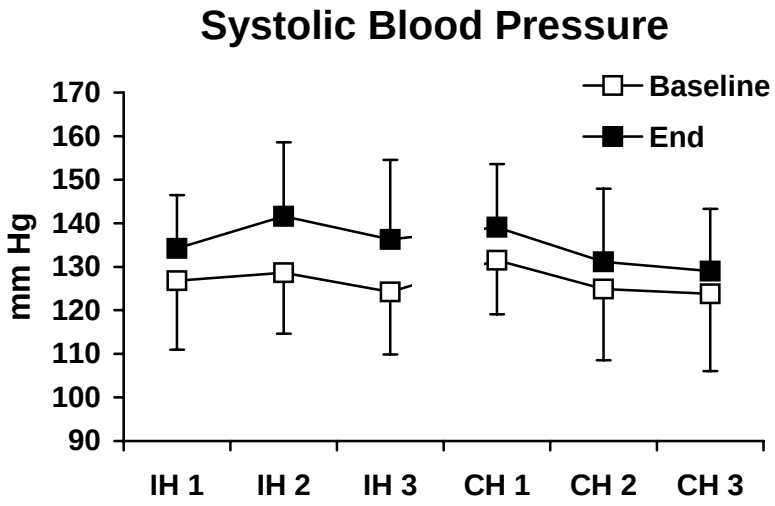

Figure 6 Systolic blood pressure

4.3.5. Diastolic Blood Pressure

When comparisons were made between data obtained at baseline and end exposure to IH-day 1, IH-day 2, IH-day 3, and CH-day 1, CH-day 2, and CH-day 3, there was a statistically significant ($p=0.02$) increase in DBP, irrespective of exposure type (IH vs. CH) on day 1, day 2, and day 3 (Figure 7). The magnitude of the DBP increase was not significantly different between the two exposure protocols. In summary, as with both HR and SBP, DBP increased from baseline to end exposure during both IH and CH, and the magnitude of the change was statistically similar in both exposure protocols.

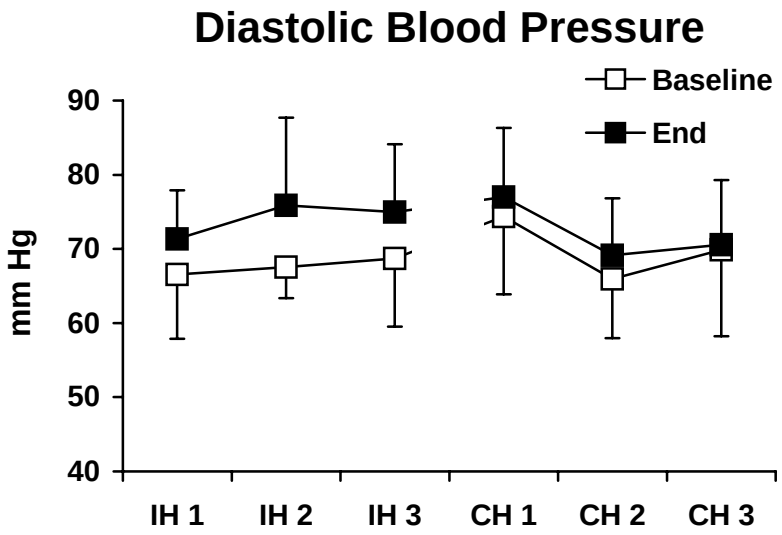

Figure 7 Diastolic blood pressure

4.4. Discussion

The main finding of our study was that, although the duration and degree of hypoxic exposure as reflected by the nadir S_pO_2 in our protocols was sufficient to elicit significant alterations of HR, SBP and DBP, the magnitude of these alterations did not change significantly over time (3 days) or differ by the hypoxia exposure profile.

Numerous studies have examined the hemodynamic response of humans to IH of different frequencies and durations (Arabi et al., 1999; Cutler et al., 2004; Morgan, Crabtree, Palta, & Skatrud, 1995; Xie, Skatrud, Puleo, & Morgan, 2001; Xie et al., 2000), and a lesser number have examined sympathetic and hemodynamic response to CH of days to weeks in duration (Calbet, 2003; Hansen & Sander, 2003). However, to the best of our knowledge, only one other study has directly compared hemodynamic responses to IH and CH in humans (Tamisier et al., 2005). While the Tamisier et al. (Tamisier et al., 2005) investigation exposed subjects to IH and CH once for 2 hours each on separate days ~1 month apart, our study had the

capability to look for hemodynamic changes over the course of 3 consecutive days of IH and CH exposures.

Changes (elicited by hypoxia) in the physiologic parameters measured in this study, over the course of days, have been of interest for some time. Some of the hemodynamic changes that occur during the IH episodes of OSA are thought to persist into the waking hours, causing e.g., daytime systemic hypertension (Fletcher, 2003). This "carryover", or long term facilitation (LTF) with potentiation of SNA outflow, may be dependent on prior hypoxic stimulation. Xie et al. (Xie et al., 2001) has speculated that this memory-like effect occurs in neurons of the rostral ventrolateral medulla (RVLM), is related to a modulation of afferent input from peripheral chemoreceptors by brain hypoxia , and is responsible for generating sympathetic "tone". Peng et al. (Peng, Overholt, Kline, Kumar, & Prabhakar, 2003) recently demonstrated that LTF of carotid body sensory discharge (in the form of progressive baseline sensory activity) increases with each successive hypoxic challenge, persisting for at least 60 min., in rats exposed to "chronic" IH (15 sec. of $F_IO_2 = 0.05$ followed by 5 min. normoxia, 9 episodes/hr., 8 hr./day for 10 days) when given "acute" IH challenges (10 episodes of 15 sec. Of 12% O_2 interspersed with 5 min. of 95% O_2), but not in animals exposed to CH ($F_IO_2 = 0.05$ for 4 hrs.) for 10 days in either acute or multiple exposures when given the "acute" IH challenge.

Aside from the issue of LTF, it must be noted that there is disagreement as to the duration of IH necessary to evoke a hemodynamic response. For example, Fletcher et al. found an elevated arterial pressure in rats after 35 days of 8 hr./day nighttime hypoxic exposure, but not after 10 days (Fletcher et al., 1992). By contrast, Sica et al. (Sica, Greenberg, Ruggiero, & Scharf, 2000) and Peng et al.(Peng et al., 2003) found that systolic arterial pressure increases in rats after 7 days of chronic IH with a pattern like that used by Fletcher et al. It should also be

noted that when SNA remains elevated during recovery from hypoxia exposure, there is no certainty that BP will remain elevated. Why BP is not elevated in some cases when sympathetic vasoconstrictor outflow remains elevated is unknown, but Xie et al.(Xie et al., 2001) suggest that the magnitude of sympathoexcitation after 20 minutes of hypoxic exposure may not be sufficient to alter total peripheral resistance. They suggest that longer periods of hypoxic exposure are likely needed to activate mechanisms such as the renin-angiotensin axis.

Adding to the debate regarding the pattern and duration of hypoxia necessary to evoke a hemodynamic and/or a SNA response, Tamisier et al. (Tamisier et al., 2005) has recently found that in 10 healthy humans, BP and muscle SNA actually show a statistically significant ($p<0.05$) increase after 2 hours of CH but not 2 hours of IH. In this study, IH was induced by alternating breathing of 100% nitrogen and room air so as to produce 30 to 40 drops in S_pO2 per hour (with 10% fluctuation in S_pO2, nadir = S_pO2 of 85%). These findings support the speculation that hemodynamic and SNA response to hypoxia depends more on the intensity of exposure than pattern of exposure, as subjects in the Tamisier et al. study exhibited significantly more profound hypoxia during 2 hrs. of CH than during 2 hrs. of IH (S_pO2 of 85.1 $\pm$1.6% for CH vs. 92.0 $\pm$ 3.4% for IH; $p<0.001$). Even though the reported range between the maximum and nadir S_pO2 was greater during IH (93.8 $\pm$ 2.7 and 67.9 $\pm$ 6.8%) compared with CH (98.8 $\pm$ 1.1 and 75.0 $\pm$ 4.5%), CH still elicited a significantly greater hemodynamic and SNA response than IH. The results of our study provide a contrast to the results of the work by Tamisier et al. We found that the average S_pO_2 during the last 5 minutes of all IH exposures was 84.4 $\pm$ 0.5%, and the average S_pO_2 during the last 5 minutes of all CH exposures was 86.4 $\pm$ 0.6%, but we cannot go beyond saying the S_pO_2 was in the 80-90% range (with a target of 85%) during the preceding minutes of the IH and CH exposures. As such, it is impossible for us to accurately speculate on the actual

difference in S_pO_2 magnitude between IH and CH during all of the study's hypoxia exposure times. However, we did find that 60 minutes of CH with the S_pO_2 in the 80-90% range prompted a statistically similar hemodynamic response to six 10 minute IH exposures with S_pO_2 in the 80-90% range, while Tamisier and coworkers found that 2 hours of much more frequent IH than ours did not evoke statistically similar hemodynamic results compared to 2 hours of CH. It is possible that the more profound differences in hypoxia levels reported during Tamisier et al.'s IH and CH exposures may account for this difference in response between our studies, but it is conceivable that other aspects of our study design (e.g., IH frequency and IH/CH duration) may have accounted for the similar hemodynamic response to IH and CH (Brooks et al., 1997).

Hence, there are several factors that should be considered when interpreting our data. While current thinking suggests the mechanism of increase in BP is related to activation of the sympathetic nervous system and concurrent activation of the renin-angiotensin system (Fletcher, Bao, & Li, 1999), the duration and/or frequency of IH necessary for BP alteration in humans is not known. This may be an important factor in considering why no statistically significant difference in day-to-day BP changes during the 3-day IH and CH sessions was noted in the present study. Perhaps the duration and/or frequency of IH and CH exposures in this study was not sufficient to sustain a significant day-to day activation of the sympathetic nervous system and the renin-angiotensin system. It is, however, worth noting that during IH exposures, SBP and DBP (Figs. 6 and 7) show a tendency (although not a statistically significant tendency) to increase over the course of the 3-day session – especially from day 1 to day 2. Additionally, it appears that in our study IH may have possibly created a more profound ventilatory stimulus, with a statistically significant baseline to end change in $P_{et}CO_2$ (in IH as compared to CH), during the last 5 minutes of each day's IH and CH exposures (Figure 2). It is not known if this

greater baseline to end exposure change persisted throughout the entirety of the IH sessions, but if it did, lower CO_2 levels during IH may possibly have influenced SNA and hemodynamic response.

It is possible that although sufficient to alter hemodynamic variables, the nature of the IH and CH profiles used our study may not have been sufficiently different and/or the sample size may have been too small to elicit detectable differences in hemodynamic responses. It is intriguing to speculate as to what change in the design and methodology of this study might be sufficient to cause a statistically significant difference in HR or BP change between IH and CH blocks or between days of exposure within each block. The 10-minute duration of IH exposures used in this study does not accurately simulate the character of the hypoxic episodes caused by the obstructive apneas seen in OSA. It is likely that voluntary apneas (creating a level of hypercapnia) lasting, e.g., ~30 seconds every 1 minute --simulating an apnea/hypopnea index of 60/hour - for 20 minutes as performed by the Cutler et al. study participants (Cutler et al., 2004), perhaps in combination with continuous breathing of mildly hypoxic air, may conceivably be the most effective way in which to simulate OSA for research purposes with human subjects. However, that it is unlikely that an experimental protocol has yet been designed, for animals or humans, to accurately simulate all of the characteristics of OSA (e.g., the Cutler et al. study ignores the potential interaction with sleep and arousal). Although the intent of the protocols in this study was not to mimic the episodes of hypoxic apnea characteristic of OSA, we nonetheless aimed to create an effective method with which it would be possible to explore the hemodynamic response to variations in the duration/frequency of hypoxia exposure in normal human subjects.

In summary, the results of this study provide insight into the potential design of future studies aiming to elucidate the mechanisms responsible for pathophysiologic changes in humans

suffering from hypoxia-related diseases. While inconsistent findings across studies reported to date may be due to differences in, e.g., experimental protocol and sample size, future studies directly comparing hemodynamic and SNA response to IH and CH will need to carefully consider how to match two different patterns of hypoxia. We chose to match the total duration of exposure and (attempted) to match the nadir of S_pO2. Further work in this area will help investigators better understand whether the sympathetic/hemodynamic response during and following hypoxia depends more on the mean S_pO2 value, total duration, or pattern of hypoxic exposure.

5. IH VS. CH: RELATIONSHIP OF HEMODYNAMIC RESPONSE TO MOLECULAR RESPONSE

5.1. Introduction

Exposure to hypoxia triggers a spectrum of biologic (cellular) and physiologic (organ system) responses. Alterations in cardiovascular parameters such as blood pressure (BP) and heart rate (HR) reflect one physiologic change that is modulated by central and peripheral chemoreceptors. Hypoxia Inducible Factor 1α (HIF-1α) represents a consequence at the cellular level. While difficult to detect under normoxic conditions because of a normoxic half-life of less than 5 minutes (Huang, Arany, Livingston, & Bunn, 1996; Wang, Jiang, Rue, & Semenza, 1995), HIF-1α "increases" as oxygen tension decreases because hypoxia slows destruction of HIF-1α (Huang & Bunn, 2003). HIF-1α has been called the most important protein regulating molecular response to hypoxia in mammals (Hopfl, Ogunshola, & Gassmann, 2003). As a transcription factor, HIF-1α activates a number of genes associated with hypoxic adaptation, including several genes important to vascular function such as vascular endothelial growth factor (VEGF), erythropoietin (EPO), and inducible nitric oxide synthase (iNOS) (G. Semenza, 2000). Nitric oxide (NO) is produced through the action of three isoforms of nitric oxide synthase (NOS); endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (Isaacson, Hampl, Weir, Nelson, & Archer, 1994; Le Cras et al., 1998; Le Cras, Xue, Rengasamy, & Johns, 1996; Muramatsu, Tyler, Rodman, & McMurtry, 1996; L. A. Palmer, Semenza, Stoler, & Johns, 1998; Resta, Gonzales, Dail, Sanders, & Walker, 1997; Resta & Walker, 1996; Russ & Walker, 1992; Shaul, Kinane, Farrar, Buja, & Magness, 1991; Wilson, Thompson, Moore, Khimenko, & Taylor, 1997; Xue & Johns, 1996).

Ni et al. (Ni, Bemanian, Kivlighn, & Vaziri, 1998) has reported that a hypoxia-induced increase in BP is accompanied by a marked reduction in urinary excretion of nitric oxide (NO) metabolites in a rat model. This suggests that hypertension induced by chronic exposure to hypoxia in the rat is associated with, and possibly due to, decreased NO availability. NO has many properties among which is as a potent mediator of vasodilation (Ignarro, Buga, Wood, Byrns, & Chaudhuri, 1987; R. M. J. Palmer, Ferrige, & Moncada, 1987). The observation that iNOS is upregulated (e.g. increased mRNA expression and iNOS protein) within the pulmonary vasculature in rats (Le Cras et al., 1998; Le Cras et al., 1996; L. A. Palmer et al., 1998; Shaul et al., 1995; Xue & Johns, 1996) with chronic hypoxic exposure has prompted speculation that it blunts hypoxic vasoconstriction in the pulmonary and other arterial beds (Resta, O'Donaughy, Earley, Chicoine, & Walker, 1999) by opposing the vasoconstrictor effects of endothelin, angiotensin II, and renal sympathetic nerve activity (Kone, 1997; Kone & Baylis, 1997). In addition, the presence of NOS in the bronchial epithelium confirms a potential distinct source of NO production outside of the vasculature (Kobzik et al., 1993; Pearl et al., 2000).

There is inconsistent evidence supporting the thinking that the pattern of hypoxia (intermittent [IH] or continuous [CH]) is a significant factor in determining physiologic response. For instance, a recent study by Tamisier et al. (Tamisier et al., 2005) reported that in 10 healthy humans, BP and muscle sympathetic nerve activity (SNA) actually show a statistically significant ($p<0.05$) increase after 2 hours of CH but not 2 hours of IH. However, another study in humans failed to demonstrate different effects of IH and CH on hemodynamic variables (Rodway et al., 2005). On the other hand, animal studies have shown marked increases in BP and sympathetic activity following IH that are not seen following exposure to a similar duration of CH (Prabhakar, 2002; Young et al., 1997).

The purpose of this study was to explore the relation between the hemodynamic and molecular responses in normal human subjects who were exposed to 2 profiles of hypoxia, IH and CH. Attempting to identify the relationship between molecular and hemodynamic responses may help to provide a better understanding of inter-individual differences in physiologic response of human subjects to different patterns of hypoxic stimulus.

5.2. Methods

5.2.1. Sample

Data were collected on 10 healthy males aged 25.3 ± 5.7 years (mean ± SD, range 20-35 years) as part of a study of the hemodynamic responses to IH and CH, described in chapter 4 of this report. Characteristics of participants are described there in more detail. In brief, none of the subjects had smoked for > 3 months; were taking prescription medications; or engaged in a formal athletic training program. None of the subjects had a history of cardiopulmonary disease and all had a resting room air SpO2 >95%, a normal electrocardiogram, and normal spirometry. Only males were studied to avoid the potentially confounding influence of cyclic estrogen and progesterone fluctuation on study variables. The study received approval from the University of Pittsburgh Institutional Review Board for Biomedical Research and all subjects provided written, informed consent.

5.2.2. Procedure

Subjects were instructed not to eat or drink for at least 2 hours and to avoid food and beverages containing caffeine for 24 hours prior to arriving for each study block. They were also asked to avoid meat, cured or smoked foods (e.g. ham, bacon, smoked fish) and leafy greens/vegetables (e.g. broccoli, kale, spinach) which may have contained degraded red blood cells and or nitrates for at least 48 hours before and during each study block. In addition,

participants were asked to avoid taking vitamin pills which may have contained anti-oxidants for at least 3 weeks. Compliance with these requests was by self-report.

The protocol for IH and CH has been described in chapter 4 and is reported briefly here: each subject participated in 3 sessions: 1) normoxic acclimatization; 2) 3 consecutive days of IH; and 3) 3 consecutive days of CH (Figure 3). Four subjects started with the IH session and 6 with the CH session. To eliminate an order effect, IH and CH sessions were separated by a washout period (≥ 7 days). During all sessions, subjects were semi-recumbent in a hospital bed for 15 minutes prior to and during data collection. Since subject's concentration on their breathing could potentially influence study variables, distraction in the form of television or music was provided during all test sessions. Subjects could not be blinded to the nature of the exposure protocol, but were blinded to the hypothesis, as none of the subjects had advanced knowledge of hypoxia/cardiovascular physiology.

5.2.2.1. Normoxic Acclimatization Session

Subjects breathed medical grade air (MGA) through a commercially available non-rebreather anesthesia facemask for 60 minutes. The mask worn by the subject was connected via a one-way valve to a 15-liter Douglas bag, out of which ran a line connected to a wall-mounted tap supplying the MGA. For the first and last 5-minute period of the 60-minute session, systolic, diastolic, and mean blood pressure (SBP, DBP, MBP), heart rate (HR), S_pO_2, and $P_{et}CO_2$ were continuously recorded.

5.2.2.2. IH Session

Subjects underwent 6 exposures to IH on each of 3 consecutive days. In order to achieve a steady state (as reflected by stable S_pO_2, $P_{et}CO_2$ and breathing pattern) and collect baseline hemodynamic data, MGA was administered to subjects for 10 minutes through the identical non-

rebreather facemask used in the normoxic acclimatization session. Data were recorded continuously for 5 minutes immediately before initiating IH exposure. A commercially available hypoxia generator (Hypoxico, Inc., New York, N.Y.) utilizing "pressure swing adsorption", with zeolite crystals used as a "molecular sieve", fed hypoxic air (F_IO_2 ~0.135) to the Douglas bag and hence to the non-rebreather facemask until the subject's S_pO_2 was 80-90%. The same research team member who monitored subject status also maintained the S_pO_2 in this range, with a target of 85%, by bleeding in MGA as needed. After 10 minutes of S_pO_2 80-90%, the IH exposure ended and MGA was administered so that S_pO_2 > 90% for 10 minutes. A total of 6 IH exposures were given in this manner during each of the 3 days. Hemodynamic variables were then recorded continuously during the last 5-minutes of each day's 6th IH exposure.

5.2.2.3. CH Session

Subjects underwent one continuous 60-minute exposure to hypoxia on each of 3 consecutive days. In order to achieve a steady state (as reflected by stable S_pO_2, $P_{et}CO_2$ and breathing pattern) and collect baseline hemodynamic data, MGA was administered to subjects for 10 minutes through the identical non-rebreather facemask used in the control and IH session. Data were recorded continuously for 5 minutes immediately before initiating CH exposure. The hypoxia generator then began to feed air (F_IO_2 ~0.135) to the Douglas bag and hence to the non-rebreather facemask in the same manner as during the IH session until the subject's S_pO_2 was 80-90%. The subject's S_pO_2 was maintained in this range for 60 minutes, with a target of 85%, by bleeding in MGA as needed. During the last 5-minutes of each day's CH session, hemodynamic variables were recorded continuously.

5.2.2.4. Instrumentation

A standard 3-lead electrocardiogram (in lead 2) was used to monitor heart rate and rhythm**.** Finger pulse oximetry was used to measure S_pO_2 (CO_2SMO *Plus*!® respiratory profile monitor, Novametrix Medical Systems, Inc., Wallingford, CT, USA). A commercial infrared capnograph with a solid state mainstream flow sensor (CO_2SMO *Plus*!® respiratory profile monitor), connected to the non-rebreather facemask, measured $P_{et}CO_2$. Before each session, The CO_2 sensor was calibrated using internally generated values of 0 and 38 mmHg. An inflated cuff placed on one or two fingers of the subject's non-dominant hand recorded SBP, DBP, and MBP (Portapres®, Finapres Medical Systems**,** Amsterdam, Netherlands). Known pressures (0, 50, 100, and 150 mmHg), generated internally by the device and verified using a standard sphygmomanometer, were used to calibrate the Portapres® before each session. The recording channels for blood pressure, S_pO_2, and $P_{et}CO_2$ were calibrated with known values from the Portapres® and CO_2SMO *Plus*!®. Labtech Notebook Pro software, version 10.1 (Andover, MA, USA), was used for data acquisition.

5.2.2.5. Blood Collection and iNOS/ HIF-1α mRNA Analysis

Venous blood was obtained prior to hypoxic exposure on day 1 of IH and CH of each study block and 2 hours after the end of the hypoxic exposure on day 3 of the IH and CH study blocks, for HIF-1α and iNOS mRNA gene expression analysis (Table 4).

Table 4 Blood collection time points

	IH / CH		
	Day 1	**Day 2**	**Day 3**
Pre-hypoxia	**X**		
2 hrs. post-hypoxia			**X**

PAXgene Blood RNA Tubes were used for blood collection and cellular RNA stabilization, and the PAXgene Blood RNA Kit was used for isolation of cellular RNA (PreAnalytiX; Hombrechtikon, Switerland). Gene expression quantification was performed in a two-step reverse transcription-polymerase chain reaction (RT-PCR) in which the PCR step is coupled with a 5' fluorogenic nuclease assay to amplify target cDNA without amplifying genomic DNA. In the reverse transcription (RT) step, cDNA is reverse transcribed from total RNA samples using random primers from a High Capacity cDNA Archive Kit (Applied Biosystems; Foster City, California USA). In the PCR step, PCR products are synthesized from cDNA samples using TaqMan Universal PCR Master Mix (Applied Biosystems; Foster City, California USA). Assays-on-Demand (Applied Biosystems; Foster City, California USA) were utilized for detection and quantitfication of nucleic acid sequences via ready-to-use 5' nuclease assays for human transcripts. The primer sequence for the 5' iNOS nuclease assay was CATAGTTTCCAGAAGCAGAATGTGA, and the primer sequence for the 5' HIF-1α nuclease assay was ACACACAGAAATGGCCTTGTGAAAA. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized as an external control assay in separate wells from the target assay in the 96-well PCR reaction plate. Relative quantification of gene expression was accomplished via the comparative count (C_T) method, taking into account that the efficiency of

the target and GAPDH amplification was nearly identical for a given amount of cDNA (slope of log input amount vs. normalized value <0.1). The normalized amount of target is a unitless number used to compare the relative amount of target in different samples, and is determined by dividing the average target value by the GAPDH value. The instrument used for data collection was an Applied Biosystems 7000 Sequence Detection System (Applied Biosystems; Foster City, California USA).

5.2.3. Data Analysis

Data are reported as the mean $\pm$ standard deviation. Statistical significance was considered at $p \leq 0.05$. SPSS statistical package version 11.5 was used for analysis. Nonparametric correlation coefficients (Spearman's rho) were examined in order to determine the relationship between the hemodynamic and molecular parameters. Because this study involved a small number of subjects and thus a normal distribution of values could not be assumed, Spearman's was utilized for the correlation analysis, rather than its parametric counterpart.

5.3. Results

Molecular data were available in 9 subjects (samples for one subject were unusable for analysis). The mean of the 3 days of baseline IH and CH hemodynamic values were correlated with the baseline IH and CH molecular values, and the mean of the 3 days of end IH and CH hemodynamic values were correlated with the end IH and CH molecular values. Nonparametric correlation coefficients for the relation between hemodynamic variables and iNOS mRNAare presented in Table 5. There were significant correlations between iNOS mRNAand DBP ($r = -0.89$, $p < 0.01$, Figure 8) and MBP ($r = -0.87$, $p < 0.01$, Figure 9), but not SBP ($r = -0.61$, p=NS, Figure 10) at the end of the day 3 IH session. The range of r values for iNOS mRNA/ BP

correlation prior to day 1 IH session was -0.24 to -0.07, and none of the values were statistically significant.

Table 5 iNOS / hemodynamic nonparametric correlation coefficients. Nonparametric correlation coefficients for n=9. ** Correlation is significant at p<.01 level

	IH 1	IH 3	CH 1	CH 3
$etCO_2$	-.54	-.30	-.27	-.25
HR	.24	-.35	-.42	-.27
SBP	-.07	-.61	.33	.15
DBP	-.24	**-.89 ****	-.04	.12
MBP	-.10	**-.87****	.12	.43

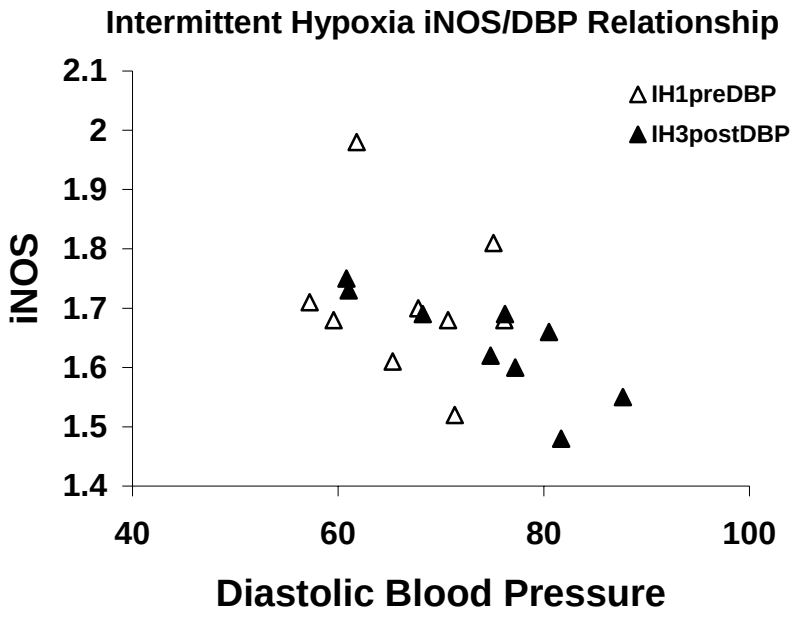

Figure 8 Plot of iNOS / Diastolic BP Relationship; IH1 r = -0.24, p = NS; 2 hour post IH3 r = -0.89, p<0.01.

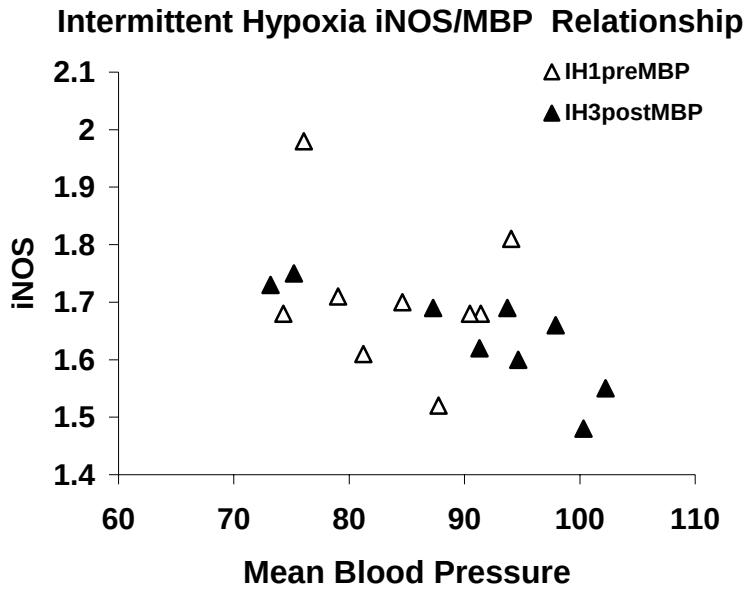

Figure 9 Plot of iNOS / Mean BP Relationship; IH1 r = -0.10 p = NS; 2 hour post IH3 r = -0.87, p<0.01.

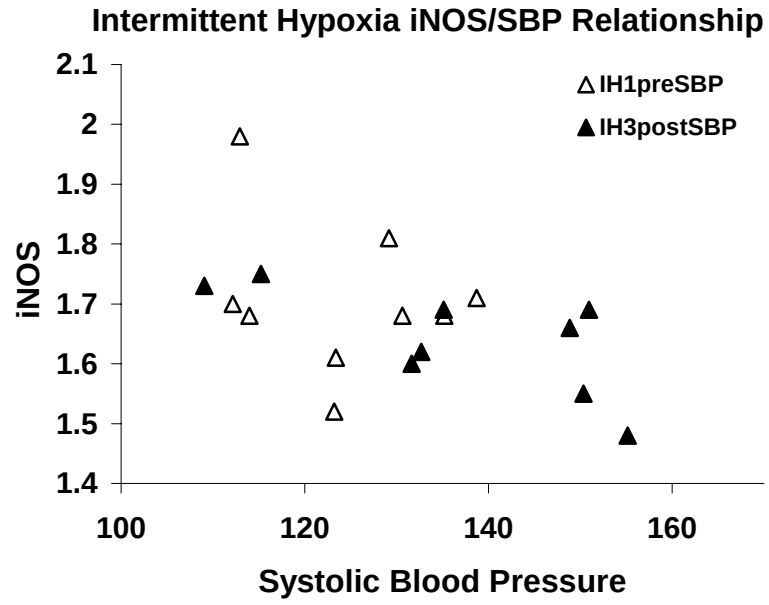

Figure 10 Plot of iNOS / Systolic BP Relationship; IH1 r = -0.07, p = NS; 2 hour post IH3 r = -0.61, p = NS.

The range of r values for iNOS mRNA/ BP correlation was -0.04 to 0.33 before CH, and 0.12 to 0.43 after CH; none of these values were statistically significant (see Figures 11 - 13 for correlations of iNOS mRNA with DBP, MBP, and SBP for CH, respectively). No other statistically significant correlations were seen between hemodynamic variables and iNOS mRNA before or after IH or CH.

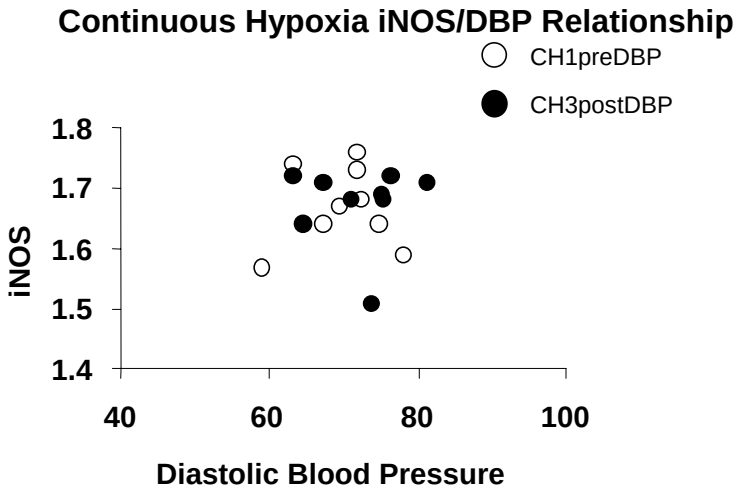

Figure 11 Plot of iNOS / Diastolic BP Relationship; CH1 r = -0.04, p = NS; 2 hour post CH3 r = 0.12, p = NS.

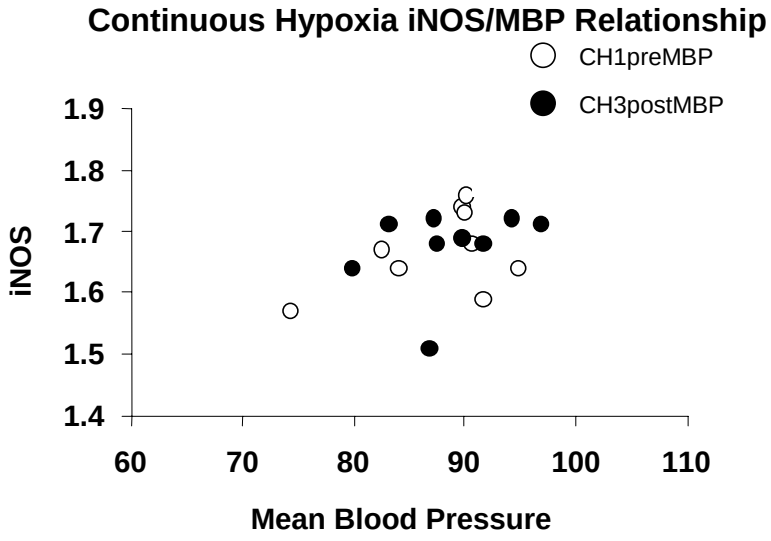

Figure 12 Plot of iNOS / Mean BP Relationship; CH1 r = 0.12, p = NS; 2 hour post CH3 r = 0.43, p = NS.

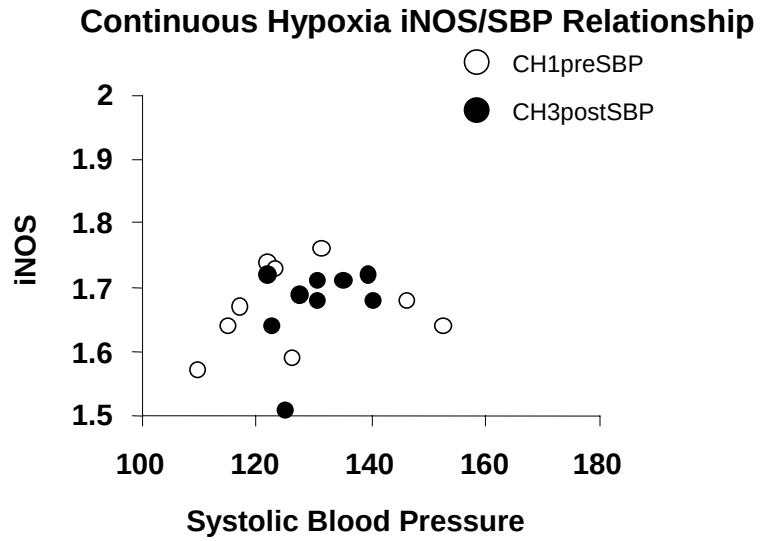

Figure 13 Plot of iNOS / Systolic BP Relationship; CH1 r = 0.33, p = NS; 2 hour post CH3 r = 0.15, p = NS.

A significant correlation involving HIF-1α mRNA was noted only for $PetCO_2$ at the end of the day 3 CH session ($r = -0.68$, $p<0.05$, Table 6). Before IH, the range of r values for HIF-1α mRNA/ BP correlation was -0.39 to -0.31, and after IH, -0.06 to 0.10. The range of r values for HIF-1α mRNA/ BP correlation was -0.32 to 0.0 before CH, and -0.07 to 0.26 after CH. None of these correlation values were statistically significant. No other statistically significant correlations were seen between hemodynamic variables and HIF-1α mRNA before or after IH or CH.

Table 6 HIF-1α / hemodynamic nonparametric correlation coefficients. Nonparametric correlation coefficients for n=9. * Correlation is significant p<.05 level.

	IH 1	IH 3	CH 1	CH 3
$etCO_2$	-.36	-.15	.05	**-.68***
HR	-.09	.02	-.55	.04
SBP	-.33	.10	.00	.12
DBP	-.39	-.04	-.32	-.07
MBP	-.31	-.06	-.14	.26

5.4. Discussion

Our results show a significant negative correlation ($p<0.01$) of iNOS mRNA with DBP and MBP after the third day of IH. It is conceivable that in our study the IH stimulus may have been potent enough to influence iNOS expression, at least as far as the relationship to DBP and MBP response was concerned. Hence, it follows that the stimulus from CH may not have been strong enough to influence iNOS expression as a mediator of vascular response. We found that the average S_pO_2 during the last 5 minutes of all IH exposures was $84.4 \pm 0.5\%$, and the average S_pO_2 during the last 5 minutes of all CH exposures was $86.4 \pm 0.6\%$, but we cannot go beyond saying (in regard to the magnitude of hypoxia) that during the preceding minutes of the IH and CH exposures the S_pO_2 was in the 80-90% range (with a target of 85%). It is thus not possible for us to speculate as to the actual difference in S_pO_2 between IH and CH during all of the study's hypoxia exposure times. We can, however, indicate that the total hypoxic time (time of IH $S_pO_2 < 90\%$ compared to CH $S_pO_2 < 90\%$) was, by design, identical. Although the results of this study

may provide some support the thinking that IH is a more potent physiological stress than CH in regard to iNOS expression and its mediation of BP, important questions remain.

Why this same relationship with BP was not seen in the case of HIF-1α may be due in large part to the study design. The timing of blood collection for analyzing gene expression may have accounted for the results. Venous blood was obtained immediately prior to the start of the exposure protocol on day 1 of IH and CH, as well as 2 hours after the end of IH and CH on day 3, for HIF-1α and iNOS mRNA analysis. Transcriptional activation of iNOS by HIF-1α presumably occurred at some time prior to the collection of blood 2 hours after the end of IH and CH on day 3, but nonetheless, an up-or-down regulated HIF-1α response to hypoxia would have been virtually impossible to detect after 2 hours of normoxia because of HIF-1α's normoxic half-life of less than 5 minutes (Huang et al., 1996; Wang et al., 1995), Additionally, the gap in HIF-1α and iNOS mRNA data points between day 1 baseline of IH and CH and 2 hours post IH and CH on day 3 makes speculation as to a time sequence for expression rather difficult. Collection of blood immediately before and immediately after each day of IH and CH may have provided enough data to increase the confidence of such speculation.

Our results are both suportive of, and in conflict with, other investigations of the reponse to IH and CH. Barton et al. (Barton, Zhenmin, & Vaziri, 2003) reported that chronic hypoxia-induced hypertension in rats is associated with marked downregulation of NOS isotypes over the course of 21 days. Their data demonstrated a consistent pattern of early increase (within 48 to 72 hours) followed by a steady decline in iNOS in the kidney and cardiovascular tissues during exposure to hypoxia. There is also some suggestion that hypoxia impairs the output of endongenous NO (Haight & Djupesland, 2003). Haight & Qian (Haight & Qian, 2000) found that changes in blood oxygen levels, achieved either through breath-hold or by lowering the

oxygen content of the gas flowing through the nose while the subject mouth breathes, quickly lowers exhaled NO output (but the inevitable washout of nasal NO with this method may influence the results). Other studies have suggested that in patients with obstructive sleep apnea (OSA), circulating NO reduction may be due to impaired function of the vascular endothelial cells (Ip et al., 2000; Kato et al., 2000; Schulz et al., 2000). Ip et al. (Ip et al., 2000) and Schulz et al.(Schulz et al., 2000) found that CPAP reversed the suppression of NO output in OSA, supporting the the view that hypoxia is responsible for depressed NO synthesis in these patients (McQuillan, Leung, Marsden, Kostyk, & Kourembanas, 1994). Furthermore, the realtionship of HIF-1α to iNOS and synthesis of NO is not completely understood. However, a study by Yin et al. (Yin, Yang, Ku, & Hsu, 2000) demonstrated that NO generated by iNOS expression inhibits HIF-1α DNA binding activity in a hypoxic cell culture, suggesting a possible negative feedback loop in the HIF-1α/iNOS cascade.

Hypoxic regulation of iNOS in humans is a phenomenon about which relatively little is known (Pitt & St. Croix, 2002). In contrast to the thinking that hypoxia is responsible for depressed NO synthesis in the above-mentioned studies, elevation of iNOS was found in cardiac biopsies in cyanotic children with congenital heart disease (Ferreiro et al., 2001). Dweik et al. (Dweik et al., 1998) provided support for iNOS as a mediator of vascular response to oxygen in a study of the effect of hypoxia on NO production in normal human volunteers. Likewise, exhaled NO is elevated in healthy high altitude natives living in Tibet and Bolivia, compared to normals at or near sea level (Beall et al., 2001). A decrease in exhaled NO is seen in sojourners to high altitude known to be susceptible to high altitude pulmonary edema (HAPE), but not in HAPE-resistent sojourners (Busch et al., 2001). These studies suggest that changes in NO production induced by hypoxia may be a homeostatic response helping to maintain a low vascular resistance

during such a stimulus (Pitt & St. Croix, 2002), and our findings seem to support this thinking. Hypoxia seems to be an important factor in the elevation or depression of iNOS and NO. However, given the lack of consensus among studies, it is conceivable that the frequency, duration and intensity of hypoxic stress is influential in regulating NO in ways that has yet to be fully elucidated.

It is possible that HIF-1α may also play a significant role in inter-individual variability in cardiovascular responses to hypoxia. The development of pulmonary artery hypertension and pulmonary artery medial hypertrophy with hypoxic exposure are thought to be influenced by HIF-1α (G. L. Semenza, 2001). Mice experienced delayed development of pulmonary artery hypertension and right ventricular (RV) hypertrophy during CH exposure when the gene encoding for HIF-1α was inactivated (G. Semenza, 2000; G. L. Semenza, 2001; Yu et al., 1999). Analysis of knockout mice has also demonstrated that HIF-1α is required for embryonic development and survival. Conversely, mice that are heterozygous for the knockout allele, and thus partially HIF-1α deficient, develop normally (G. L. Semenza, 2001). Yu et al.(Yu et al., 1999) compared the physiological response of partially HIF-1α deficient heterozygous mice to the response of their normal (wild-type) littermates after breathing 10% oxygen for 1 to 6 weeks. The partially HIF-1α deficient heterozygous mice demonstrated significantly delayed development of polycythemia, RV hypertrophy, pulmonary hypertension, and pulmonary vascular remodeling compared with their normal littermates.

In summary, the significant negative correlation seen in this study between diastolic/ mean BP and iNOS mRNA in conjunction with IH, but not CH, exposure at the end of the day 3 IH session implies that IH may elicit a compensatory biologic response. The results of this study support the thinking that IH is a more potent stressor than CH in regard to influencing iNOS

expression and its subsequent mediation of BP, but variability in the response may be attributable to variability in iNOS induction. The lack of consensus among studies, however, suggests that e.g., the frequency, duration and intensity of hypoxic stress is influential in regulating hypoxia inducible genes in ways that have yet to be fully elucidated. Nonetheless, ongoing efforts to identify and understand the relationship between molecular and cardiovascular response to hypoxia may help to provide improved comprehension of the inter-individual differences in the physiologic response of humans to different patterns of hypoxic stimulus.

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