Mechanisms of Sympathoexcitation via Hyper-Acute Intermittent Hypoxia in Humans: Implications for Obstructive Sleep Apnea

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DISSERTATION

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ABBREVIATIONS

ABP	Arterial Blood Pressure
AHI	Apnea Hypopnea Index
Ang II	Angiotensin II
ANOVA	Analysis of Variance
ARB(s)	Angiotensin Receptor Blocker(s)
ATR1a	Angiotensin II Type 1a Receptor(s)
BBB	Blood Brain Barrier
CBR	Carotid Baroreflex
CIH	Chronic Intermittent Hypoxia
CNS	Central Nervous System
CO_2	Carbon Dioxide
COX-2	Cyclooxygenase 2
CPAP	Continuous Positive Airway Pressure
CVO(s)	Circumventricular Organ(s)
DAP	Diastolic Arterial Pressure
ECG	Electrocardiogram
EPR	Electron Paramagnetic Spectroscopy
$ETCO_2$	End-Tidal Carbon Dioxide
ETO_2	End-Tidal Oxygen
F_iO_2	Fraction of Inspired Oxygen
HR	Heart Rate

IH	Intermittent Hypoxia
IHT	Intermittent Hypoxia Training
LDIH	Long Duration Intermittent Hypoxia
LSNA	Lumbar Sympathetic Nerve Activity
LTF	Long Term Facilitation
LVC	Leg Vascular Conductance
MAP	Mean Arterial Pressure
MSNA	Muscle Sympathetic Nerve Activity
N-AC	N-Acetyl Cysteine
NO	Nitric Oxide
NTS	Nucleus of the Solitary Tract
O_2	Oxygen
OSA	Obstructive Sleep Apnea
P_aO_2	Partial Pressure of Arterial Oxygen
PVN	Paraventricular Nucleus
ROS	Reactive Oxygen Species
RVLM	Rostral Ventral Lateral Medulla
SAP	Systolic Arterial Pressure
SDIH	Short Duration Intermittent Hypoxia
SEM	Standard Error of the Mean
SFO	Subfornical Organ
sLTF	Sensory Long-Term Facilitation
SNA	Sympathetic Nerve Activity

TLR2	Toll-like Receptor 2

CHAPTER I

DISSERTATION OVERVIEW

Obstructive Sleep Apnea (OSA) is a form of sleep-disordered breathing characterized by frequent obstructive apneas and hypopneas that produce intermittent hypoxic hypercapnia, intrathoracic pressure swings and arousals from sleep (7). OSA is highly prevalent in the United States, and has been estimated to affect approximately 5% of the total population (36, 49, 50), although this is likely a gross underestimate due to the significant underdiagnosis of OSA within the population (36). Furthermore, OSA has been demonstrated to cause cardiovascular disease independently of often co-morbid obesity and metabolic syndrome, mainly through co-morbid hypertension frequently observed in OSA patients (10). Hence, rigorous biomedical research is needed to investigate the pathophysiology of this disorder to develop novel therapeutic approaches to reduce OSA-mediated hypertension. Indeed, continuous positive airway pressure (CPAP) treatment, which effectively splints the airway open to prevent obstructions during sleep and is currently considered the "gold standard" of OSA treatment, only modestly impacts OSA-mediated hypertension (11, 15-17, 27, 28, 44, 45). Furthermore, sleep clinicians often encounter significant obstacles to CPAP compliance, as many OSA patients describe CPAP treatment as obtrusive and uncomfortable (19, 39, 40).

To develop novel therapies that target OSA-mediated cardiovascular disease, an evidence base that is built on basic science research in animal and human subjects must be developed. For the past quarter century, primarily animal investigations have produced evidence to link OSA-mediated cardiovascular disease and hypertension with the phenomenon of intermittent hypoxia (IH), which is experienced by OSA patients as they sleep secondary to the frequent respiratory disturbances. Indeed, Fletcher *et al.* (9) were the first group of investigators to expose rodents to cyclical hypoxia over a prolonged period, and to demonstrate the generation of hypertension in these animals, compared to control animals. Numerous animal and human investigations since then have reproduced those findings, and have demonstrated that IH alone produces a sustained hypertension that persists from periods of IH exposure during sleep into the normal, awake periods (12, 13, 18, 38, 41, 46, 47).

Fletcher *et al.* (8) and Lesske *et al.* (22) went on to identify the critical role of the peripheral chemoreflex and the sympathetic nervous system (SNS) in the hypertensive response to IH. These investigators demonstrated that carotid body denervation effectively blocked the IH-mediated hypertensive response in rodents exposed to cyclical hypoxia of at least 7 days in duration. Furthermore, blocking the sympathetic *nerve* response by ablating sympathetic nerve terminals with 6-hydroxy-dopamine, and blocking the sympathetic *humoral* response by performing surgical adrenal de-medullation also abolished the increase in arterial pressure after IH exposure (22). Hence, peripheral chemoreflex control of sympathetic nerve activity (SNA) is a critically important pathophysiological feature of IH-mediated hypertension. This conclusion is clinically relevant as OSA patients commonly exhibit very high baseline muscle SNA (MSNA) burst

frequency and amplitude (43). Furthermore, decreasing tonically active chemoreflex inputs via hyperoxic exposure decreases elevated MSNA burst frequency in OSA subjects compared to healthy subjects in which no such decrease is appreciable, regardless of baseline nerve activity (30). As a point of comparison, obesity, metabolic syndrome and fragmented sleep architecture in human experimental models have not been able to recapitulate the sympathetic phenotype observed in OSA patients (2, 14, 25, 29), once again implicating IH as a critical pathophysiological link between OSA and subsequent hypertension.

However, the mechanistic link between IH and increased SNA is currently not well understood. Generating mechanistic evidence of this kind is necessary to identify novel therapeutic targets to reduce OSA-mediated cardiovascular risk. Once again, animal research during the past 15 years has helped to understand, in part, the neural mechanisms at-play with regard to IH-mediated sympathoexcitation. Generally speaking, adaptations within the carotid body, in the central nervous system (CNS) structures that are components of the chemoreflex arc, and within the adrenal medulla are pivotal to the development of hypertension secondary to chronic IH exposure in rodents (21, 25, 37, 48). For example, the rodent carotid body when exposed to chronic IH, exhibits an augmented neural response to modest, singular bouts of hypoxia and also develops a sensory long term facilitation (sLTF), which is defined as a continued firing in the absence of the original stimulus (32-35). These findings mirror what occurs in OSA patients, who exhibit exaggerated sympathetic responses to modest hypoxia and also have a tonically active chemoreflex-mediated sympathoexcitation, insofar as silencing chemoreceptor firing via hyperoxia reduces MSNA, as aforementioned (30, 42). Peng *et al.* has demonstrated that these

phenomena are mediated in rodents by reactive oxygen species (ROS) and angiotensin II (Ang II), and blocking these mediators using oral antioxidants and angiotensin II receptor type 1a (ATR1a) blockers prevents the development of these maladaptations (31, 34, 35). Allen *et al.* identified that ATR1a are instrinsic to carotid body glomus cells and subserve critical signaling functions (42). Furthemore, Peng *et al.* also demonstrated that the nucleus of the solitary tract (NTS) and the rostral ventral lateral medulla (RVLM), nuclei which participate in the chemoreflex neural arc, generate an abundance of ROS locally in response to IH (35). Kumar *et al.* identified that IH-mediated ROS production contributes to the secretion of catecholamines via sympathetic efferent interactions with the adrenal medulla, and they effectively abrograted the elevation in catecholamines with antioxidant administration (20). Finally, numerous animal studies have demonstrated that injection of antioxidant compounds into the cerebral ventricles reduces central sympathetic outflow (3).

An important translational step would be to test the hypotheses that a: (i) CNS-permeable antioxidant and (ii) an ATR1a receptor blocker will prevent the sympathoexcitation observed with IH in *human* subjects. The experiments that test these core hypotheses, and the data and conclusions derived thereof, form the basis of the dissertation that follows.

In humans, SNA can be directly measured using the technique of microneurography, which measures the activity of the sympathetic efferent nerves that innervate the vasculature of skeletal muscle (1). This is called muscle SNA (MSNA) and the burst activities (expressed in this dissertation as burst frequency i.e. bursts/minute, and incidence, i.e. bursts/100 heart beats) of MSNA directly correlates with central sympathetic outflow in humans as determined by ECG auto-

correlation analyses (6). However, this technique can be challenging, particularly in terms of obtaining and maintaining these recordings for long periods of experimentation. Requiring human subjects to expose themselves to chronic IH as performed in animal studies, would involve 6-8 hours exposures for 2-28 days presenting serious logistical (and sometimes ethical) challenges. Hence, few investigators have undertaken human studies of chronic IH. The investigations of human chronic IH that have been performed have been tremendously valuable to this field, however — for example, Tamisier *et al.* (47) and Gilmartin *et al.* (13) reported that 14 and 28 days of IH, respectively, elevate arterial pressure and MSNA burst frequency and incidence. However, these investigations did not (and could not) maintain MSNA recordings throughout the IH exposure, so it is unclear when the acute and/or long-lasting sympathetic effects of IH begin to take place. This technical difficulty in assessing MSNA during prolonged IH conditioning is a key reason we focused on short-term IH conditioning to test our hypotheses.

In a study in which MSNA recordings were maintained throughout a shorter IH period, Morgan *et al.* demonstrated that exposure to intermittent asphyxia for only 20 minutes produces a sustained increase in MSNA (i.e. lasting into the post-experimentation period) (26). Cutler *et al.* (4, 5) and Leuenberger *et al.* (23, 24) demonstrated a similar effect on MSNA and arterial pressure using intermittent 20-30 second hypoxic apneas lasting for approximately 20 minutes. These investigations have demonstrated that this very short IH exposure (what we term, "hyper-acute" IH) produces a transient hypertension and a persistent elevation in baseline MSNA and in MSNA responses to hypoxia for at least 3 hours post IH (4, 5) . Hence, even IH exposures of less than 1 hour can transiently recapitulate the elevated arterial pressure, persistently elevated MSNA and

enhanced MSNA responses to hypoxia observed in OSA patients. We contend that exploring hyper-acute IH-mediated sympathoexcitatory and hypertensive mechanisms in healthy humans provides valuable insight into OSA pathophysiology, and this model, although not without limitations, forms the core of this dissertation.

Therefore, the present dissertation focuses on the model of hyper-acute IH in healthy, young human subjects and the sympathetic responses using microneurographic recordings of MSNA. Furthermore, the mechanistic underpinnings of hyper-acute IH-mediated sympathoexcitation are explored by testing the hypotheses that: (i) a CNS-permeable antioxidant and (ii) a ATR1a receptor blocker can abrogate the MSNA response to hyper-acute IH, implicating a role for ROS and Ang II in IH pathophysiology. To test the first hypothesis, human subjects ingested the anti-oxidant, N-Acetylcysteine (N-AC), and were exposed to hyper-acute IH, and had their MSNA and arterial pressure responses compared to subjects who ingested a vehicle placebo (independent group design). To test the second hypothesis, human subjects ingested either the ATR1a receptor blocker Losartan or placebo and were exposed to hyper-acute IH. Arterial pressure and MSNA responses were compared via repeated-measures analysis of Losartan vs. placebo treatment.

Before presenting the findings of these studies, a review of the literature is necessary. Firstly, examination of the experimental model of human hyper-acute IH versus human studies of chronic and acute IH will be explored to provide evidence of the validity of the experimental model used in these experiments conducted in this dissertation. Secondly, the molecular mechanisms of IH-mediated sympathoexcitation in rodents will be discussed to provide a basis for exploring the mechanisms of ROS and ATR1a activation via IH in humans.

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LITERATURE REVIEW – PART I

Human Intermittent Hypoxia as a Model for Obstructive Sleep Apnea Mediated Hypertension: Methods and Mechanisms

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Introduction

Obstructive Sleep Apnea (OSA) is characterized by frequent apneas and hypopneas that produce intermittent hypoxia (IH), profound swings in intrathoracic pressure, and frequent arousals from sleep that fragment sleep architecture. Numerous investigations have linked OSA with increased cardiovascular risk, which is imparted mainly through the development of hypertension (18). The etiology of OSA-derived hypertension mostly emanates from IH (22), although other pathophysiological mechanisms (e.g. obesity and other components of the metabolic syndrome) certainly contribute (45). For example, multiple studies have identified that OSA-related cardiovascular disease is dependent on the apnea/hypopnea index (AHI; (50)) and is independent of Body Mass Index (BMI), illustrating a dose-response relationship of IH and development of cardiovascular disease. Furthermore, OSA-dependent cardiovascular risk reverses, in part, with continuous positive airway pressure (CPAP) therapy, which eliminates obstructions to airflow during sleep and presumably abrogates exposure to IH (38). Moreover, rodents exposed to cyclical hypoxia develop a sustained hypertension that persists into waking hours, consistent with what is observed in OSA patients (7). Hence, it appears that IH is an important pathophysiological factor in OSA-mediated hypertension. Early animal studies have identified elevated sympathetic nerve activity (SNA) as the primary etiology of IH-mediated hypertension, as animals without an intact neural (i.e. nerve terminals) and humoral (i.e. adrenal medulla) sympathetic nervous system failed to develop hypertension after chronic IH exposure (22). Extensive animal and human studies have also demonstrated that increased SNA and circulating catecholamines mediate many of the vascular and cardiac responses to IH (40), including

impaired endothelial function, and significant left ventricular remodeling and dysfunction (5), mirroring the pathophysiological manifestations of the clinical condition of OSA.

Hence, exploration into the mechanistic relation of IH to elevated SNA in humans is needed to develop effective, novel adjunctive therapies in OSA-mediated hypertension.

The gold standard treatment for OSA is CPAP; however, CPAP therapy only modestly decreases arterial pressure in OSA patients (13, 14, 31), and sleep clinicians often encounter significant issues with CPAP compliance (19). The development of new adjunctive therapies relies on rigorous experimentation in human models to investigate the mechanisms of IH-mediated increases in SNA and arterial pressure. However, significant disparity exists in the literature in models of IH-conditioning in human subjects with regard to time course (8, 11, 16, 42), use of apneas (23, 35) and control of arterial carbon dioxide (4, 8). The present discussion will review these methods and their physiological consequences with particular emphasis to how they accurately model OSA cardiovascular pathophysiology. Importantly, although human IH studies necessarily rely on mechanistic evidence derived from other mammalian investigations of IH, this review will not discuss methods in animal models in depth; excellent reviews may be found elsewhere (21, 28, 32, 39). Furthermore, this review will focus on the cardiovascular sequelae of IH in humans, particularly related to elevated SNA and arterial pressure, and current knowledge of relevant mechanistic factors. Outstanding reviews on IH-mediated alterations in cerebrovascular homeostasis (10) and respiratory function (27) are to be found elsewhere, as indicated.

Methodological Considerations

Time Course

Perhaps the most disparate methodological distinction in human investigations of IH regards the duration of the IH exposure. Generally, human IH studies have three categories of time course: chronic (1-28 days), acute (1-24 hours) and what we term "hyper-acute," which consists of IH protocols lasting <1 hour. Although there may be additional physiological information that could be obtained by enhancing the temporal resolution between these categories, the current human literature is limited in this regard. Discussion will first focus on chronic IH paradigms, as these most closely mirror what occurs in animal studies and in OSA patients. Consideration will then be given to acute, and finally to the newer designation of "hyper-acute" IH. Furthermore, discussion of the pattern of hypoxic exposure will be offered in the concluding chapter, giving particular reference to the frequency of hypoxia/reoxygenation cycles.

Chronic IH

Chronic IH (1-28 days of IH exposure), which arguably most closely approximates human OSA and most animal models of IH, is rarely used in human studies due to the logistical challenge it poses to experimental design. However, investigations discussed here using chronic IH contribute profoundly to the mechanistic insight of IH-mediated pathophysiology. Foster *et al.*. (9) identified that a cyclical IH protocol of 2 minutes of hypoxia and 2 minutes of normoxia for 6 hours over 4 days elevated baseline arterial pressure, and increased the pressor and

cerebrovascular resistance responses to hypoxia. Moreover, also in humans, Tamisier *et al.* (42) and Gilmartin *et al.* (11) found that 14 and 28 days of IH (consisting of 3 minute exposures), increased arterial pressure as well as MSNA burst frequency and incidence. Pialoux *et al.* (36) also demonstrated that baseline ventilation and ventilatory responses to hypoxia were enhanced with chronic IH in humans (consisting of 2 minute exposures) (11, 36), which mimics rodent models of chronic IH where ventilatory sLTF and augmented ventilatory responses to hypoxia are observed (21). The same investigators (36) also found that humans exposed to 4 days of IH for 6 hours per day elevated plasma markers of oxidative stress (e.g. lipid and DNA oxidation) which is consistent with what is observed in rodent models of chronic IH (20). The long-term IH (4 weeks) utilized by Gilmartin *et al.* (11) also induced endothelial dysfunction as indicated by reduced forearm flow mediated dilation (FMD), in line with a rodent study by Marcus *et al.* (26). Furthermore, Tamisier *et al.* (42) found that their 14 day IH protocol significantly reduced baroreflex gain of SNA, also consistent with CIH studies in rodents which produce decrements in baroreflex sensitivity (48).

Hence, the similarity of these findings illustrate a remarkable resemblance of the physiology of IH in humans and in rodents, giving mutual credence to both of these experimental models as clinically applicable and useful.

Acute IH

Protocols that utilize acute IH (1-24 hours) are more commonly used in humans, although direct measurements of SNA using microneurography are likely still impractical, since these recordings are difficult to obtain and maintain for such long periods of time; for example, Gilmartin *et al.* (11) and Tamisier *et al.* (42) repeated measurements of MSNA in their protocol and did not maintain recordings throughout the entire IH period, consisting of 6 hours.

Foster *et al.* (9) were the first investigators to demonstrate in humans that short-duration intermittent hypoxia (SDIH), consisting of 5 minute episodes of 12% O_2 for 1 hour causes a significant elevation in mean arterial pressure (MAP). This is in contrast to long-duration intermittent hypoxia (LDIH), which consisted of 30 minute episodes of hypoxic exposure, which did not elevate arterial pressure in the same study. In a later investigation, Foster *et al.* demonstrated that a single 6 h exposure of cycles of 90s of hypoxia and normoxia elevated diastolic arterial pressure (8), and Pialoux *et al.* (35) reported an increase in plasma oxidative stress (e.g. DNA and lipid oxidation) in the same protocol. Importantly, Foster and colleagues effectively blocked the hypertensive response to IH by blocking Angiotensin II Type 1a Receptors (ATR1a) with Losartan, thereby identifying the important role of ATR1a in IH-mediated hypertension. Furthermore, Beaudin and coworkers (1) blocked cyclooxygenase-2 (COX-2) using Celecoxib and attenuated the hypertensive response to IH, thereby identifying a role for inflammatory processes via prostaglandins in this response as well. Similarly, Polotsky *et al.* (37) demonstrated that 5 hours of IH, with approximately 21 events per hour, produces a two-fold increase in Toll-Like Receptor-2 (TLR2, which binds an array of microbial and

endogenous antigens) gene expression in human peripheral blood mononuclear cells, further emphasizing the importance of inflammatory processes in the physiological response to IH. With regard to ventilation, Beaudin *et al.* (1) found that a single 6 h exposure of IH was sufficient to increase resting ventilation, as well as the ventilatory response to hypoxia and hypercapnia. Hence, it appears that shorter exposures of IH also induce LTF and other adaptations to the ventilatory peripheral chemoreflex as demonstrated with more chronic IH protocols. Recently, Tremblay *et al.* (44) exposed subjects to 6 hours of IH and measured vascular strain (a measure of arterial stiffness) and carotid-baroreflex (CBR) function using a variable-pressure neck chamber, and demonstrated that IH: (a) reduced vascular strain (and hence increased arterial stiffness) in the carotid and the femoral artery; and (b) shifted the CBR curve of HR and BP upwards and rightwards while blunting the leg vascular conductance (LVC) response to neck suction, analogous to a hypertensive stimulus. These investigators found that acute IH did not affect brachial artery endothelial function using FMD or shear rate; however, they identified a reduction in femoral artery antegrade shear rate compared to the sham condition (44). This regional change in vascular function in response to acute IH could be attributed to lower-limb specific increases in α-receptor density or enhancement of sensitivity (44), emphasizing the important role of elevated SNA in responses to acute IH.

Collectively, these findings indicate that acute IH causes an elevation in MAP (8). This hypertensive response is mediated, in part, by ATR1a that produce increases in plasma oxidative stress (8, 35) as well as, by inflammatory processes that are modulated through COX-2 inhibition (1). Furthermore, increases in resting ventilation and the ventilatory response to hypoxia and

hypercapnia are observed with acute IH (36). These findings are similar to what is observed in rodent models of chronic IH, with regards to elevation of ROS (20), activation of ATR1a (25), and induction of inflammatory processes (15). Most importantly, acute IH recapitulates the hypertensive response observed with chronic IH in healthy humans (11, 42) rodents (7), and OSA patients (33). Furthermore, studies of acute IH in humans demonstrate similar findings with regard to vascular and carotid baroreflex function (44) as chronic IH studies in rodents (26, 48). Hence, the acute IH model provides a more convenient experimental design for testing IH-mediated pathophysiology in human subjects compared with the longer duration chronic models.

Hyper-Acute IH

The paradigm of hyper-acute IH (i.e. <1 hour) is best suited for human studies involving MSNA measurements, as these recordings are difficult to obtain and to maintain for periods beyond 2-3 hours. Xie *et al.* (47) first demonstrated that 20 minutes of intermittent 20 second exposures of hypoxic hypercapnia (i.e. asphyxia) per minute causes a persistent increase in MSNA burst activity that lasted for approximately 25 minutes after the exposure. Interestingly, unlike the MSNA response, the transient hyperventilatory response quickly returned to baseline after intermittent asphyxia (47), which also was observed by Leuenberger and colleagues using a similar protocol (23). This stands at odds to what is observed in rodents (21) and humans (46) in which acute and chronic IH consistently elevates resting ventilation and ventilatory responses to hypoxia and hypercapnia in the post-exposure period as aforementioned. However, hyper-acute

IH paradigms that lengthen the hypoxic exposures to 4 minutes and use free-breathing methods (versus apneas) show progressive augmentation and LTF of ventilation (27), indicating that the dose of 20 seconds of hypoxia per minute is not sufficient to induce long-lasting increases in ventilation. Similar to Xie *et al.*, Cutler and co-workers (4) found that 20 seconds of IH per minute for 20 minutes caused a long-lasting elevation in MSNA. However, while Xie and coworkers (47) only observed a transient increase in systolic arterial pressure with their intermittent asphyxia protocol, Cutler *et al.* (3, 4) and Leuenberger *et al.* (23) using an IH paradigm that included apneas (hence, an intermittent asphyxia with periodic cessations of lung inflation), demonstrated that short 20-30 minute IH exposures cause a transient increase in MAP. This finding was replicated more recently in a similar study in humans (16) which found that increases in MAP were mostly driven by elevations in systolic and not diastolic pressure. These mixed data representing elevations in arterial pressure after IH are at odds with the findings of human and animal investigations of acute and chronic IH, where elevations in arterial pressure are more consistent (6, 11, 42)

Collectively, these findings indicate that very short bouts of IH cause a long-lasting increase in MSNA and, variably, arterial pressure without producing elevations in ventilation. The persistent elevation in MSNA is possibly caused by a LTF-like effect of chemoreflex control of MSNA, similar to that observed after chronic IH in humans (11). Interestingly, induction of carotid body sLTF after hyper-acute IH is not likely as most human paradigms of hyper-acute IH do not elevate minute ventilation into the post-IH recovery period (23, 47). The absence of post-hypoxia hyperventilation indicates that a central nervous system mechanism is most likely responsible for

the persistent elevation in MSNA; if the carotid body were involved, ventilation should be elevated in tandem with MSNA after hyper-acute IH. Indeed, Peng *et al.* demonstrated that at least 3 days of IH are necessary to induce sLTF of carotid body neural activity in rodents (34), and Yamamoto *et al.* found that intermittent optogenetic stimulation of rodent nucleus tractus solitarius (NTS) neurons alone produced similar increases in renal SNA as hyper-acute IH (49).

Apnea vs. Free-Breathing

IH protocols in humans differ with regard to the use of apneas versus freely breathing paradigms in which the F_iO_2 is adjusted to achieve the desired hypoxic target. This dichotomy is particularly true for the shorter duration protocols that we describe as hyper-acute IH. One would assume that employing apneas in IH experimental models produces a greater sympathoexcitatory effect, insofar as lung inflation, hypoxia and hypercapnia synergize to increase SNA (17). Furthermore OSA is a disorder characterized by obstructive apneas, and it would seem that an IH paradigm using apneas would more similarly approximate OSA pathophysiology. However, it appears that the use of hypoxic apneas versus freely breathing IH paradigms not significantly affect the sympathetic dysregulation and the hypertension observed. For example, Leuenberger *et al.* (23, 29) demonstrated that repetitive room-air apneas do not elevate MSNA in the same fashion as hyper-acute IH with apneas. Furthermore, Cutler *et al.* (3, 4) identified that hyperacute IH with and without hypercapnia produced similar elevations in MSNA and arterial pressure as with hyperacute IH with apneas. Hence, the chemoreflex dysregulation imparted via

IH is apparently a function exclusively of the hypoxia, which further supports the validity of the animal and human IH models as a model of OSA.

Isocapnic vs. Hypercapnic Intermittent Hypoxia

A controversial point regarding IH protocols is the question of controlling for fluctuations in arterial and end-tidal CO_2. For example, investigators of acute IH in humans often carefully control the delivery of IH to ensure isocapnia (8, 9). Indeed, our laboratory (17), as well as others (30, 41), have demonstrated the important synergism of hypoxia and hypercapnia in elevating MSNA and arterial pressure. However, Cutler *et al.* (3, 4) demonstrated that hyper-acute IH with hypercapnia produced similar elevations in MSNA and arterial pressure as isocapnic hyper-acute IH. Moreover, animal studies also demonstrate the lack of an added hypertensive effect with the addition of hypercapnia to chronic IH protocols (22). Controlling end-tidal CO_2 may be necessary for studies assessing the effect of IH on cerebrovascular homeostasis (10), although it can be argued that true OSA is intermittent hypercapnic hypoxia; thus, using this approach would be a more clinically relevant paradigm. While hypoxia produces a hyperventilatory response that will, in turn, produce hypocapnia, no human investigations to date have tested the hypothesis that intermittent *hypocapnic* hypoxia produces a *decrease* in either MSNA or arterial pressure versus isocapnic or hypercapnic IH. However, we have demonstrated that the MSNA response to hypocapnic hypoxia is not different from isocapnic hypoxia in humans (17), indicating that inhibitory modulation of chemoreflex control of MSNA does not extend to

hypocapnia. These findings decrease enthusiasm for the idea of hypocapnic IH as an inhibitory MSNA and pressor stimulus.

Summary

Chronic, acute, and (mostly) hyper-acute IH protocols increase arterial pressure and MSNA. These findings recapitulate findings in rodent models of chronic IH and OSA patients. Although acute and chronic IH produce changes to resting ventilation and in the ventilatory response to hypoxia and hypercapnia, similar responses in ventilation are not observed with hyper-acute IH. The lack of a persistent ventilatory response to hyperacute IH is most likely because longer IH exposures are required to induce sLTF of the carotid body or in central ventilatory control, such that the characteristic respiratory plasticity of IH can be manifested (27). In contrast, LTF of chemoreflex control of MSNA or a similar phenomenon is prevalent after hyper-acute IH, implicating a potential central nervous system mechanism for the elevations in SNA and arterial pressure observed with these paradigms. Futhermore, chronic IH paradigms in humans have been shown to blunt baroreflex sensitivity (43), whereas hyper-acute and acute IH only resets the CBR operating point pressure (23, 44), a response remarkably similar to that seen in rodents (48). Recent human investigations have demonstrated IH-mediated vascular impairments, as acute IH produces significant alterations in lower-limb hemodynamics (44). Mechanistically, acute IH produces increases in oxidative stress (35), and increases arterial pressure through an ATR1a

mechanism (8), insofar as Losartan blocks the hypertensive response observed after acute IH. Table 1 and Figure 1 summarize these findings.

Tables

Table 1: Table 1: Review of Physiological Effects of IH Time Course in Human Subjects

Physiological Variable	**Hyper-Acute** <1 hour	**Acute** 1 hour-24 hours	**Chronic** 24 hours-28 days
Sustained Arterial Pressure Elevation	• Xie *et al.* (47): no increase • Increases only in SAP and MAP but not DAP (16) • Transient increases in SAP but sustained increases in DAP (3, 4) • Sustained increase in all arterial pressures (SAP, DAP, MAP) (29)	• Consistently demonstrate an increase in MAP (8) • Increases pulse pressure (44)	• Increases daytime arterial pressure (11, 42, 43)
Ventilation and Ventilatory Responses to Hypoxia and Hypercapnia	• No changes in baseline ventilation and in ventilatory responses to hypoxia (12, 23)	• Consistently increases baseline ventilation and ventilatory responses (2, 36)	• Increases hypoxic and hypercapnic ventilatory response gain (42)
Oxidative/Inflammatory Stress	• No increases in superoxide measured via electron paramagnetic spectroscopy (EPR) (16)	• Consistently increases markers of oxidative stress (35, 36) • Increases TLR gene expression within immune cells (37)	• Inflammatory markers were not changed (43)
Acute and Sustained MSNA Burst Frequency, Incidence and Total Activity	• Consistently increased without regard for use of apneas or for controlling end-tidal CO_2 (3, 4, 16, 23, 24, 29, 47)	• No data available	• Increased after 14 (43) and 28 (11) nights of IH
Endothelial and Vascular Function	• No data available	• No change in upper-limb endothelial function (44) • Increased carotid and femoral stiffness (44) • Altered shear rate of femoral artery (44)	• Increase forearm vascular resistance (11) • Decreased forearm blood flow response to ischemia (11)
Carotid-Baroreflex Function	• Resets operating point pressure without changing gain (29)	• Resets OP pressure without changing gain, but reduced control of leg vascular conductance (44)	• Reduction in baroreflex sensitivity (43)

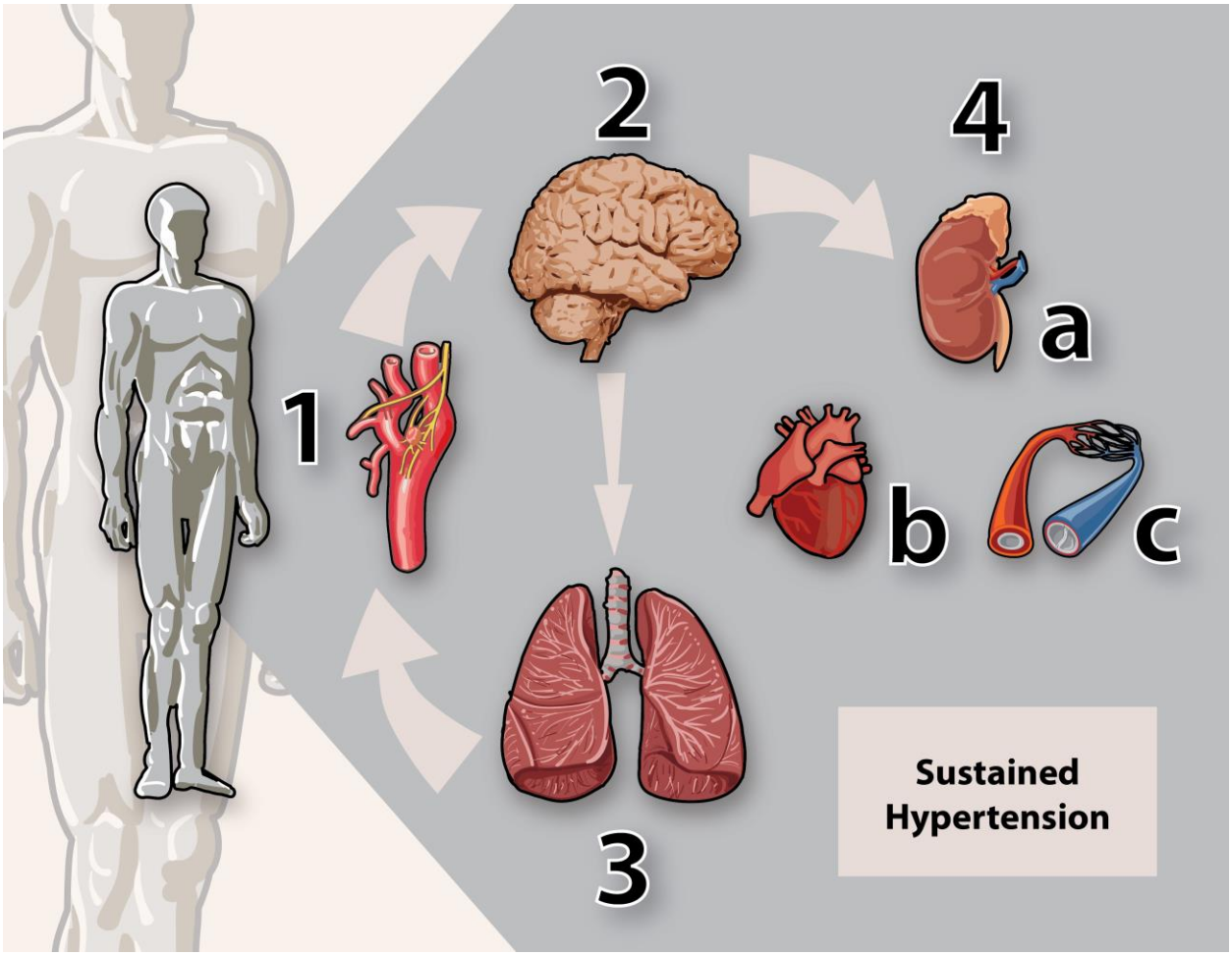

Figure 1: Physiological model for the development of IH-mediated hypertension in the human model. Intermittent Hypoxia (IH) interacts with the peripheral carotid body chemoreceptor (1) to increase its afferent discharge to the medullary cardiovascular control centers (2). This, in turn, alters central control of ventilation (3), e.g. by altering loop gain. Centrally, IH induces increased sympathetic outflow to end organs, such as the kidney (4a), which engages the renin-angiotensin axis, the heart (4b) which alters ventricular filling and cardiac electrical activity, and the blood vessels (4c), which produces a sustained increase in systemic vascular resistance, resulting in sustained hypertension.

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LITERATURE REVIEW – PART II

Human Intermittent Hypoxia as a Model for Obstructive Sleep Apnea Mediated Hypertension: Methods and Mechanisms

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Introduction

When exposed to the intermittent hypoxia (IH) frequently encountered by patients with Obstructive Sleep Apnea (OSA), healthy human and animal subjects consistently develop elevated arterial pressure and increased sympathetic nerve activity (SNA). This hypertensive and sympathoexcitatory response to IH contrasts with other pathophysiological components of OSA, including obesity, metabolic syndrome and frequent arousals, all of which do not independently increase arterial pressure or baseline SNA to the same extent as observed with OSA patients. Over the last 15 years, investigations, primarily in rodent models, have identified important adaptations within the carotid body chemoreceptors, the central controllers of SNA, baroreflexes, and the vascular endothelium. Many of these adaptations are mediated by production of reactive oxygen species (ROS) and activation of ATR1a. Testing the hypotheses that antioxidants and angiotensin receptor blockers (ARBs) can reduce sympathoexcitatory and arterial pressure responses to IH in human subjects is paramount to this dissertation. However, a thorough review of the mechanistic basis and rationale for employing blockade of the biochemical mediators, primarily derived from animal models, is warranted.

Mechanisms of IH Mediated Sympathoexcitation and Hypertension in Animal Models

IH Produces Functional Changes in the Carotid Body Chemoreceptor

The carotid body chemoreceptors play a central role in the hypertensive response to chronic IH. Indeed, animals with carotid body denervation do not develop hypertension with exposure to IH

(4, 18), and medullary nuclei that exhibit synaptic connections to the chemoreflex arc undergo profound IH-mediated alterations in neuronal excitability and firing (20, 31). In particular, two important adaptations occur at the carotid body chemoreceptor in response to IH that deserve attention. Firstly, upon exposure to IH, the carotid body increases its excitability in response to varying degrees of hypoxia (25). Several investigations have demonstrated that this process is mediated by ROS (25) and by endothelin-1 (25, 34), as blockade of these biochemical mediators attenuates this IH-mediated carotid body hypersensitivity. This adaptation has profound clinical significance, as OSA patients exhibit exaggerated increases in MSNA to modest quantities of hypoxic stresses normally encountered in everyday life (12, 24), which certainly increases their cardiovascular risk. Secondly, IH induces sensory long-term facilitation (sLTF) of the carotid body (26), which is characterized by continued neuronal activity in the absence of stimulus. Peng *et al.* (26) identified that angiotensin II and ROS mediate this adaptation, as the ARB Losartan reduces in parallel the sLTF response to IH and attenuates the oxidative stress at the carotid body. IH-induced sLTF has important clinical ramifications, as OSA patients have an increased muscle SNA (MSNA) burst frequency during waking hours despite normal P_aO_2 (22). Hence, continued firing of the carotid body in the absence of stimulus may, in part, drive this day-time increase in MSNA. This hypothesis is supported by the fact that OSA patients exhibit large decreases in MSNA in response to hyperoxia, which presumably silences carotid body signaling, whereas normal control subjects do not exhibit such a decrease (23). This indicates that tonic chemoreflex hyperactivity may contribute to the daytime increases in MSNA in OSA.

IH Alters Central Sympathetic Regulation and Outflow

Rodents exposed to IH also exhibit profound changes in sites of central sympathetic regulation. Indeed, the induction of carotid body sLTF and chemoreflex hypersensitivity cannot explain the hypertension caused by chronic IH alone (34), as important adaptations within these central sites of sympathetic control are critically involved. For example, even very short term IH (i.e. <1 hour) has been shown to bias neurons within the nucleus of the solitary tract (NTS) towards increased discharge (36). Particularly intriguing is the role of the rodent forebrain-hindbrain interactions mediated by IH induction of plasma angiotensin II (20, 31). Longer term IH in rodents increases renal sympathetic outflow which initiates the renin-angiotensin axis. Plasma angiotensin II then interacts with the forebrain lamina terminalis to increase excitatory input into paraventricular nucleus (PVN) of the hypothalamus, which sends projections to NTS neurons to elevate central sympathetic outflow (20, 31). Furthermore, angiotensin II released via IH also interacts with nuclei within the circumventricular organs (CVO), such as the subfornical organ (SFO), to further enhance central sympathetic outflow and elevate arterial pressure (31). Hence, IH induces a positive feedback mechanism, by which initial increases in SNA cause further increases in SNA via plasma angiotensin II interacting with CVOs (20). Importantly, in addition to angiotensin II, ROS are formed throughout the central chemoreflex arc, including the NTS and the rostral ventrolateral medulla (RVLM) in response to chronic IH (28, 29). Human (13) and animal (15, 29) investigations have demonstrated that centrally-active antioxidants have the ability to blunt the increases in SNA observed after IH. Furthermore, OSA patients have an

increased oxidative internal mileu (9). Hence, centrally active ARBs and antioxidants exhibit promising clinical potential to ameliorate sympathetic dysfunction in OSA patients.

Progressive Decrements in Baroreflex Function with Increasing IH Exposure

Rodent models of IH have demonstrated a progressive impairment of the baroreflex impairment as IH exposure becomes more chronic. For example, rats exposed to chronic IH for 5-17 days do not develop decrements in the baroreflex sensitivity, despite an increased resting arterial pressure (38). Yamamoto *et al.* (35) demonstrated that this phenomenon is mediated by a resetting of the operating point of the arterial pressure/renal SNA baroreflex, without a change in baroreflex sensitivity. However, as IH becomes more chronic, several animal investigations show that the gain of baroreflex control of heart rate (HR) becomes blunted (17), possibly through a central mechanism (8). The long-term changes in baroreflex sensitivity mirror what occurs in OSA patients, who display profound decrements in baroreflex control of arterial pressure and sympathetic nerve activity (21). Interestingly, the baroreflex dysfunction observed in OSA appears to be ameliorated with surgical (10) and CPAP (27, 32) treatment, indicating that this process is possibly reversible, perhaps because of the reduced competing input of the peripheral chemoreceptors (1).

IH Induces Vascular and Endothelial Dysfunction

Animal models clearly demonstrate the detrimental effect of IH on endothelial and vascular function (37). Indeed, chronic IH has been shown to reduce endothelial-dependent dilation (2). This process is most likely mediated through IH-dependent increases inROS and oxidative stress in general via ATR1a activation (19), as well as increases in inflammatory mediators and cell infiltration (11). The IH-mediated activation of ATR1a and subsequent generation of oxidative stress cause the scavenging of nitric oxide (NO) and alters the activities of NO synthase isoform (14). These animal studies recapitulate the findings of endothelial dysfunction observed in OSA patients (3, 33), and constitute a very clinically relevant research direction, as endothelial dysfunction is a primary cardiovascular risk factor and independent predictor of cardiovascular morbidity and mortality (30).

Summary

Early rodent studies identified that chronic IH exposure causes persistent elevations in arterial pressure (6). Importantly, Lesske *et al.* (18) and Fletcher *et al.* (5) demonstrated the critical role of the sympathetic nervous system, as animals with neuro-humoral sympathectomies did not develop hypertension after chronic IH exposure (18). Furthermore, the hypertensive response appears to be mediated, in part, by the peripheral, oxygen-sensitive carotid body chemoreceptor (e.g. the phenomena of sLTF and carotid body hypersensitivity mediated by angiontensin II, ROS and endothelin-1), as rodents with carotid body denervation similarly do not develop

increases in arterial pressure with chronic IH (16, 18). Moreover, central controllers of SNA, including the NTS and RVLM as well as CVOs, contribute to IH mediated hypertension via a ROS and angiotensin II linked mechanism (31, 34). The maladaptations of the chemoreflex arc also extend to baroreflex control of arterial pressure, as the sensitivity of the baroreflex is substantially reduced with chronic IH (7). Finally, IH also causes vascular and endothelial dysfunction in animal models (19). Figure 1 summarizes the experimental findings in animal studies.

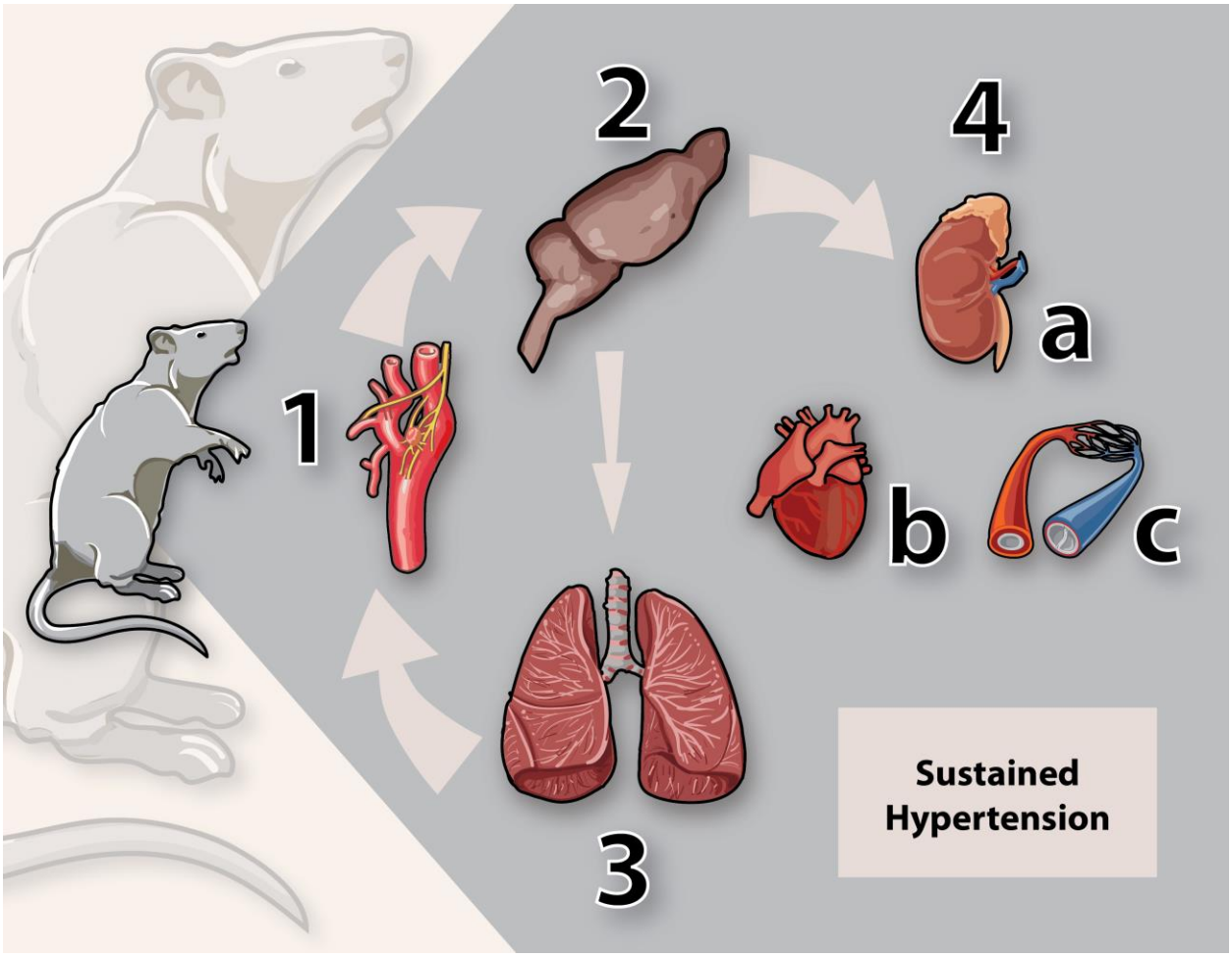

Figure 1: Pathophysiology of intermittent hypoxic conditioning as described in rodent models. Intermittent Hypoxia (IH), much like the human model, produces increases in carotid body afferent discharge (1), which enhances central sympathetic efferent transmission (2) and alteration of central control of respiration (3). Similar to humans, rodent CIH produces central sympathetic activation which affects end-organs as described in the previous chapter.

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OVERALL HYPOTHESIS AND SPECIFIC AIMS:

Overall Hypothesis: That reduction of ROS via oral ingestion of an anti-oxidant (N-Acetylcysteine) and blockade of ATR1a via oral ingestion of an ATR1a antagonist (Losartan) will abrogate the sympathetic and arterial pressure responses to hyper-acute IH.

Specific Aim I

To demonstrate that reducing ROS via oral ingestion of an anti-oxidant (N-Acetylcysteine) will attenuate the sympathetic activation and elevation in arterial pressure observed during hyper-acute IH.

Specific Hypothesis I

That the anti-oxidant N-acetylcysteine will reduce MSNA burst frequency and incidence as well as arterial pressure during hyper-acute IH in human subjects.

Specific Aim II

To demonstrate that oral ingestion of an ATR1a antagonist (Losartan) will reduce the acute and sustained sympathetic activation and elevation in arterial pressure observed during hyper-acute IH.

Specific Hypothesis II

That ATR1a antagonist Losartan will reduce the acute and sustained elevation in MSNA burst frequency and incidence as well as arterial pressure during and after hyper-acute IH in human subjects.

CHAPTER II

The Anti-Oxidant N-Acetyl Cysteine Reduces Hyper-Acute Intermittent Hypoxia Induced Sympathoexcitation in Human Subjects

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Abstract

This investigation tested the hypotheses that N-Acetyl Cysteine (N-AC) attenuates hyper-acute intermittent hypoxia induced sympathoexcitation without elevating superoxide measured in peripheral venous blood. Twenty-eight healthy human subjects were recruited to the study. One hour prior to experimentation, each subject randomly ingested either 70 $mg \cdot kg^{-1}$ of N-Acetyl Cysteine (N-AC, n =16) or vehicle placebo (n =12). Three lead electrocardiogram (ECG) and arterial blood pressure (ABP), muscle sympathetic nerve activity (MSNA, n = 17) and whole blood superoxide concentration using electron paramagnetic resonance (EPR, n =12) spectroscopy were measured. Subjects underwent a 20 minute hyper-acute intermittent hypoxia (hyper-acute IH) protocol consisting of cyclical end-expiratory apneas with 100% N_2. N-AC decreased MSNA after hyper-acute IH compared to placebo ($P < 0.02$). However, N-AC did not alter superoxide concentrations compared to placebo ($P > 0.05$) in venous blood. Moreover, hyper-acute IH did not increase superoxide concentrations in the peripheral circulation as measured by EPR ($P > 0.05$). Based on these findings, we contend that hyper-acute IH and the actions of N-AC in hyper-acute IH are primarily centrally vs. peripherally mediated, although central measurements of ROS are difficult to obtain in human subjects thus making this assertion difficult to verify. This investigation suggests the possibility of developing a pharmaceutical therapy for inhibiting the sympathoexcitation associated with OSA.

Introduction:

Obstructive Sleep Apnea (OSA) is associated with a significant increase in cardiovascular morbidity and mortality due to intermittent hypoxia (IH) induced sympathetic activation (16, 33). Animal studies have elucidated the causal role of reactive oxygen species (ROS) in this phenomenon. The findings of these studies demonstrate that ROS generated at the carotid body, in the nucleus of the solitary tract (NTS) and within the rostral ventral lateral medulla (RVLM) in response to chronic intermittent hypoxia (CIH; i.e. 1-14 days of IH exposure) elicits increases in sympathetic nerve activity (SNA) and hypertension (23, 25, 26). Further, it has been reported that antioxidant administration substantially reduces the efflux of catecholamines from *ex-vivo* adrenal medullae in rats conditioned to CIH (12). In addition, CIH has been found to reduce the activities of critical antioxidant enzymes in the carotid body, the NTS and RVLM (23, 25, 26). Human (4, 27) and animal (17) studies have demonstrated that oxidative stress is also elevated after acute intermittent hypoxia (AIH; i.e. 4-24 hours of IH exposure), and plays a crucial role in the blood pressure (BP) response to AIH through activation of Angiotensin II Type Ia (AT1a) receptors. It is also known that hyper- acute IH (hAIH; i.e. <1 hour of IH exposure) alters chemoreflex control of SNA and arterial pressure in human subjects (1, 14, 21); yet, the extent of the elevation in systemic ROS and the causal relationship of ROS to elevated SNA are poorly understood in human subjects exposed to hyper-acute IH. Animal investigations have reported that 20 minutes to 1 hour of IH produces sensory long-term facilitation (sLTF) of ventilation and enhances neural transmission to central cardiovascular control centers via a superoxide dependent mechanism (5, 15). Furthermore, Yamamoto *et al.* (38) demonstrated that activating NTS neurons hyper-acutely

and intermittently via optogenetic mechanisms produces similar increases in renal SNA compared to hAIH. These findings indicate that hypoxia, chemoreceptor stimulation or peripherally generated oxidative stress is not necessary to produce the sympathetic dysregulation observed with hAIH. Indeed, at least 3 days of IH are required to induce carotid body sensory plasticity and hAIH stimulation is not sufficient to elicit changes in carotid body sensory activity (28). These investigations suggest that hAIH mediated increases in sympathetic nerve discharge can be reduced with an antioxidant such as N-Acetyl Cysteine (N-AC) (6-9), without altering systemic ROS. The present investigation tested the hypothesis that N-AC, which interacts within the CNS (e.g. NTS and RVLM) tissues, will reduce the sympathoexcitation associated with hyper-acute intermittent hypoxia, while not altering circulating superoxide concentrations measured in the peripheral venous circulation.

Methods:

Ethical Approval

The research described herein was approved by the University of North Texas Health Science Center Institutional Review Board (IRB #2011-079). Written informed consent was obtained from all participants in this study in accordance with the Declaration of Helsinki.

Subjects:

Twenty-eight human subjects (11 females, 17 males) participated in the protocol. Female subjects were tested during the early follicular phase (days 1-4 post menses) of their menstrual cycle. All subjects completed a medical history questionnaire and physical examination prior to experimentation and were free of cardiovascular, metabolic or respiratory diseases including hypertension. Two subjects were excluded due to mild hypertension and a high pre-test probability of OSA present at the time of experimentation. Both subjects were advised to consult with their primary care physician. Subjects were randomly assigned into placebo (n =11) or N-AC (n =15) groups prior to experimentation. Out of these 26 subjects, a reliable muscle sympathetic nerve activity (MSNA) recording was obtained on 17 (N-AC n = 10, Placebo n =7), and blood samples were successfully obtained on 13 (N-AC n = 6, Placebo n = 7). Hemodynamic data were recorded and analysed for all 26 subjects

Cardiovascular and Hemodynamic Measurements:

We measured MSNA with standard microneurographic techniques (n = 17) as described elsewhere (31, 32). Briefly, a sterile tungsten microelectrode was inserted into a fascicle of the peroneal nerve near the fibular head. The nerve signals were amplified, filtered (700 to 2,000Hz), rectified and discriminated. The nerve signals were then integrated (time constant = 0.1s) to produce a mean voltage display for quantitative analysis (10, 37). Muscle sympathetic neural

bursts were readily recognized by their tight temporal relationship to the cardiac cycle, their increasing frequency during Valsalva maneuvers and their failure to respond to arousal stimuli or stroking of the skin (10, 37).

A three-lead electrocardiogram (ECG) was obtained (Hewlett-Packard, Inc.) along with beat-to-beat photoplethsymographic measurements of arterial pressure (Finometer, Finapres, The Netherlands). Pulse-oximetry (Nellcor, Inc.) was performed to measure the nadir of O_2 saturation while ventilation was measured with a Ventilation Measurement Module (VMM, Laguna Hills, CA). All data were digitally recorded in data acquisition software (WinDaq, Dataq, Akron, OH) and stored offline for later analysis.

Measurements of Superoxide in Peripheral Venous Blood:

Superoxide concentrations were measured experimentally (n = 13) via electron paramagnetic resonance (EPR) spectroscopy. An intravenous catheter was inserted into an antecubital fossa vein to withdraw 5 mL samples of venous blood. 200 μL of whole blood samples for each time point (pre-ingestion, post-ingestion and hyper-acute IH, see '*Experimental Protocol'* below) were incubated in a buffer solution: 3.5 mM deferoxamine methanesulfonate salt (DF), 9.08 mM of diethyldithiocarbamic acid sodium (DETC) and Krebs-HEPES buffer (Noxygen Science Transfer & Diagnostics GmbH, Elzach, Germany) containing the superoxide-sensitive methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH) spin probe at 37^{o}C for 15 min (3, 35). 50 μl samples of

whole blood for each time point in duplicate were then loaded into a 1-ml syringe and flash frozen using liquid nitrogen between buffer solutions to form a continuous frozen plug. Samples were then stored at −80°C and shipped to the University of Nebraska Medical Center's EPR Spectroscopy Core for analysis (3, 35). EPR amplitude was measured using a Bruker e-Scan EPR Spectrometer and was averaged for all duplicate data to generate an individual subject's average for each time point. One subject (placebo) was excluded due to unequal variation among duplicate samples between time points (Levene's test of equal variance $P = 0.004$, final EPR n=12).

Experimental Protocol:

After instrumentation, baseline MSNA and arterial blood pressure data were obtained for a 5 min period (pre-ingestion, Figure 1). After the baseline period, a 5 ml blood sample was withdrawn. Subjects then ingested either 70 $mg.kg^{-1}$ of N-AC (PharmaNAC, BioAdvantex Pharma, Mississauga, Ontario, Canada) dissolved in a fruit-tasting drink as a vehicle or vehicle placebo (same volume of fruit-tasting drink). After a 1 hour ingestion period to reach peak N-AC plasma concentrations (2), another 5 min baseline period (post-ingestion) commenced where experimental data were recorded and another blood sample was obtained. Subjects then underwent a 20 min hyper-acute IH protocol (Figure 2 illustrates 1 minute of the 20 minute protocol). Subjects respired 2-3 breaths at normal tidal volume of 100% N_2 prepared in a Douglas bag, and then initiated a 20-second end-expiratory apnea. Subjects were allowed to recover on

room-air for 40 seconds, at which point the N_2 breathing and apnea cycle was repeated and continued for 20 minutes. Desaturations below 90% were targeted for each apnea. The apneic frequency of this protocol approximates severe OSA (AHI ~ 60) for 20 minutes, which is sufficient to elicit significant alterations of sympathetic regulation in human subjects (1). A final 5 ml venous blood sample was obtained immediately after hyper-acute IH to capture the ROS, particularly superoxide, in circulating venous blood post-intermittent hypoxia.

Data Analysis:

Data were sampled at 500Hz and recorded directly to a computer with data acquisition software (WINDAQ, Dataq Instruments, Akron, OH). Data were then analyzed using a commercially available biomedical analysis software program (WinCPRS, Absolute Aliens, Turku, Finland). R-waves generated from the ECG were detected and marked at their occurrence in time while diastolic and systolic pressures were marked from the Finometer for arterial blood pressure (20). MSNA bursts were identified by a single investigator and average MSNA burst frequency (bursts/min) and burst incidence (bursts/100 heart beats) were calculated during the entire hyper-acute IH period and the entire baseline periods according to published guidelines (37). Briefly, MSNA bursts were selected by the investigator if they occurred within 1-1.4 s of an R-wave on ECG and they exhibited a 3:1 signal-to-noise ratio (37). Hemodynamic and MSNA variables were averaged over the entire 5 minute period to generate averages for the pre-ingestion and post-ingestion periods. Hemodynamic data during hyper-acute IH were averaged over the last five

minutes of the protocol, since it has been established that hemodynamic variables require at least 15 minutes to increase (1, 14). In contrast to the increase in MAP, MSNA increases almost immediately during hyper-acute IH and remains elevated to a similar extent throughout the hyper-acute IH protocol (1). Hence, MSNA was averaged over the entire 20 minute period to generate an hyper-acute IH average for MSNA burst frequency and incidence.

Statistical Analysis:

All data were analyzed with commercially available statistical software (SigmaPlot, Systat Software Inc., California, USA). Microneurographic and EPR data were compared for each time point using a three (time point: pre-ingestion, post-ingestion or hyper-acute IH)-by two (treatment: N-AC or placebo) ANOVA, which was followed by Student-Newman-Keuls *post-hoc* tests. Hemodynamic data were first compared pre-ingestion and post-ingestion by Student's t-test, where no statistical differences were observed ($P > 0.05$). Subsequently, a two (time point: post-ingestion or hyper-acute IH)-by two (treatment: N-AC or placebo) ANOVA was performed with subsequent Student-Newman-Keuls *post-hoc* tests. Statistical normality was verified using a Kolmogorov-Smirnov test. Significance was set at $\alpha = 0.05$. Results are reported as means ± SEM unless otherwise stated.

Results:

Subject Demographics and Representative Subject:

Subject demographics are provided in Table 1. Placebo control and N-AC subjects did not differ in age, height, weight or body mass index (BMI) (all $P > 0.05$). Furthermore, there were no statistically significant differences in baseline measures of arterial blood pressure (systolic, diastolic or mean) or HR between placebo and N-AC subjects ($P > 0.05$, Table 1). One minute of experimental recordings from a representative subject ingesting N-AC for each of the time points are provided in Figure 2. The hyper-acute IH protocol with apnea elicited increases in MSNA as described previously (32) and caused substantial (11%-30%) oxyhemoglobin desaturation.

Hemodynamic Changes During hyper-acute IH and the effect of N-AC on Hemodynamics during hyper-acute IH:

Hemodynamic data during the pre-ingestion, post-ingestion and hyper-acute IH periods are provided in Table 2. Hemodynamic variables were not statistically different between pre-ingestion and post-ingestion, hence the arterial blood pressure and heart rate averages were compared from post-ingestion to hyper-acute IH (see 'Data Analysis' and 'Statistical Analysis' sections). Systolic blood pressure (SBP) increased significantly during hyper-acute IH compared to post-ingestion (ANOVA main time effect, $P = 0.04$), while mean arterial blood pressure (MAP) trended towards an increase during hyper-acute IH (ANOVA main time effect, $P = 0.07$). Diastolic blood pressure (DBP) was unaffected by hyper-acute IH (ANOVA main time effect, P =

0.17). N-AC significantly reduced SBP and MAP (ANOVA main effect, $P < 0.03$) and subjects ingesting N-AC had lower SBP and MAP during hyper-acute IH compared to placebo control subjects during hyper-acute IH ($P < 0.05$). N-AC did not affect DBP (ANOVA main effect, $P = 0.30$) and no interaction of treatment (e.g. N-AC vs. vehicle placebo control) and time point were observed for any form of arterial pressure or heart rate (ANOVA interaction effect, $P > 0.05$). Subjects ingesting N-AC increased HR significantly during hyper-acute IH compared to post-ingestion ($P = 0.05$), while subjects ingesting the vehicle placebo did not change their HR significantly ($P > 0.05$).

Alterations in MSNA during hyper-acute IH and the effect of N-AC on MSNA during hyper-acute IH:

Hyper-acute IH increased MSNA burst frequency and burst incidence significantly (ANOVA main time effect, $P < 0.001$), while N-AC reduced the MSNA response to hyper-acute IH (ANOVA main treatment effect, $P < 0.047$). MSNA burst frequency did not display significant interaction between treatment (i.e. N-AC vs. vehicle placebo) and time point (ANOVA interaction effect, $P > 0.05$). Subjects ingesting the vehicle placebo significantly increased MSNA burst frequency with hyper-acute IH ($P<0.01$). In contrast, subjects ingesting N-AC did not significantly increase MSNA during hyper-acute IH ($P > 0.05$). Furthermore, MSNA (both burst frequency and incidence) was significantly less after hyper-acute IH in subjects who ingested N-AC compared to subjects who ingested placebo ($P < 0.03$, Figure 3).

Changes in Superoxide Concentrations during hyper-acute IH and N-AC effects:

Two-way ANOVA demonstrated no main effects of hyper-acute IH or N-AC and no interaction effects on superoxide concentrations in whole blood (all ANOVA main and interactions effects $P > 0.05$, Figure 4). Furthermore, neither N-AC nor placebo subjects nor all subjects combined had significant changes in blood concentrations of superoxide in response to hyper-acute IH (one-sample t-test, all $P > 0.05$), indicating that hyper-acute IH did not significantly elevate superoxide concentrations in the peripheral venous circulation.

Tables

Table 1: Anthropometric and Hemodynamic Variables of Study Sample

Group	**Placebo**	**N-AC**	**P-Value**
Age (years)	32 ± 3	31 ± 3	0.54
Sex (M/F)	6/5	9/6	---
Height (cm)	174.2 ± 3.5	174.5 ± 2.4	0.95
Weight (kg)	83.1 ± 5.9	76.5 ± 3.2	0.30
BMI (kg/m^2)	27.2 ± 1.5	25.1 ± 0.80	0.23
SBP (mm Hg)	129.2 ± 5.2	127.8 ± 3.1	0.81
DBP (mm Hg)	74.4 ± 3.0	72.5 ± 1.6	0.54
MAP (mm Hg)	96.6 ± 3.7	93.2 ± 1.9	0.39
HR (beats per minute)	65.6 ± 2.6	65.3 ± 1.4	0.86

Table 2: Hemodynamic Data Throughout the Study

Variable	**Placebo**			**N-AC**		
	Pre Ingestion	*Post Ingestion*	*HYPER-ACUTE IH*	*Pre Ingestion*	*Post Ingestion*	*HYPER-ACUTE IH*
SBP (mm Hg)	133.9 ± 3.2	132.0 ± 2.9	138.9 ± 2.9	129.3 ± 2.8	127.5 ± 2.1	131.5 ± 2.1 †
DBP (mm Hg)	75.5 ± 2.2	73.9 ± 2.2	77.8 ± 2.2	72.5 ± 2.2	73.1 ± 2.1	74.6 ± 1.6
MAP (mm Hg)	99.0 ± 2.8	96.7 ± 2.5	102.3 ± 2.5	93.1 ± 2.4	93.1 ± 1.8	95.8 ± 1.8 †
HR (bpm)	64.1 ± 2.6	68.2 ± 3.3	72.3 ± 3.3	65.4 ± 2.3 *	66.0 ± 2.3 *	72.7 ± 2.3

*: different from HYPER-ACUTE IH within group, P≤0.05

†: different from placebo condition, P≤0.05

Systolic arterial pressure: time point, P=0.04; treatment, P=0.03; time point x treatment, P=0.58

Diastolic arterial pressure: time point, P=0.17; treatment, P=0.30; time point x treatment, P=0.53

Mean arterial pressure: time point, P=0.07; treatment, P=0.03; time point x treatment, P=0.58

Heart rate: time point, P=0.07; treatment, P=0.76; time point x treatment, P=0.65

Figure 1

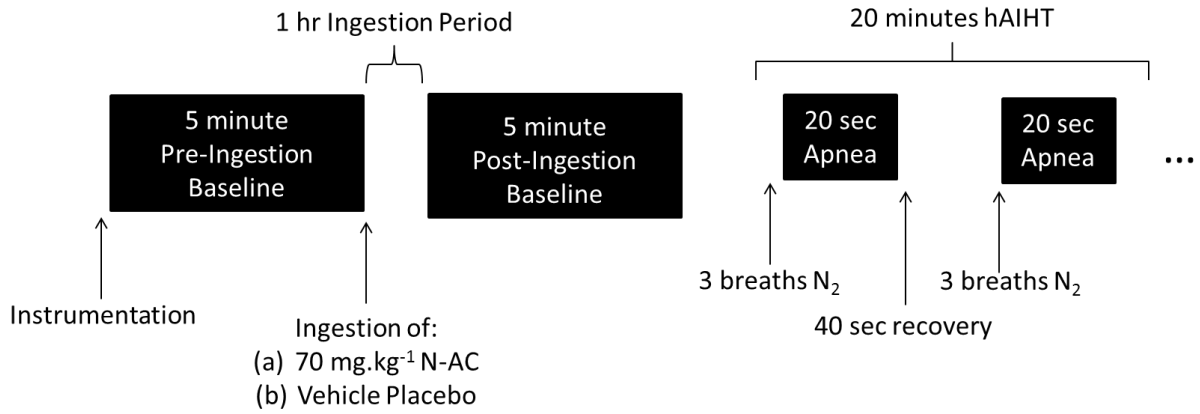


Figure 1: Representation of the experimental protocol over time. Black boxes represent measurement of hemodynamic, microneurographic and EPR data. Experimental data were averaged over the entire 5 minute period for the pre- and post-ingestion periods. MSNA data were averaged over the entire 20 minute hyper-acute IH period, while hemodynamic data were averaged only during the last 5 minutes of the hyper-acute IH period (see 'Data Analysis' for rationale).

Figure 2

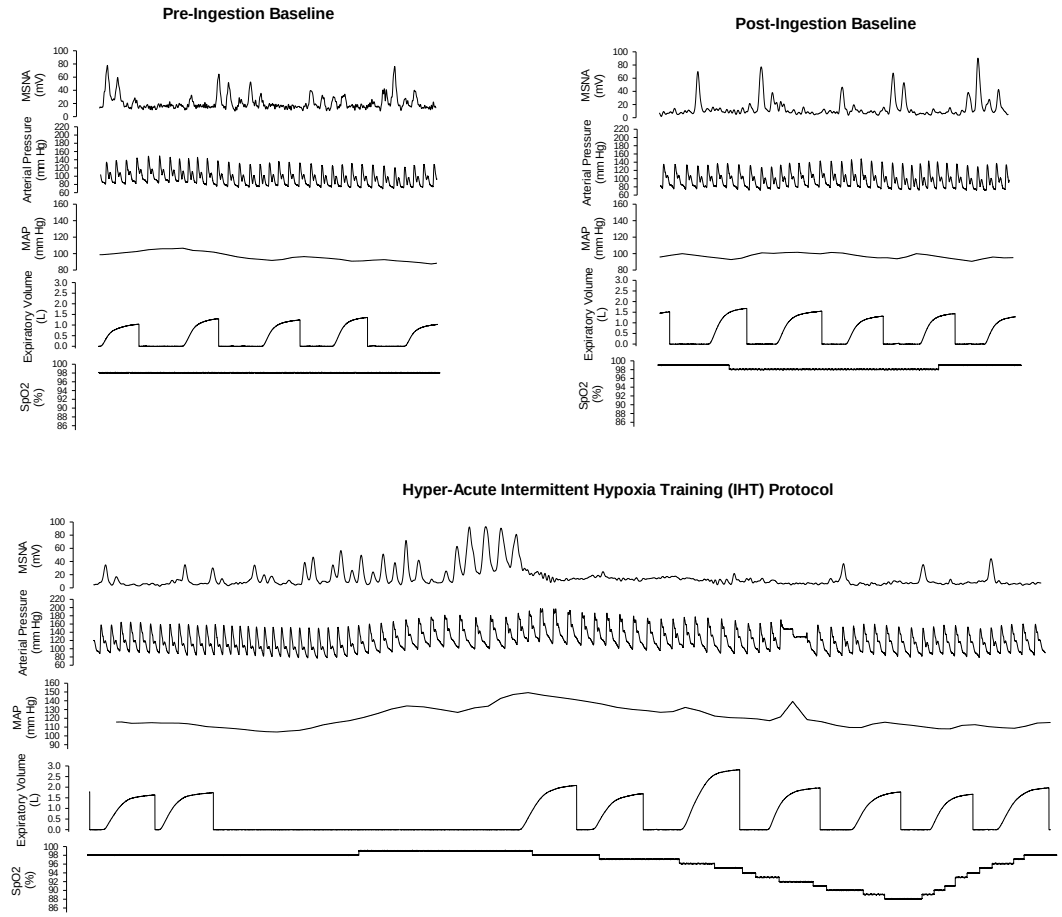


Figure 2: Data from a representative subject ingesting N-AC. Thirty seconds of continuously recorded data from pre-ingestion and post-ingestion baseline periods are provided, while a full cycle (1 minute) of apnea/recovery data are provided in this figure. N-AC did not alter baseline MSNA, however, hyper-acute IH with apnea produced substantial sympathoexcitation. (note: the sudden increase in MAP during the 4th breath after the apnea represents the re-calibration period of the beat-to-beat blood pressure monitor)

Figure 3

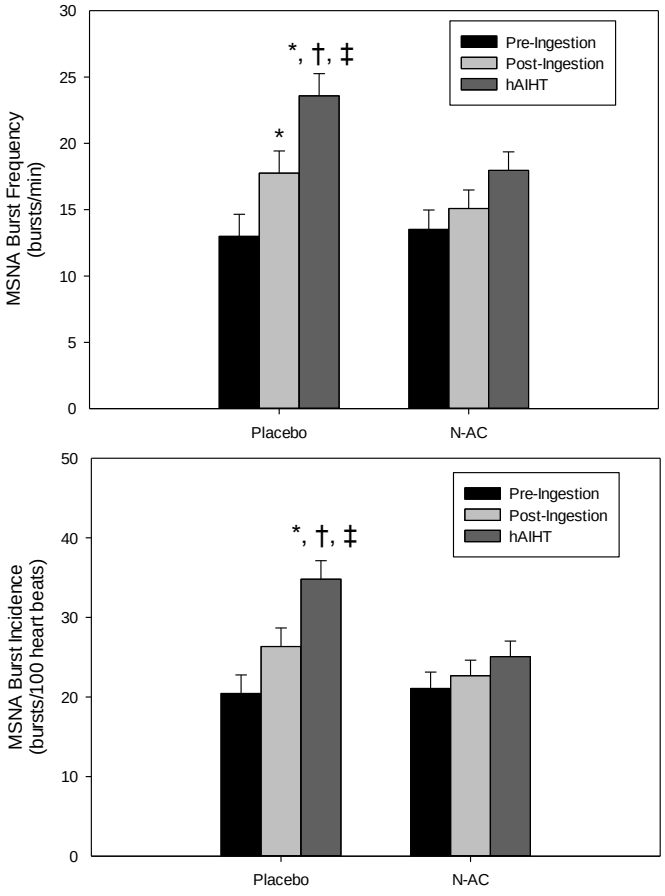
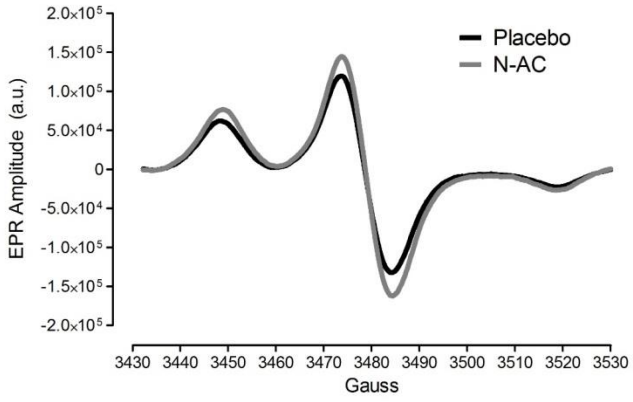


Figure 3: MSNA burst frequency (bursts/minute, top) and incidence (bursts/100 heart beats, bottom) absolute data during the pre-ingestion, post-ingestion and hyper-acute IH time points. *, different from pre-ingestion; †, different from post-ingestion; ‡; different from N-AC corresponding timepoint. N-AC substantially reduced hyper-acute IH sympathoexcitation. MSNA bursts/min: time point, $P<0.001$; treatment, $P=0.047$; time point x treatment, $P=0.15$. MSNA bursts/100 heart beats: time point, $P<0.001$; treatment, $P=0.02$; time point x treatment, $P=0.07$.

Figure 4

A



B

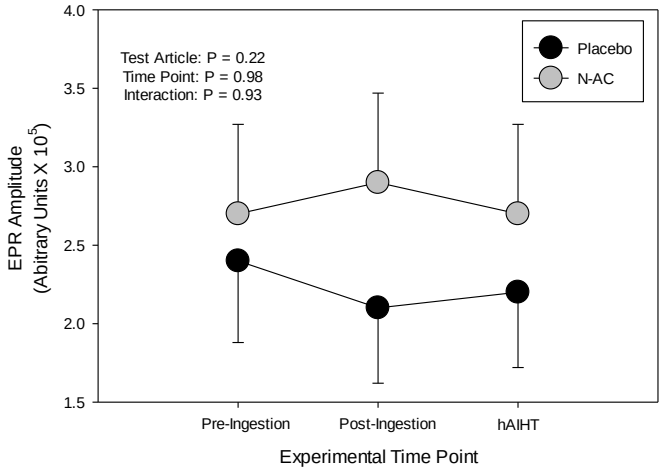


Figure 4: (A) Representative EPR spectra from blood collected from subjects that received either placebo or N-AC. The EPR amplitude (arbitrary units) is directly proportional to the amount of free radicals in the sample. (B) Summary data of EPR amplitude from subjects that received placebo or N-AC at the pre-ingestion, post-ingestion, and hyper-acute IH time-points. ANOVA main effects P values are provided in the top left. Neither hyper-acute IH nor N-AC altered the concentrations of circulating free radicals in peripheral venous blood in the study subjects.

Discussion:

This study demonstrated that N-AC attenuated the MSNA response to hyper-acute IH but did so without altering peripherally circulating superoxide concentrations as measured by EPR spectroscopy. Furthermore, peripherally circulating superoxide concentrations did not increase after hyper-acute IH. Therefore, we contend that hyper-acute IH and the actions of N-AC in hyper-acute IH are likely to be mediated centrally rather than peripherally, although we were limited in identifying across-the-brain effects of the antioxidant, as discussed below.

Hemodynamic Responses to hyper-acute IH

Our hyper-acute IH protocol elevated arterial blood pressure in a manner consistent with that of Cutler *et al.* (1) and Leuenberger *et al.* (14), who utilized a similar hypoxic apnea protocol to the present study. However, arterial blood pressure during hyper-acute IH has been shown to normalize to baseline shortly after the IH exposure while MSNA remains elevated (1). Animal studies utilizing IH exposures indicate that at least 24 hours of IH is required to elicit sustained increases in MAP that persist beyond the IH exposure (18, 19), while human studies suggest that as little as 4-6 hours is required (4). Hence, we postulate that sustained arterial blood pressure elevations between hyperacute versus longer IH protocols are likely due to slow elevations in circulating neurohormones, such as endothelin and catecholamines (11). The increases in HR that we observed only with N-AC during IH are probably baroreflex mediated, as subjects ingesting

N-AC had significantly decreased MAP during IH compared to subjects ingesting vehicle placebo (Table 2).

Sympathetic Responses during hyper-acute IH and the Effect of N-AC:

Which structures in the cardiovascular neural and humoral arc mediate the hyper-acute IH-dependent increases in MSNA? Although human studies do not permit us to achieve such a distinction, an intact carotid body (13, 30) and neural chemoreflex arc (23, 26) are required to mediate the hypertension observed in CIH conditioned rodents. However, it has been reported that hyper-acute intermittent optogenetic stimulation of NTS neurons increase renal SNA in a fashion similar to hyper-acute IH (38), indicating that neither hypoxia, chemoreceptor stimulation nor peripherally generated oxidative stress are required to produce the sympathetic dysregulation observed with hyper-acute IH. Especially considering that at least 3 days of IH exposure are required to alter carotid body responses to hypoxia (24, 28), it is likely that only central (e.g. NTS) components of the chemoreflex mediate sympathetic responses to hyper-acute IH (19, 29).

Furthermore, studies in CIH conditioned rodents have identified that both free radicals and ROS generated in the carotid body and the chemoreflex neural arc mediate the carotid body responses to CIH, thereby, activating SNA (23, 25, 29, 30). Other animal studies have demonstrated that antioxidants reduce the efflux of catecholamines from the adrenal medulla (12) and reduce the carotid body response to CIH (23, 25, 26). Furthermore, Pialoux and colleagues (27) demonstrate that acute intermittent hypoxia (AIH; i.e. 1-24 hours of IH exposure) in human subjects increase

markers of oxidative stress, identifying an ATR1a dependent mechanism, suggesting that shorter exposures of IH can also increase oxidative stress and arterial blood pressure (4). However, we have demonstrated that neither hyper-acute exposure to IH nor N-AC altered peripheral superoxide; yet, N-AC significantly lowered the MSNA response to hAIH. We contend that this finding may be explained by N-AC crossing the blood-brain-barrier (BBB) (6-8) and decreasing efferent SNA without altering peripheral redox balance (Figure 4, since hyper-acute IH did not significantly alter peripheral ROS to begin with). Putatively, N-AC could reduce efferent SNA by rescuing NO, which is rapidly degraded by superoxide and therefore preserves its important sympathoinhibitory function (22, 36, 39, 40).

Indeed, our finding that hyper-acute IH did not increase circulating superoxide concentrations as measured by EPR spectroscopy and that N-AC had no effect on superoxide concentrations versus placebo in the peripheral circulation suggest that the sympathetic dysregulation and alterations in redox balance during hAIH are primarily centrally-mediated (5, 15). These findings are supported by reports that changes in carotid body activity require at least 3 days of IH exposure (24), and stimulation of NTS neurons alone produces similar changes in MSNA and following hAIH (38). However, one of the limitations of the present study is that superoxide is a highly reactive molecule and likely experiences substantial chemical decay during sample collection, hence, we were limited by time in not exploring other measures of oxidative stress suggested by Pialoux *et al.*. (27).

Conclusions:

These results indicate that N-AC reduces the MSNA response to hyper-acute IH without altering circulating concentrations of free radicals, particularly superoxide. Moreover, peripherally circulating venous superoxide concentrations do not appear to be increased with hyper-acute IH. Since N-AC crosses the BBB these data may be explained by centrally mediated effects of N-AC consistent with previously reported animal studies. Mechanistically, we suggest that N-AC rescues central NO, thereby, decreasing efferent MSNA (36). Thus, these findings indicate that centrally active antioxidants, perhaps in combination with angiotensin receptor blockers, can provide sympathoinhibitory benefit in clinical conditions of intermittent hypoxia, such as OSA (33, 34)

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CHAPTER III

The Angiotensin Receptor Blocker Losartan Reduces the Immediate and Sustained Increases in Muscle Sympathetic Nerve Activity after Hyper-Acute Intermittent Hypoxia.

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Abstract

Obstructive sleep apnea (OSA) is characterized by intermittent hypoxemia (IH), which produces elevations in sympathetic nerve activity (SNA) and associated hypertension in experimental models that persist well beyond the initial exposure. We tested the hypotheses that angiotensin receptor type 1a blockade in humans using Losartan attenuates the immediate and sustained increases in SNA discharge and mean arterial pressure (MAP) after hyper-acute intermittent hypoxia (IH) using a randomized, placebo controlled repeated-measures experimental design. We measured ECG and photoplethysmographic arterial pressure in 9 healthy human subjects, while muscle sympathetic nerve activity (MSNA) was recorded in 7 subjects using microneurography. Subjects were exposed to a series of hypoxic apneas in which they inhaled 2-3 breaths of N_2, followed by a 20-second apnea and 40 seconds of room air breathing every minute for 20 minutes. Hyper-acute IH produced substantial and persistent elevations in MSNA burst frequency during the placebo condition (baseline: 15.3 ± 1.8, IH: 24 ± 1.5, post-IH 20.0 ± 1.3 bursts/min, all $P < 0.01$ between time points) and MAP (baseline: 89.2 ± 3.3, IH: 92.6 ± 3.1, post-IH: 93.8 ± 3.1 mm Hg mm Hg, all $P < 0.02$ between time points). Losartan attenuated the immediate and sustained increases in MSNA (baseline: 17.3 ± 2.5, IH: 18.6 ± 2.2, post-IH 20.0 ± 1.3 bursts/min, all $P < 0.001$) and MAP (baseline: 81.9 ± 2.6, IH: 81.1 ± 2.8, post-IH: 81.3 ± 3.0 mm Hg, all $P >0.70$). This investigation confirms the role of angiotensin II type 1a receptors in the immediate and sustained sympathoexcitatory and pressor responses to hyper acute IH.

Introduction:

Obstructive Sleep Apnea (OSA) is characterized by intermittent apneas and hypopneas that produce intermittent hypoxemia and sleep disruption, and has been shown to cause cardiovascular disease independently from obesity and metabolic syndrome (29), common co-morbidities associated with OSA. Numerous investigations have demonstrated that exaggerated sympathetic nerve activity (SNA) drives the observed cardiovascular risk in OSA (43). Elevated SNA and subsequent hypertension is consistently and reliably produced in human (10, 11, 18, 25, 45) and animal (22, 39) models of intermittent hypoxia (IH), the latter of which have convincingly identified that the IH-mediated elevation in SNA is dependent on an intact chemoreflex arc (17, 39). Hence, it is apparent from these studies that IH-dependent chemoreflex dysfunction is a critical pathophysiological feature of OSA. Mechanistically, angiotensin II binding to angiotensin II type 1a receptors (ATR1a) mediates, in part, the IH induced adaptations at the chemoreceptor (21, 33) and in brainstem nuclei that control central sympathetic outflow (42). In addition, these adaptations include the circumventricular organs that are known to have a poorly developed blood-brain-barrier (BBB) and hence, are exposed to substances in the peripheral circulation (42). Marcus *et al.* (21) demonstrated that oral administration of the angiotensin receptor blocker (ARB) Losartan in rodents exposed to chronic IH (CIH) reduced both basal lumbar SNA (LSNA) and LSNA in response to acute hypoxia. Furthermore, Peng *et al.* elucidated the role of angiotensin II in the sensory long-term facilitation (sLTF) of chemoreceptor discharge after chronic IH in rodent models (32, 34), which is defined as a sustained increase in neural discharge after cessation of the original stimulus. sLTF potentially represents an important mechanism that

could explain the elevation in daytime muscle SNA (MSNA) observed in OSA patients during baseline room air breathing with normal P_aO_2 (28). In human subjects, Foster *et al.* (10) demonstrated that Losartan attenuated the hypertension observed after acute (~6 hours) IH. However, no human investigations to date have addressed whether Losartan treatment prevents the increase in MSNA observed after shorter (hyper-acute, e.g. 20 minutes to 1 hour) bouts of IH (13). Furthermore, we have previously documented that a short paradigm of IH also produces a prolonged elevation of SNA in human subjects (6) consistent with a sLTF-like effect of SNA control. However, it is unclear if Losartan modifies the sustained MSNA during room-air breathing observed immediately after a short bout of IH in human subjects, similar to what was observed by Cutler *et al.* (6). Indeed, although most of the aforementioned animal and human studies have investigated chronic IH, our laboratory and others have demonstrated that only a 20 minute bout of IH is required to recapitulate, in healthy humans, the hypertension, sustained sympathetic nerve discharge, and augmented chemoreflex responses to hypoxia observed in OSA patients (5, 6, 18, 19, 25, 48). This indicates that only short bouts of IH are required to facilitate changes in chemoreflex control of SNA in a similar fashion to what occurs in OSA. Hence, we tested the hypotheses that 100 mg of Losartan will attenuate the increases in MSNA during and immediately after a hyper-acute IH protocol and will be accompanied by lower arterial pressures during and immediately after IH.

Methods:

Subjects:

We recruited 9 human subjects (1 female, 8 males) to participate in the protocol after providing informed consent. The female subject was tested during the early follicular phase (days 1-4 post menses) of her menstrual cycle for both treatment conditions. All subjects completed a medical history questionnaire prior to experimentation and were free of cardiovascular, metabolic or respiratory diseases including hypertension (13). The research described herein conforms to the Declaration of Helsinki and was approved by the University of North Texas Health Science Center Institutional Review Board (IRB #2016-007). Subjects repeated the protocol in a randomized cross-over subject-blinded design in two separate visits separated by at least 1 week.

Cardiovascular and Hemodynamic Measurements:

MSNA was performed using standard microneurographic techniques (47) and successful measurements were obtained on 7 subjects. Briefly, a sterile tungsten microelectrode was inserted into a fascicle of the peroneal nerve near the fibular head. The nerve signals were then amplified, filtered (700 to 2,000Hz), rectified, discriminated and were then integrated with a time constant of 0.1s to produce a mean voltage display for quantitative analysis (14, 47). Muscle sympathetic neural bursts were readily recognized by their tight temporal relationship to the cardiac cycle, their responses to voluntary apnea, and their failure to respond to arousal stimuli or stroking of the skin (14, 47).

A three-lead electrocardiogram (ECG) was obtained (Hewlett-Packard, Inc.) along with beat-to-beat photoplethsymographic measurement of arterial pressure (Finometer, Finapres, the Netherlands). Pulse-oximetry (Nellcor, Inc.) was performed to measure the nadir of O_2 saturation while ventilation was measured with a Ventilation Measurement Module (VMM, Laguna Hills, CA) (13). Photoplethysmographic arterial pressure data were calibrated against an automated brachial oscillometric arterial pressure measurement for each experiment, which was measured on the opposite arm (Tango+ BP Stress, SunTech Medical Systems). End-tidal O_2 and CO_2 (ETO_2 and $ETCO_2$) were measured with a commercially available gas analyzer (AD Instruments Gas Analyzer, Melbourne, Australia), which was calibrated prior to each experiment. Physiological data were digitally recorded at 500 Hz in data acquisition software (WinDaq, Dataq, Akron, OH), which were stored offline for analysis.

Experimental Protocol:

Prior to instrumentation, subjects were seated comfortably for approximately 20 min to minimize the effect of anxiety on measured variables and to establish a baseline oscillometric arterial pressure measurement. Subjects then ingested either cellulose placebo or 100 mg of Losartan for each study visit. Subjects were then observed for adverse responses to study medication for an additional 20 minutes while additional brachial blood pressure readings were taken. After the observation period, an MSNA recording was obtained and a total of 1 hour was allowed from Losartan administration to the first baseline recording period to allow the medication to reach

peak plasma concentration (20). Subsequently, subjects were instrumented with the rest of the aforementioned devices to obtain physiological data. Figure 1A illustrates the basic experimental design of the protocol. Data were recorded during the baseline, pre-IH period that lasted 5 min. The IH protocol then commenced in which the subject completed 20 hypoxic apnea cycles in the following manner: the subject breathed 2-3 breaths of 95-100% N_2, and then initiated a 20 s voluntary, end-expiratory apnea. The subject then recovered on room air for 40 seconds, at which time the next apnea/recovery cycle began. A total of 20 hypoxic apneas were performed over a period of approximately 20 minutes, with each apnea achieving a target SaO_2 nadir between 85-90%. After the final apnea/recovery cycle, a post-IH recovery period was recorded for 5 minutes to observe the immediately persistent effects of IH on hemodynamic and microneurographic data.

Data Analysis:

Data were analyzed using physiological analysis software (WinCPRS, Absolute Aliens, Turku, Finland). R-waves generated from the ECG were detected and marked at their occurrence in time while diastolic and systolic pressures were marked from the Finometer waveform for arterial pressure (13, 24). MSNA bursts were identified by a single investigator (NPJ) and average MSNA burst frequency (bursts/min) and burst incidence (bursts/100 heart beats) were calculated during the entire IH period and the entire baseline periods according to published guidelines (13, 47). Briefly, MSNA bursts were selected by the investigator if (a) they occurred within 1-1.4 s following an R-wave on ECG and (b) they exhibited a 3:1 signal-to-noise ratio (13, 47). Because

MSNA recordings were obtained on different days (placebo vs. Losartan treatments), analysis of MSNA total activity would not be appropriate, since the distance from the microelectrode tip to the sympathetic efferent nerves, which is a primary determinant of burst amplitude, cannot be adequately controlled between recordings (47). Arterial pressure data (mean, systolic and diastolic arterial pressure) were averaged during the last five minutes of the 20-min IH protocol, where we have demonstrated arterial pressure reaches its peak (13).

Statistical Analysis:

All data were analyzed with commercially available statistical software (SigmaPlot, Systat Software Inc., California, USA). Hemodynamic and microneurographic data were compared for each time point (i.e. baseline, IH and post-IH) and drug treatment (e.g. placebo vs. Losartan) using a two-way ANOVA with repeated measures, which was followed by Student-Newman-Keuls *post-hoc* tests. Significance was set at $\alpha = 0.05$. Results are reported as means ± SEM unless otherwise stated.

Results:

Subject Demographics and Representative Subjects:

Table 1 provides anthropometric data for our study sample, which consisted of young, healthy, non-overweight or obese subjects. Figure 1B illustrates one cycle of the apnea/recovery periods that constituted the IH protocol, which produced profound sympathoexcitation with oxygen desaturation. Furthermore, as evidenced by a separate representative subject completing the cross-over design protocol, compared to the placebo condition (Figure 2A), Losartan greatly reduced the MSNA and arterial pressure response to IH (Figure 2B), despite a similar degree of hypoxemia (~88% in both exposures).

Immediate and Sustained Changes in MSNA after IH and Effects of Losartan:

Two-way ANOVA with repeated measures demonstrated a significant interaction between time point and drug for MSNA burst frequency and incidence (all $P < 0.01$; Figure 3A-B)). *Post-hoc* testing, identified that Losartan did not alter baseline MSNA burst frequency or incidence (all $P > 0.40$. Compared to the placebo condition, in which IH profoundly increased MSNA burst frequency compared to baseline (baseline: 15.3 ± 1.8 vs. IH: 24 ± 1.5 bursts/min, $P < 0.001$; Figure 3A), IH with prior Losartan did not elevate MSNA burst frequency (baseline: 17.3 ± 2.5 vs IH: 18.6 ± 2.2 bursts/min, $P = 0.46$; Figure 3A). A similar effect was observed for MSNA burst incidence, where IH with placebo raised MSNA burst incidence compared to baseline (baseline: 26.8 ± 3.7 vs. IH: 38.0 ± 3.5 bursts/100 heart beats, $P < 0.001$; Figure 3B); however, Losartan

blocked this effect (baseline: 28.6 ± 3.8 vs. IH: 28.4 ± 3.3 bursts/100 heart beats, P = 0.95; Figure 3B). Furthermore, placebo IH produced a sustained elevation in MSNA burst frequency and incidence that persisted throughout the post-IH period both for burst frequency (baseline: 15.3 ± 1.8 vs. post-IH 20.0 ± 1.3 bursts/min, P < 0.001) and incidence (baseline: 26.8 ± 3.7 vs. post-IH 36.3 ± 4.6 bursts/100 heart beats, P < 0.001). This effect was similarly blocked with Losartan treatment for MSNA burst frequency (baseline: 17.3 ± 2.5 vs. post-IH 17.4 ± 2.3 bursts/min, P = 0.87; Figure 3A) and burst incidence (baseline: 28.6 ± 3.8 vs. post-IH 28.1 ± 3.9, P = 0.85; Figure 3B). Interestingly, MSNA burst frequency decreased during the post-IH period compared to IH with placebo (IH: 24 ± 1.5 bursts/min vs. post IH 20.0 ± 1.3 burst/min, P = 0.02; Figure 3A). However, this effect was not observed when MSNA was normalized to 100 heart beats (IH: 38.0 ± 3.5 vs. post-IH 36.3 ± 4.6 bursts/100 heart beats, P = 0.40; Figure 3B). Losartan treatment did not produce differences in IH MSNA burst frequency or incidence compared to the post-IH period (all P > 0.85; Figure 3A-B).

Immediate and Sustained Effects of IH and of Losartan on Subject Hemodynamics:

Two-way repeated measures ANOVA for the mean arterial pressure (MAP) data demonstrated a significant interaction between Losartan and IH (P = 0.04). Subsequent *post-hoc* testing demonstrated that IH with placebo produced an elevated MAP compared to baseline (89.2 ± 3.3 vs. 92.6 ± 2.6 mm Hg, P = 0.005; Figure 4). Furthermore, IH produced a sustained elevation in MAP during the post-IH recovery period compared to baseline (89.2 ± 3.3 vs. 93.8 ± 3.1 mm Hg,

P = 0.02); however, post-IH MAP was not different from IH (92.6 ± 2.6 vs. 93.83 ± 3.1 mm Hg, P = 0.39). In contrast, IH with prior Losartan ingestion failed to elevate MAP (81.9 ± 2.6 vs. 81.1 ± 2.8 mm Hg, P = 0.84) and did not produce a sustained elevation in MAP as observed with the placebo condition (81.89 ± 2.6 vs. 81.3 ± 3.0 mm Hg, P = 0.70). An identical pattern was observed for systolic arterial pressure (SAP) within and between conditions (interaction effect P = 0.03). IH with prior Losartan treatment failed to elevate SAP compared to baseline and also failed to produce a sustained increase in SAP, as observed with placebo treatment (placebo condition: baseline: 119.8 ± 4.8 mm Hg, IH: 125.6 ± 3.6 mm Hg, post-IH: 127.7 ± 3.7 mm Hg; all P < 0.02 except for IH vs. post-IH where P = 0.38; Losartan condition: baseline: 110.5 ± 4.1 mm Hg, IH: 108.2 ± 3.4 mm Hg, post-IH: 108.8 ± 3.5 mm Hg; all P > 0.48; Figure 4). However, DAP did not display a similar effect, with only a main effect for drug (P = 0.02) and not for time point or interaction (all P > 0.89; Figure 4). Heart rate was only increased with IH (ANOVA main time effect, P = 0.006), and did not show a main effect of time point or interaction (all P > 0.60; Figure 4)

Tables

Table 1: Characteristics of Study Sample

Group	**Value**
Age (years)	23.3 ± 1.3
Sex *(M/F)*	8/1
Height (cm)	177.4 ± 3.6
Weight (kg)	79.4 ± 7.4
BMI (kg/m^2)	25.0 ± 1.4

Figure 1A

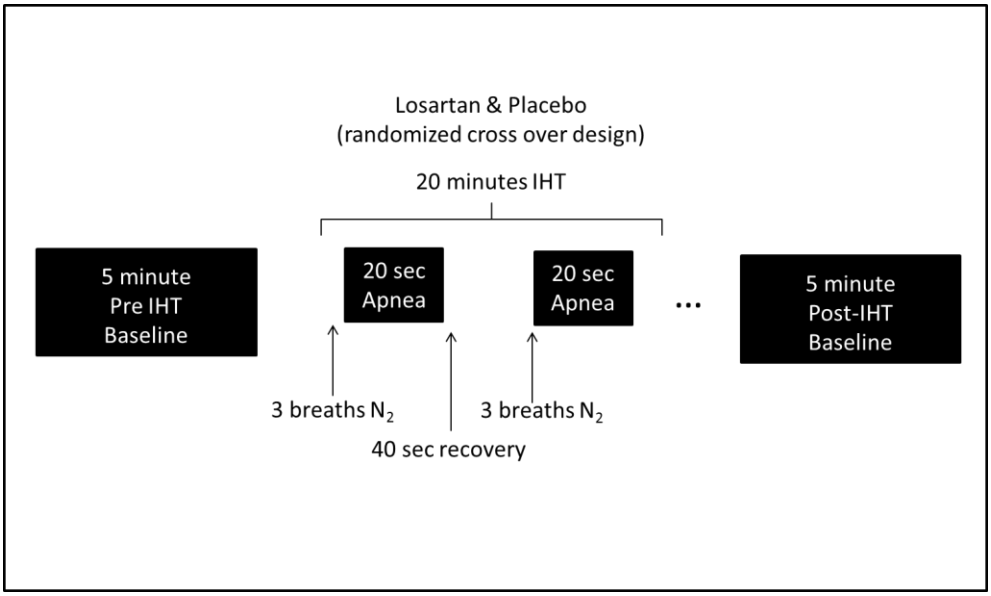

Figure 1A: Schematic of the the experimental protocol. Subjects completed the protocol in a randomized, subject-blinded cross-over design, where subjects ingested both placebo and 100 mg of Losartan on separate experimental days.

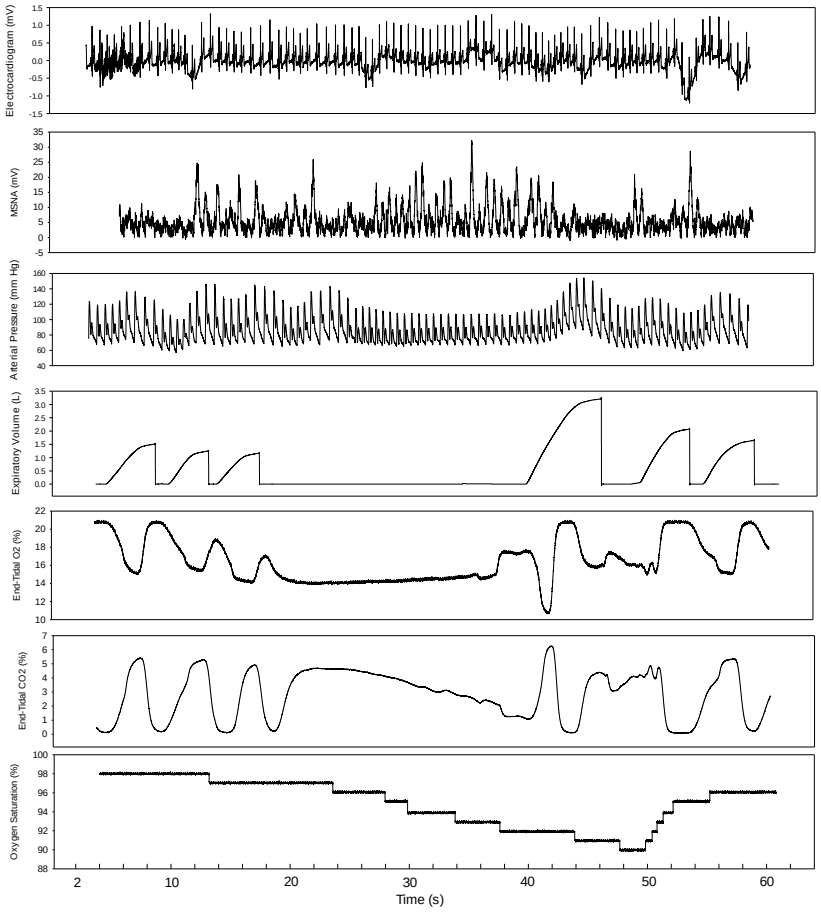

Figure 1B: Data from a representative subject depicting one cycle of the IH protocol, which produced substantial sympathoexcitation, elevations in arterial pressure, hypoxemia and hypercapnia as well as oxyhemoglobin desaturation mimicking an apnea-hypopnea index (AHI) of 60 events/hour.

Figure 2A

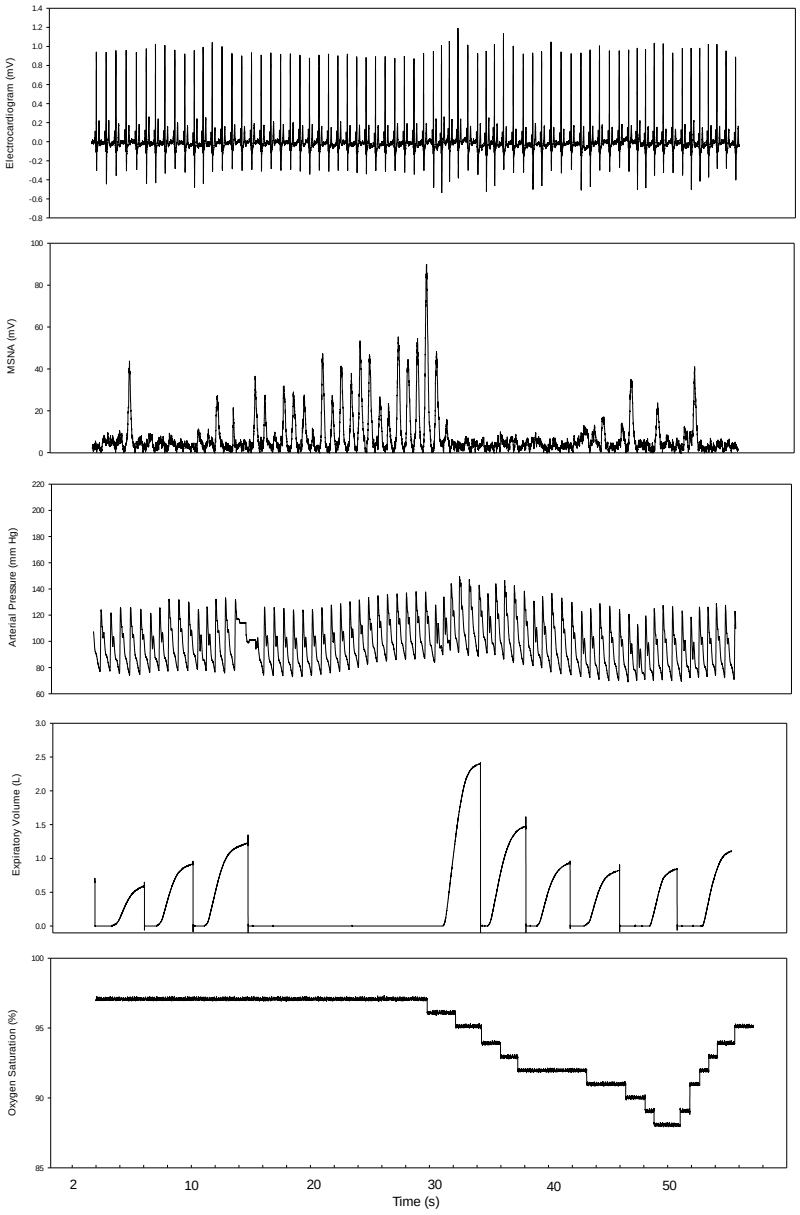
Electrocardiogram (mV)
MSNA (mV)
Arterial Pressure (mm Hg)
Expiratory Volume (L)
Oxygen Saturation (%)
Time (s)

Figure 2B

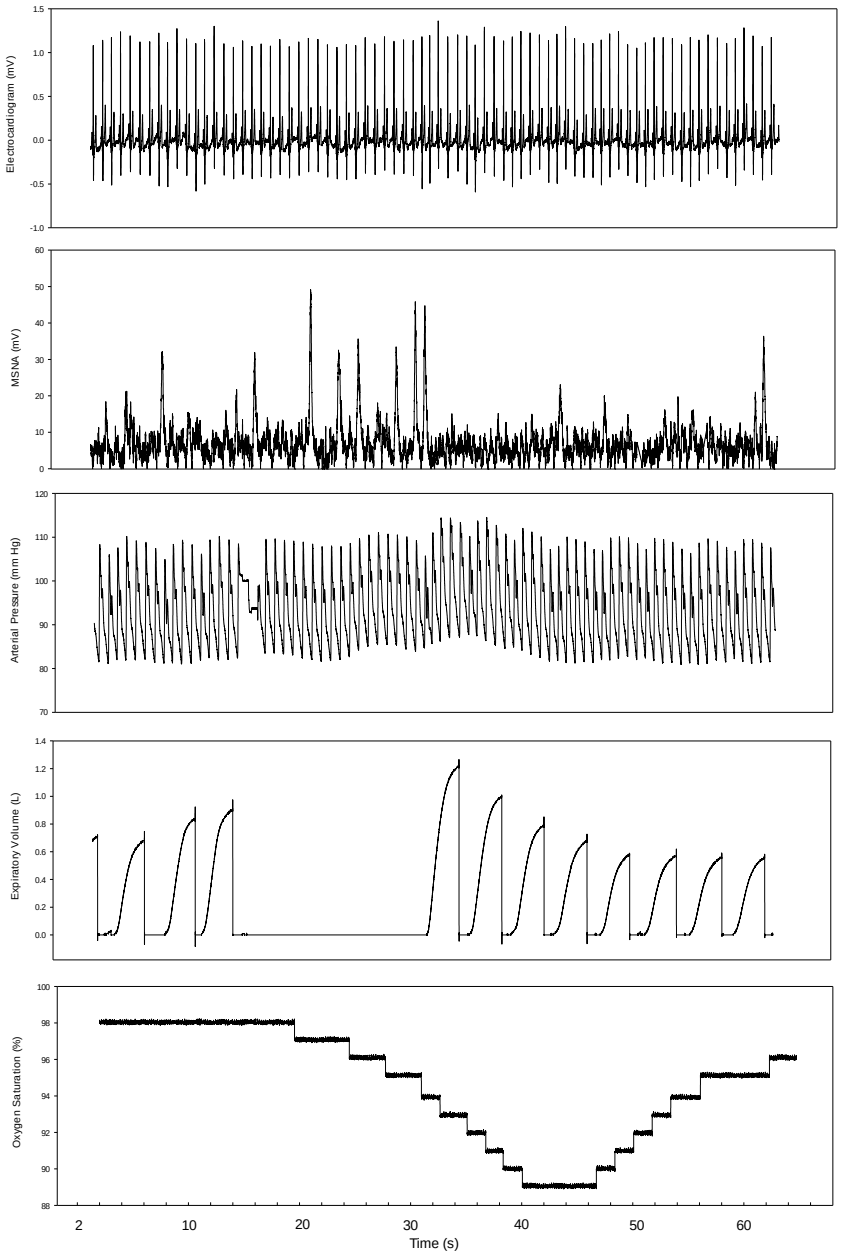
Electrocardiogram (mV)
MSNA (mV)
Arterial Pressure (mm Hg)
Expiratory Volume (L)
Oxygen Saturation (%)
Time (s)

Figure 2A-B: Data from a representative subject during IH after ingesting cellulose placebo (A) and after ingesting 100 mg of Losartan (B) on separate experimental days. One cycle of apnea and subsequent recovery data are shown in this figure. Note that, although Losartan did not alter baseline MSNA (see results) it significantly reduced the MSNA burst frequency and arterial pressure response to IH, despite a similar nadir of oxygen saturation.

Figure 3

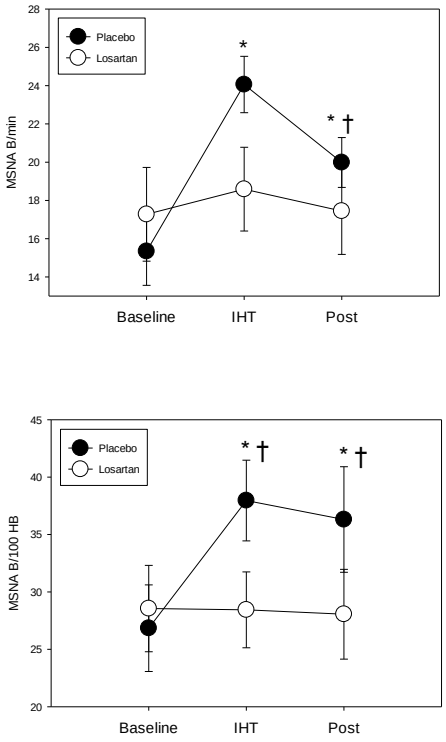

Figure 3: MSNA data (expressed in burst frequency (A) and burst incidence (B)) during each time point of the experimental protocol. *, different from baseline; †, different from Losartan (B), different from IH (A). IH: hyperacute intermittent hypoxia, Post: Post IH baseline period.

Figure 4

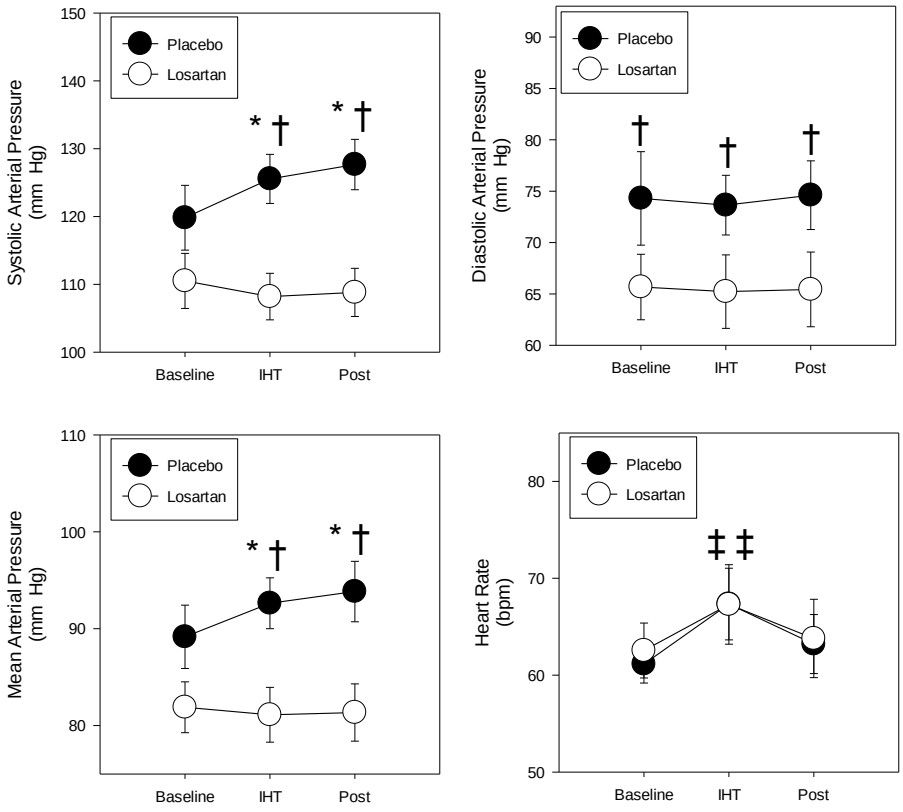

Figure 4: Hemodynamic data during each time point of the experimental protocol. *, different from baseline; †, different from Losartan. IH: hyperacute intermittent hypoxia, Post: Post IH baseline period.

Discussion:

The present investigation confirmed that IH produces significant elevations in MSNA and arterial pressure that persist with normal, room-air breathing consistent with previous studies (6, 13). Furthermore, this study demonstrated, for the first time in humans, that Losartan attenuated both the immediate and sustained elevations in MSNA and arterial pressure in response to hyper-acute IH. Hence, this study represents an important translational step to confirm the role of ATR1a in the sympathoexcitatory and pressor responses of IH, providing evidence for future clinical investigations of ATR1a blockade in OSA patients.

Intermittent Hypoxia, Chemoreflexes and Sympathetic Outflow:

IH consistently produces hypertension and sympathetic activation in animal (22, 41) and human (10, 18) models. Importantly, Lesske *et al.* demonstrated that surgical denervation of the carotid chemoreceptors was sufficient to prevent increases in arterial pressure after IH in rodents (17), indicating that an intact chemoreflex arc is necessary to produce the hypertension observed in IH. Furthermore, the same investigators chemically denervated sympathetic nerve terminals and performed simultaneous adrenal demedullation in another subset of animals. IH-exposed rodents without a neuro-humorally intact sympathetic nervous system also failed to develop hypertension, demonstrating the important pathophysiological role of SNA in IH-mediated increases in arterial pressure. More recently, Peng *et al.* (35) also demonstrated the critical role of the carotid body and sympathetic innervation of the adrenal medulla in the hypertensive response to chronic IH.

Hence, it is clear from these studies that IH produces chronically elevated sympathetic activation, but the molecular mediators have only recently been elucidated in both animals and humans. Indeed, animal investigations have shown the important role of angiotensin II, working through the ATR1a, in producing substantial IH-mediated maladaptations in the carotid chemoreceptor (32, 39), and also in the forebrain and hindbrain central controllers of SNA that exhibit synaptic connections to the chemoreflex neural arc (42). Losartan treatment in rodents exposed to IH has been shown to attenuate the increase in basal SNA and SNA responses to hypoxia (21) as well as the subsequent hypertension (7), while the same medication blocks the hypertension observed after ~6 hours of IH in human subjects (10). However, the present investigation, to our knowledge, is the first to demonstrate that Losartan reduces MSNA (both burst frequency and incidence) after brief exposures to IH. These novel findings solidify the role of ATR1a and subsequent sympathoexcitation in IH pathophysiology in humans, and the contribution of ATR1a to a sLTF-mediated adaptation of neural control accompanying short-term IH conditioning.

Indeed, consistent with previous studies, IH produced a sustained elevation in MSNA that persisted into periods of room-air breathing (6). This finding could be related to sLTF, a phenomenon in which repeated activation of neurons produces a sustained elevation in discharge frequency even after cessation of the initial stimulus (32). This effect has been observed both with IH (6), and also with sustained hypoxic hypercapnia (i.e. asphyxia), as demonstrated by Morgan *et al.* (25). Hence, asphyxia itself (e.g. not in concert with absent lung inflation (14)) could produce this sLTF-like effect of sympathetic control in human subjects. Regardless, convincing animal investigations have demonstrated the role of ATR1a in this phenomenon observed in IH-

conditioned rodents (34), providing a potentially important mechanistic explanation for the persistent elevation in daytime MSNA observed in OSA patients (27). Our study demonstrates that the persistent elevation in MSNA observed with IH in the placebo condition is blocked with Losartan treatment, and supports the possibility of this phenomenon occurring in human subjects. However, it is not clear if Losartan is actually reducing the sLTF-like effect of SNA control after IH, or simply reducing the strength of the sympathetic stimulus required to produce such an effect.

The pharmacological properties of Losartan and its absorptive kinetics in humans indicate that its actions are mostly peripheral in nature (4). ATR1a have important roles at the carotid body, a peripherally located structure, in altering its response to basal discharge and responses to hypoxia in the setting of IH (16, 26, 38). Hence, actions at the carotid chemoreceptor alone could possibly explain our results. However, Losartan has been shown to affect neural tissue in humans and in rodent models of Alzheimer's disease and other neurological disorders (2, 31). This paradox may be explained by the blood-brain barrier (BBB) alterations that occur in these diseases (3, 31) or, in the setting of normal physiology, by Losartan interacting with circumventricular organs (CVOs), which have a poorly developed BBB. The CVOs have been shown to play critical roles in developing the hypertensive responses to IH in rodents, and these processes are, in part, mediated by angiotensin II working on ATR1a (22, 42). Hence, although Losartan's pharmacokinetics indicate a predominantly peripheral mechanism at the level of the carotid chemoreceptor, its interaction (even with oral administration) with CVOs cannot be excluded from consideration. Furthermore, it is interesting that despite Losartan producing clear reductions

in diastolic pressure throughout the protocol (especially during baseline), there was **not** a baroreflex-mediated compensatory increase in MSNA. These actions of Losartan may be mediated by a central mechanism of action and/or by the medication altering the control of baroreceptor afferent discharge.

Hemodynamic Responses to IH

Importantly, as in previous studies from our laboratory, the IH protocol elevated arterial pressure (5, 6, 13). We contend that the mechanism lies predominantly with an elevation in sympathetic nerve activity, particularly considering the short time frame of our IH paradigm. Indeed, the elevation in MAP observed with the present study was mostly driven by increases in SAP, as DAP did not change in response to IH, consistent with prior observations of hyper-acute IH (13). However, investigations using human models of longer duration IH consistently demonstrate elevations in DAP (10). We propose that the disparity in the DAP responses may be a function of elevations in slowly secreted neurohormones such as epinephrine, which is a critical feature of CIH-mediated hypertension, and elevates DAP in longer-term IH protocols (e.g. 6 hours to 28 days (15)). The short time frame of our protocol would most likely prevent significant elevations in catecholamines, and hence, prevent effects on DAP. As mentioned, we observed a substantial and persistently elevated increase in SAP as a result of IH. As indicated from other studies in our laboratory, the acute SAP response to hypoxic apnea is mostly a function of SNA, as the ΔSAP response correlates very well with the MSNA responses to hypoxic apneas (12). Hence, we

contend that the arterial pressure responses to the IH protocol used in this investigation is explained, in part, by elevated SNA, which could potentially be the result of a variety of molecular and physiological factors including activation of ATR1a and subsequent generation of oxidative stress (10, 37).

The heart rate response to IH was profound, but was unaffected by Losartan. The heart rate response that we observed is most likely an acute effect of hypoxia, which is very well established (1, 8). Since resting heart rate is almost solely controlled by beat-to-beat fluctuations in vagal nerve activity (46), the fact that Losartan did not produce an effect indicates that its mechanism in IH is separate from modifying cardiovagal function, as the elevations in heart rate observed with IH could be mediated by vagal withdrawal. However, the possibility that Losartan did not modify the cardioacceleratory actions of the SNA response to IH cannot be excluded from consideration.

Limitations:

The present investigation is limited by not examining a longer time window for the post-IH recovery period. We only investigated the effects of IH after 5 minutes, whereas other studies evaluated much longer time frames (5, 6, 25). Future investigations should examine the effects of Losartan on longer IH recovery periods. Nevertheless, the induction of sLTF-like control of MSNA after IH in humans has been repeated many times in different laboratories, and the sustained increase in MSNA observed in our study would likely persist into a longer recovery

period, as Cutler *et al.* demonstrated that MSNA can remain elevated for up to 180 minutes post IH (5, 6).

Further, our model of hyper-acute IH does not reproduce the frequent respiratory events that occur over months and years as occurs in many OSA patients. However, as mentioned, our laboratory and others recapitulate the hypertension, sustained elevation in MSNA, and altered chemoreflex control of MSNA that occurs in OSA patients in healthy subjects after only 20 minutes of IH, albeit in a transient fashion (5, 6, 18, 19, 25, 48). This indicates that much shorter time periods of IH are required to affect sympathetic chemoreflexes, which was the central focus of the present study. Indeed, animal and human investigations have demonstrated that as little as a single day of IH exposure is required to elevate arterial pressure and MSNA with only incremental increasse in the response after that time-frame (9-11, 36, 45). However, hyper-acute IH may be a poor models for other pathophysiological mechanisms of OSA apart from altered chemoreflex control of SNA (e.g. vascular, hematologic, cerebrovascular).

Our study is also limited with regards to differentiating the effects of Losartan between central versus peripheral sites of action, an inherent limitation to most human studies. Furthermore, since the route of delivery of Losartan was oral, there is possibly of substantial variance in absorptive kinetics between subjects. However, the data that we have presented indicate a clear effect of the study medication, regardless of this potential variability. Additionally, Losartan- and IH-mediated baroreflex alterations were not examined in this study. However, previous human investigations identified that the reductions in arterial pressure observed with Losartan and increases in arterial pressure produced via hyper-acute IH is caused by a resetting of the baroreflex operating point

without compromising sensitivity (23, 40). Furthermore, our experimental model of IH features voluntary apneas, which differ physiologically from the obstructive apneas that occur in OSA patients, primarily in regards to swings in intrathoracic pressure. However, Somers *et al.* compared the cardiovascular and MSNA responses of a Müller maneuver (which is similar to an obstructive apnea) to a voluntary end-expiratory apnea in humans and showed that the pattern of increases in arterial pressure and MSNA burst frequency were similar, albeit the Müller maneuver produced a greater magnitude of responses (44). Moreover, our IH protocol with apneas is actually an intermittent hypoxic hypercapnia, which may exert effects independent of IH. However, animal investigations have demonstrated that the addition of hypercapnia has no added hypertensive effect (17), and human studies have identified no additional sympathetic effect of IH with hypercapnia versus IH with and without apnea (5, 6). Furthermore, we did not measure peripherally circulating angiotensin II—although animal studies have demonstrated that only chronic IH stimulates renal ang II production (42) so we would likely not uncover any differences in our study. Regardless, our data exhibit substantial effects of Losartan, indicating significant IH-mediated effects via ATR1a activation. Finally, although other human studies had subjects ingest Losartan over several days (10, 37), we elected to give subjects a single dose. It has been shown, however, that a single 100 mg dose of Losartan peaks within the plasma 1 hour post-ingestion, and produces a therapeutic plasma concentration for 2-3 hours (30), which was within the time frame for experimentation in the present study.

Conclusions:

We conclude that hyper-acute IH produces significant sympathoexcitation and an arterial pressor response to IH, which are blocked by antagonism of ATR1a via Losartan. This investigation confirms the role of the ATR1a in IH-mediated increases in SNA and underscores the important treatment potential of this particular class of medication in OSA.

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CHAPTER IV

PERSPECTIVE AND SIGNIFICANCE

The intermittent hypoxia (IH) of Obstructive Sleep Apnea (OSA) produces sustained hypertension and sympathoexcitation (4, 12). Investigations studying animal models over the past 15 years have elucidated some of the mechanisms that are responsible for this elevation in arterial pressure and sympathetic nerve activity (SNA) (4, 5, 9, 11). Indeed, adaptations that occur within the carotid body chemoreceptor and the central controllers of SNA are, in part, responsible for the altered chemoreflex control of SNA (9, 12). Several of these adaptations have been demonstrated to be mediated by reactive oxygen species (ROS) and by activation of angiontensin II type 1a receptors (ATR1a) (6-8). Animal investigations have demonstrated that scavenging ROS using oral antioxidants attenuate the efflux of catecholamines from the adrenal medulla (2). Many animal investigations have also shown that injection of antioxidant compounds into the cerebral ventricles reduces central sympathetic outflow (1, 10). Furthermore, similar animal studies employing blockade of ATR1a using Losartan demonstrate an attenuation of the lumbar SNA response to chronic IH (3). Within this dissertation I presented experimental evidence that blockade of these important molecular mediators abrogate the sympathetic and arterial pressure responses to a very short bout of IH in humans. The results of these projects will be summarized here, followed by a discussion of their significance with regard to future clinical application

Oral Antioxidant Administration Reduces Sympathetic Neural and Arterial Pressure Responses to Hyper-Acute Intermittent Hypoxia

The first project tested the hypothesis that ingestion of N-Acetylcysteine (N-AC), a CNS-permeable antioxidant, would abrogate the sympathetic and arterial pressure response to hyper-acute IH in humans. The data identified that subjects who ingested N-AC did not show an elevation in muscle SNA (MSNA) burst frequency and incidence in comparison to subjects who ingested vehicle placebo. Furthermore, subjects in the N-AC group had lower MAP responses to hyper-acute IH compared to the placebo group. However, measurement of superoxide in whole venous blood using electron paramagnetic spectroscopy (EPR) did not identify differences in response to either hyper-acute IH or N-AC ingestion. Although superoxide is highly transient, these data may be explained by a primarily central mechanism of action, which is in accordance with animal models of IH of a similar time course. Measurements of downstream markers of oxidative stress constitutes a future research direction to investigate oxidative stress mediated elevations in hyper-acute IH. These data indicate that oral, lipid-permeable antioxidants have treatment potential for the IH encountered in OSA patients; however, further research of human experimental models of acute and chronic IH is needed.

Oral Administration of the Angiotensin Receptor Blocker Losartan Abrogrates the Acute and Sustained Sympathetic and Arterial Pressure Response to Hyper-Acute Intermittent Hypoxia.

The second project tested the hypothesis that Losartan (an angiotensin II receptor type 1a (ATR1a) antagonist) reduces the acute and sustained MSNA and arterial pressure responses to hyper-acute IH in human subjects. The major findings of this project are that Losartan virtually abolished the acute and sustained hypertension and significantly attenuated the acute and sustained MSNA burst frequency and incidence with hyper-acute IH. As Losartan is poorly permeable across the blood brain barrier, these findings may be explained by interactions with the carotid body chemoreceptor, or by mitigating the pre-synaptic angiotensin II-mediated modulation at sympathetic nerve terminals. Furthermore, interaction with circumventricular organs (CVOs) cannot be excluded from consideration, or that hyper-acute IH may introduce some transient damage to the blood-brain-barrier. These findings indicate thatATR1a blockade could be a potentially important treatment avenue to reduce sympathetically-mediated cardiovascular risk in OSA patients.

Perspectives

The results of these experiments indicate that antioxidant and ATR1a blockade in humans prevent the increases in arterial pressure and MSNA in response to hyper-acute IH, which is probably mediated through a primarily central mechanism. These data represent important first

steps in translating the work conducted in the rodent model of IH to human subjects, and eventually, to OSA patients.

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CHAPTER V

FUTURE DIRECTIONS

Future Directions for Investigation of Intermittent Hypoxia in Humans: Implications for Obstructive Sleep Apnea

There are numerous questions involving human IH that have been, heretofore, unanswered:

1. As we contend that the mechanism of hyper-acute IH is primarily central, future study should examine downstream markers of oxidative stress that are putatively generated by hyper-acute IH, with particularly emphasis on examining peripheral (e.g. antecubital venous blood) versus central (e.g. internal jugular venous blood) circulations This is especially important to consider based on the findings of acute IH in humans (20, 21), which show a substantial IH-mediated peripheral oxidative stress.
2. The mechanism for the discrepancy of hyper-acute IH-mediated LTF of sympathetic versus ventilatory control should be examined,e.g. what neural pathways and central modes of regulation are involved, and why does chemoreflex control of SNA quickly develop LTF whereas ventilatory LTF requires a longer bout of IH?
3. Future human and rodent investigations should focus on the mechanisms of the decrements in baroreflex sensitivity, and why longer exposures of IH (e.g. 14-28 days) are required to develop reductions in baroreflex gain.
4. Future studies of human IH should also fill in important knowledge gaps regarding: (1) vascular and endothelial function after hyper-acute IH; and (2) elevations in MSNA burst frequency, incidence and total activity after acute IH (Table 1).

5. Clinical trials involving ARBs such as Losartan show excellent promise in treatment of OSA-mediated hypertension, and larger, multi-center trials should be explored to determine if these medications should be included as a standard-of-care.
6. A particularly intriguing future direction of human IH research is to further investigate the beneficial vs. maladaptive mechanisms of intermittent hypoxia, which is likely related to the dose of IH (19). For example, high frequency, high amplitude (i.e. severe hypoxic exposures) and low wavelength (i.e. short hypoxic exposures) IH generally produce sympathoexcitation, hypertension and decrements in vascular function, whereas lower frequency, moderate-to-high amplitude (i.e. not as severe hypoxic exposures) and higher wavelength IH (i.e. longer hypoxic exposures) exposures produces beneficial plasticity in ventilatory control (e.g. in spinal cord injury patients), hypotension, and beneficial effects on cerebrovascular homeostasis (2, 7, 12, 13, 22). Particularly with regard to IH wavelength, the discrepancy of beneficial versus maladaptative responses may be related to the limitation of reoxygenation frequency, although more study is required to investigate this physiology. Indeed, future human investigations should examine the differential effects of these protocols on generation of oxidative/inflammatory stress, angiotensin II, hypoxia-inducible factor 1 (HIF-1) versus HIF-2α (2), MSNA burst frequency and incidence and finally, arterial pressure.

Clinical Implications

It is well-documented that treatment of OSA with PAP does not fully reverse hypertension or elevated MSNA with OSA (1, 4-6, 17, 18). In addition, an important challenge of effective treatment of OSA is patient compliance with treatment interventions (8, 9). The current Medicare standards only require 4 hours of treatment/night and only 5 days/week to be designated as effective treatment (16). The data presented within this disseration, and those from previous studies using this model, show that even short periods of intermittent apneas can produce the neurally-mediated adaptations that contribute to both the short-term and long-term pathophysiologic conditions of elevated MSNA and altered chemoreflex control that are common to OSA (7, 10, 11, 14, 15, 23). Moreover, these findings demonstrate that blockade of ATR1a receptors have the potential to provide significant adjunctive support in the treatment of OSA, and this may include compensating for the adverse effects of inadequate compliance with other forms of treatment.

Conclusions and Perspectives

CPAP only produces modest decreases in arterial pressure (5, 6, 17) and does not completely correct the increases in MSNA observed in OSA patients (18). As OSA increases cardiovascular risk independentfrom common co-morbidities such as obesity and metabolic syndrome (3), investigations into the connection between IH as the primary pathophysiological feature of OSA and subsequent sympathoexcitation and hypertension is warranted. Rodent models provide a

convenient and rigorous method to evaluate these mechanisms, and studies of chronic IH in the rodent model have provided invaluable insight into IH-mediated pathophysiology. However, human studies of IH pathophysiological mechanisms are absolutely necessary to: (a) provide important translation of findings derived from animal studies; and (b) justify pursuing clinical studies in relevant patient populations. Indeed, investigations of human IH must continue, as nearly a quarter century of OSA-related research has not yet produced meaningful clinical benefit to OSA patients.

Figure 1

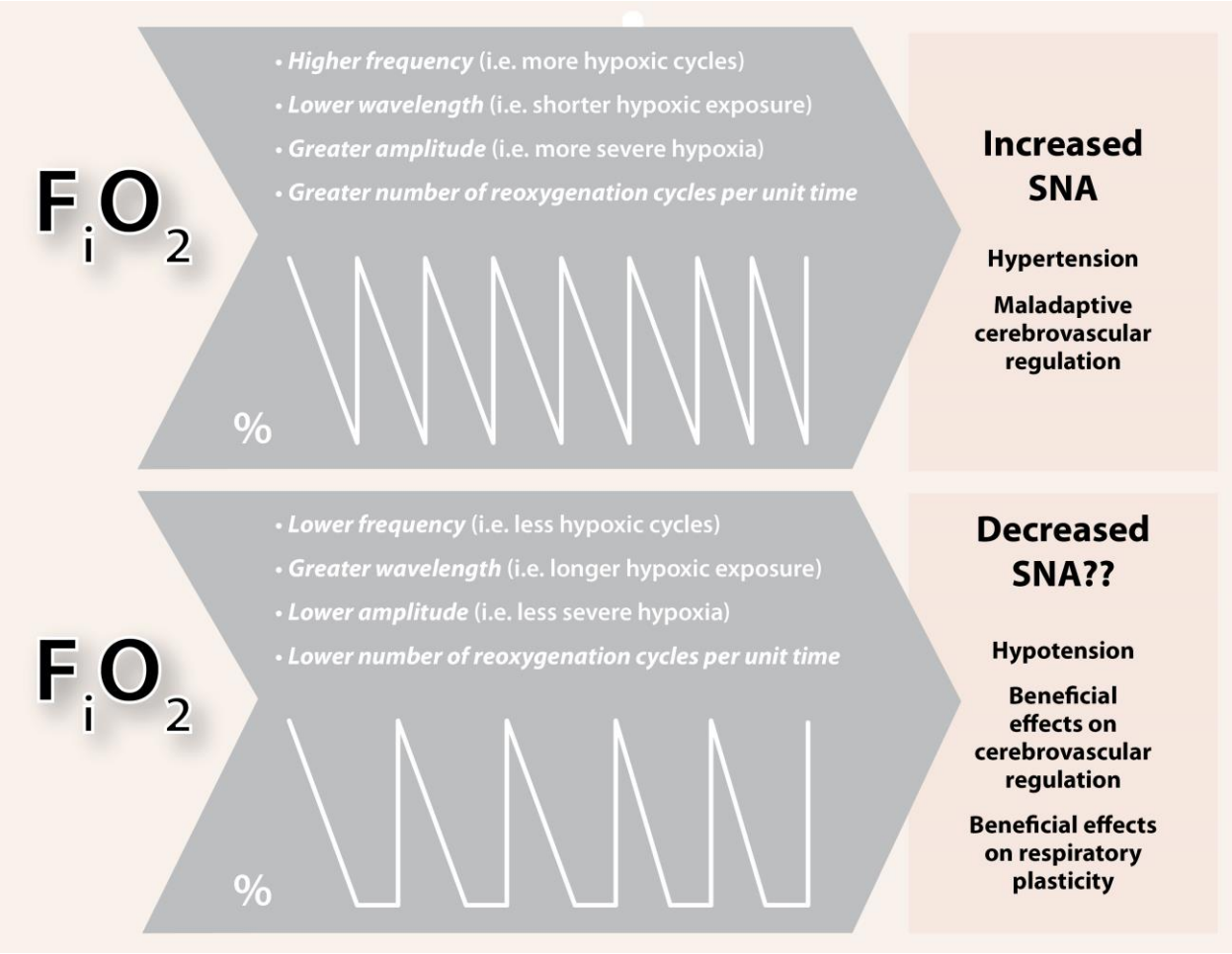

Figure 1: Maladaptive IH (top) vs. beneficial IH (bottom) experimental paradigms.

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