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UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Roles of Inflammation and Reactive Oxygen Species in the Medulla during
Ventilatory Acclimatization to Hypoxia

A dissertation submitted in partial satisfaction of the requirements
for the degree of Doctor of Philosophy

in

Biomedical Sciences

by

Daniel Alexander Popa

Committee in charge:

Professor Frank L. Powell, Jr., Chair
Professor Laura Dugan
Professor Gabriel Haddad
Professor Michael Hogan
Professor Jason Yuan

2012

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The Dissertation of Daniel Alexander Popa is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2012

Dedication

Dedicated to my family,

Marcel, Maria, and Theodore Papa and

Christine and Richard Forest,

And to all of my friends whose support and love allowed 8 years

to fly by with more joy than I had previously thought possible.

“Two roads diverged in a wood, and I—

I took the one less traveled by,

And that has made all the difference.”

- Robert Frost, “The Road Not Taken” (1915)

“Ever tried. Ever failed. No matter. Try again. Fail again. Fail better.”

-Samuel Beckett, *Worstward Ho* (1983)

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List of Abbreviations

AMS	Acute Mountain Sickness
ANOVA	Analysis of Variance
B2M	β -2 Microglobulin
BW	Body Weight
cDNA	Complimentary Deoxyribonucleic Acid
CH	Chronic Sustained Hypoxia
CIH	Chronic Intermittent Hypoxia
CNS	Central Nervous System
CO ₂	Carbon Dioxide
COX	Cyclooxygenase
CSN	Carotid Sinus Nerve
CuZnSOD	Cytoplasmic Superoxide Dismutase
DAPI	4',6-diamidino-2-phenylindole
DCFHDA	2',7'-dichlorofluorescein-diacetate
DHE	Dihydroethidium
DMV	Dorsal Motor Nucleus of the Vagus Nerve
EcSOD	Extracellular Superoxide Dismutase
PET _{CO₂}	End Tidal Carbon Dioxide Partial Pressure
f	Frequency
f _R	Frequency of Respiration (i.e. Respiratory Rate)

GABA	Gamma-Aminobutyric Acid
GFAP	Glial Fibrillary Acidic Protein
HIF-1 α	Hypoxia Inducible Factor-1 α
HVD	Hypoxic Ventilatory Decline
HVR	Hypoxic Ventilatory Response
I κ B	Inhibitor of Nuclear Kappa-Light-Chain-Enhancer of Activated B Cells
IH	Intermittent Hypoxia
IKK- β	I κ B Kinase
IL-1 β	Interleukin-1 β
IL-6	Interleukin-6
LTF	Long Term Facilitation
LTP	Long Term Potentiation
MnSOD	Mitochondrial Superoxide Dismutase
MnTMPyP	Manganese(III) Tetrakis(1-Methyl-4-Pyridyl)Porphyrin
mRNA	Messenger Ribonucleic Acid
N	Normoxic
N ₂	Nitrogen
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NF- κ B	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NMDA	N-Methyl-D-Aspartic Acid
NOX	NADPH Oxidase
NSAID	Nonsteriodal Anti-Inflammatory Drug

NTS	Nucleus Tractus Solitarius
O ₂	Oxygen
O ₂ ⁻	Superoxide
Pa _{O₂}	Arterial Oxygen Partial Pressure
Pa _{CO₂}	Arterial Carbon Dioxide Partial Pressure
qPCR	Quantitative Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
R	Respiratory Exchange Ratio
ROI	Region of Interest
ROS	Reactive Oxygen Species
RPL13A	Ribosomal Protein L13a
SOD	Superoxide Dismutase
TNF α	Tumor Necrosis Factor α
TASK	<u>T</u> WIK-related <u>a</u> cid <u>s</u> ensitive <u>K</u> ⁺ channels
TWIK	<u>T</u> andem of Pore Domains in a <u>W</u> eak <u>I</u> nward Rectifying <u>K</u> ⁺ Channel
VAH	Ventilatory Acclimatization to Hypoxia
\dot{V}_{CO_2}	CO ₂ Production
\dot{V}_I	Inspired Ventilation
VLM	Ventrolateral Medulla
\dot{V}_{O_2}	O ₂ Production
V _T	Tidal Volume

XII Cranial Nerve XII-the Hypoglossal Nerve
5-HT Serotonin

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Popa D, Fu Z, Go A, Powell FL. Ibuprofen blocks time-dependent increases in hypoxic ventilation in rats. *Respir Physiol Neurobiol*. 2011 Sep 30;178(3):381-6. Epub 2011 Mar 30.

Johnson PL, Popa DA, Prisk GK, Edwards N, Sullivan CE. Non-invasive positive pressure ventilation during sleep at 3800 m: Relationship to acute mountain sickness and sleeping oxyhaemoglobin saturation. *Respirology*. 2010 Feb;15(2):277-82.

Abstract of the Dissertation

The Roles of Reactive Oxygen Species and Inflammation in Ventilatory
Acclimatization to Hypoxia

by

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Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2012

Professor Frank L. Powell, Jr., Chair

Ventilatory acclimatization to hypoxia (VAH) is a time dependent increase in ventilation that develops after hypoxic exposures of hours to days. VAH consists of both a change in chemosensitivity to hypoxemia in the carotid body and a change in the central nervous system sensitivity to afferent carotid body signals (Nielsen *et al.*, 1988; Dwinell & Powell, 1999). My dissertation tests the hypotheses that

inflammation and reactive oxygen species (ROS) each plays an essential role in the respiratory control centers of the medulla during VAH.

Inflammatory cytokines have recently been established as important in the increased O₂-sensitivity of the carotid body during VAH using ibuprofen to block this increased sensitivity (Liu *et al.*, 2009). Using a similar experimental design to Liu *et al.* (2009), we examined the effect of systemic ibuprofen administration on the hypoxic ventilatory response and cytokine expression in the dorsal medulla, which contains respiratory control centers. Ibuprofen blocked VAH in rats breathing 10%O₂ and further analysis showed this result cannot be explained simply by the published effect of ibuprofen on the carotid body effect but it must involve the central nervous system as well. Measurements of cytokines in the dorsal medulla indicate that, inflammatory signals are increased with chronic hypoxia and this increase is blocked by ibuprofen, supporting our hypothesis of the signaling role inflammation plays in VAH.

In a second set of experiments, we tested the hypothesis that ROS decrease in CNS respiratory centers with hypoxic exposure and these changes in ROS contribute to VAH in mice. To test this, we measured ROS in normoxia, acute hypoxia, chronic sustained hypoxia, and acute intermittent hypoxia under control conditions and under chronic anti-oxidant treatment. Results show a trend for decreased ROS in acute hypoxia and a return toward normoxic levels with chronic hypoxia. Chronic anti-oxidant treatment tended to decrease ROS more in normoxia and chronic hypoxia than in acute hypoxia. In acute intermittent hypoxia, ROS showed an unexpected trend to increase. Chronic anti-oxidant treatment had no effect on the HVR or VAH except that

normoxic mice breathing room air hyperventilated. While there were no clear correlations between ROS levels and ventilation, the results are consistent with certain species or sources of ROS stimulating ventilation and such ROS increasing in chronic hypoxia, in normoxia after chronic antioxidant treatment, and in intermittent hypoxia after chronic antioxidant treatment. Further experiments are necessary to test this, as well as the hypothesis that the transcription factor, NF- κ B, may link the effects of inflammation and ROS on VAH.

Chapter 1: Introduction

1.1 Dissertation Overview

This dissertation focuses on signaling mechanisms that produce ventilatory acclimatization to chronic sustained hypoxia. The two studies included here investigate the roles of inflammation and of reactive oxygen species (ROS) as signaling molecules in the brain stem respiratory control centers known to have a role in that acclimatization process.

Hypoxia represents both a pathological and physiological condition affecting many individuals. Chronic obstructive pulmonary disease (COPD) is a disease largely caused by smoking that afflicts 12 million people and is the fourth leading cause of death in the United States and the world (National Heart, Lung, and Blood Institute 2008). COPD patients live their lives in CH, unable to properly perform gas exchange and often require supplemental O₂ therapy. Many more people live at high altitude or venture to high altitude either for work or for recreation. With tens of millions of people worldwide experiencing chronic hypoxemia from cardiopulmonary disease or environmental factors, understanding the physiological mechanisms of acclimatization to hypoxia is an important medical problem. The effects of chronic hypoxia on ventilatory control are being studied intensely and many important advances have been made on this problem, such as identification of molecular mechanisms of plasticity in carotid body chemoreceptors (involving HIF-1 α , (Kline *et al.*, 2002) and our own laboratory's demonstration of plasticity in central processing of arterial chemoreceptor afferent input (Dwinell & Powell, 1999). However, the signals for these adaptations to chronic hypoxia remain unknown. Inflammation has only recently

been recognized as a set of signaling pathways important in physiologic adaptations. In addition, an exciting field has opened up with new methods available for quantifying ROS levels *in vivo*. ROS have emerged as prominent targets of investigation to better understand sensory signaling and even aging. By better understanding the interplay between inflammation and ROS with hypoxia, approaches to clinical therapies in pathological conditions causing hypoxemia can be developed and clinical outcomes improved.

1.2 Control of Breathing

1.2.1 Control of Breathing Overview (Feldman & McCrimmon, 2003)

The purpose of breathing is primarily gas exchange with oxygen, O₂, diffusing from inhaled gas into the blood stream while CO₂ diffuses in the opposite direction in the alveoli of the lung. While O₂ is transported primarily by being bound to hemoglobin, CO₂ has no such transporter. Rather, CO₂ exists in equilibrium with carbonic acid (H₂CO₃) and also hydrogen ion with bicarbonate (H⁺ + HCO₃⁻), implicating CO₂ in acid/base regulation of the blood. Each gas, furthermore, is partially soluble in arterial blood and the partial pressures of each gas (Pa_{CO₂} and Pa_{O₂}) are maintained at appropriate levels by regulating breathing. No known physiologic detector of oxyhemoglobin saturation exists.

The act of breathing is a balance between skeletal muscle activation and tissue elastic properties. The primary skeletal muscle of respiration is the diaphragm, innervated by the phrenic nerve originating from the cervical spinal cord at C3-5. Upon contraction of the dome shaped diaphragm, the thoracic cavity volume increases with a corresponding pressure decrease that ultimately leads to inspiration at the naso/oropharynx. Expiration, in turn, is achieved at rest by relaxation of the diaphragm. Expiration under forced or exercising conditions occurs with the assistance

of skeletal muscle activation, particularly the internal intercostal and abdominal muscles. Skeletal muscles of the upper airway including those controlling the oropharynx and tongue also can have significant roles in breathing, primarily by changing airway caliber.

The center for respiratory control is the brainstem with multiple regulatory nuclei located in the medulla. Projections from the medullary respiratory control centers lead to the frontal cortex, allowing voluntary changes to breathing, and to the spinal cord to innervate the muscles required to breathe. The brainstem serves as an integrator of the numerous sensory inputs from chemosensors of arterial pH, PaO_2 , and PaCO_2 ; from mechanoreceptors in the thorax to facilitate coughing and clearance of airway foreign bodies; and from voluntary control by the cortex.

1.2.2 Arterial Chemoreceptor System (Gonzalez et al., 1995)

The principal chemoreceptors for the control of breathing are those within the carotid body and those within the aortic arch. Carotid body chemoreceptors represent the overwhelming source of chemosensory input under most conditions and serve as models for arterial chemosensors, hence aortic chemoreceptors will not be discussed here. Within each carotid body, pH, PaO_2 , and PaCO_2 levels are constantly being monitored and encoded into an afferent signal sent to the medullary respiratory control centers via the carotid sinus nerve (CSN). Breathing is increased in response to detected decreased pH and PaO_2 levels and increased PaCO_2 levels. The carotid chemoreceptors also represent the only major known source of hypoxemic (low PaO_2) sensory input.

Indeed, the carotid bodies' anatomical location is ideally suited for instantaneous chemosensation. Each carotid body is located at the bifurcation of the common carotid artery into external and internal carotid arteries and consists of

chemoreceptor, neural, glial, and vascular cells bundled together with connective tissue. Average arterial blood flow to the organ is estimated at 1.5 to 2 L/kg*min, among the highest of any tissue relative to size (De Burgh Daly *et al.*, 1954; Acker & O'Regan, 1981). This central vascular location allows the carotid body to accurately sense systemic changes in Pa_{O_2} , Pa_{CO_2} , and pH by ensuring consistent blood sampling, reducing the chances of exposure to local ischemia compared to more peripheral locations. There is also evidence that hypotension (Landgren & Neil, 1951), high potassium levels (Jarisch *et al.*, 1952; Linton & Band, 1985), changes in osmolarity (Carpenter & Peers, 1997), and hypoglycemia (Alvarez-Buylla & Roces de Alvarez-Buylla, 1994) are all sensed by the carotid body as well.

Within the carotid body, type I glomus cells appear to be the site of Pa_{O_2} chemosensation. These cells are electrically excitable, depolarizing and sending a signal to the central nervous system via the carotid sinus nerve (CSN). The neurotransmitter and pathway for this signaling between glomus cells and CSN afferents remain unknown. Additionally, the mechanism by which glomus cells sense hypoxemia remains largely unknown although inhibition of potassium membrane channel activity accompanied by calcium entry and excitatory neurotransmission appear to be involved. Direct interaction between O_2 and the channels as well as indirect inactivation through other O_2 sensing molecules have been hypothesized. Reactive oxygen species and enzymes that produce them such as NADPH oxidase have been proposed as molecular O_2 sensors in the carotid body.

Tonic output is maintained in normoxia (90-100 mmHg Pa_{O_2}) and increased output (i.e. carotid body stimulation) starts with moderate hypoxia (50-60 mmHg Pa_{O_2}) (López-Barneo *et al.*, 2004). These observations are consistent with the differential stimulatory effects of changes in Pa_{CO_2} and Pa_{O_2} : small increases in Pa_{CO_2}

stimulate the carotid bodies to a degree consistent with moderate decreases in PaO_2 to elicit a ventilatory response.

1.2.3 Central Chemoreceptor System

In addition to peripheral chemoreceptors, evidence exists that there are chemoreceptors in the central nervous system (CNS) that are sensitive to changes in PaCO_2 and PaO_2 .

The sensitivity to PaCO_2 , via pH differences, by neurons in the brainstem is well documented (Leusen, 1954; Pappenheimer *et al.*, 1965; Fencl *et al.*, 1966). The acidification of the central nervous system stimulates the central ventilatory chemoreflex (Guyenet *et al.*, 2008). Although many cells are sensitive to changes in CO_2 or pH, recent studies have shown that neurons in the nucleus tractus solitarius can function as intrinsic chemosensors and that the neurons' sensitivity changes with acclimatization to chronic hypoxia (Nichols *et al.*, 2009).

In addition to sensitivity to changes in PaCO_2 , experiments within the past decade have demonstrated hypoxic sensitivity in the rostral ventrolateral medulla (RVLM) and pre-Bötzinger complex with heme oxygenase enzymes playing a critical role in that sensitivity (Mazza *et al.*, 2001).

1.2.4 Central Integration of Peripheral and Central Chemosensory Input

The CNS integrates the signals it receives from both peripheral and central chemoreceptors to maintain homeostasis of PaCO_2 . Hypoxemic and acid/base inputs are also integrated along with PaCO_2 to ultimately produce the motor efferent signal that will cause the diaphragm to contract and inspiration to occur. Mechanisms describing how the CNS integrates these various signals remain undescribed. Indeed, mechanisms of how the CNS processes each individual signal are active areas of ongoing research.

The medulla is the site of integration of all of the afferent pathways affecting breathing and from where efferent signals emanate to the muscles that control respiration. Within the medulla, the nucleus tractus solitarius (NTS) receives the first synapse from the carotid bodies, according to anatomical and electrophysiologic data (Donoghue *et al.*, 1984; Housley *et al.*, 1987; Housley & Sinclair, 1988; Vardhan *et al.*, 1993; Marchenko & Sapru, 2000), and also has CO₂-sensitive chemoreceptor cells (Dean *et al.*, 1989; Coates *et al.*, 1993), placing it in an ideal location for signal integration from the peripheral and central nervous systems.

Horseradish peroxidase injections of rat carotid bodies showed labeling in the commissural and ventrolateral subnuclei of the NTS, approximately 0.24 to 0.48mm caudal to obex (Housley *et al.*, 1987). Cats showed a similar labeling pattern and location (Torrealba & Claps, 1988). Electrophysiologically, cats demonstrated chemosensitive afferents that innervated the commissural NTS from the obex to 1.5mm caudally (Donoghue *et al.*, 1984). Neurons within the caudal NTS treated with kainic acid showed functional decreases of approximately 67% in the ventilatory response to hypoxia (Housley & Sinclair, 1988). Anterograde tracers originating in the carotid body have been used to identify NTS neurons receiving chemosensitive afferents and to record from them in slice preparations (de Paula *et al.*, 2007).

In addition to receiving afferents, the NTS also has been demonstrated to have chemosensitive neurons. Acetazolamide microinjection and CO₂ microdialysis into the caudal NTS have been shown to each phrenic nerve output (Coates *et al.*, 1993; Nattie & Li, 2002). Single NTS neurons have also been demonstrated to have intrinsic sensitivity to CO₂ in slice preparations (Dean *et al.*, 1989). Based on its anatomical, electrophysiologic, and chemosensitive roles, the NTS is essential to breathing.

1.2.5 Motor Neurons of Medulla (DMV, XII)

Adjacent to the NTS is found the dorsal motor nucleus of the vagus nerve (DMV), another nucleus with a role in breathing. The vagus nerve is responsible for innervating much of the viscera.

The hypoglossal nucleus (from cranial nerve XII, here abbreviated to XII) is located at the same level of the medulla as the NTS and DMV, immediately ventral to the DMV and central canal. XII controls tongue protrusion via the genioglossus muscle and, hence, the largest airway obstruction typically found in an animal.

1.2.6 Phrenic Motor Neurons of the Spinal Cord

The phrenic motor neurons have been hypothesized as another site of plasticity during exposure to chronic sustained hypoxia. However, studies investigating this hypothesis have been unable to show plasticity in those motor neurons with bouts of acute sustained hypoxia (Baker *et al.*, 2001). They have displayed serotonin-mediated plasticity during intermittent hypoxia.

1.3 Ventilatory Acclimatization to Hypoxia

1.3.1 Ventilatory Acclimatization to Hypoxia Overview (Powell *et al.*, 1998)

Ventilation is the product of respiratory rate (f_R) and tidal volume (the volume gas per breath (V_T)). With acute exposure to hypoxia, the carotid body is stimulated and the CNS generates an increase in ventilation by increasing either f_R or V_T. This hypoxic ventilatory response (HVR) persists with continued exposure to hypoxia; although after 5 to 30 minutes of increased ventilation, a slight decrease in the hyperventilation occurs, termed hypoxic ventilatory decline (HVD) or “roll-off” (Fig.1.1). Small Pa_O₂ changes have a small effect on ventilation until an inflection point that is dependent on concurrent Pa_{CO}₂ is reached (Weil *et al.*, 1970). If returned

to normoxia at this point, persistent hyperventilation will occur up to an hour under normoxic conditions. As acute hypoxia becomes chronic for hours, days, or weeks; ventilation begins to rise even more in a process known as ventilatory acclimatization to hypoxia (VAH). This second increase from VAH, however, is far more gradual than that observed in the HVR. As chronic hypoxia continues for months and then years, ventilation decreases from its VAH peak but remains elevated with respect to baseline normoxic ventilation, known as hypoxic desensitization (HD). HVRs also become blunted with such long term chronic hypoxic exposure. The focus of the studies included in this dissertation was on the HVR and VAH with hypoxic exposures not exceeding 2 weeks.

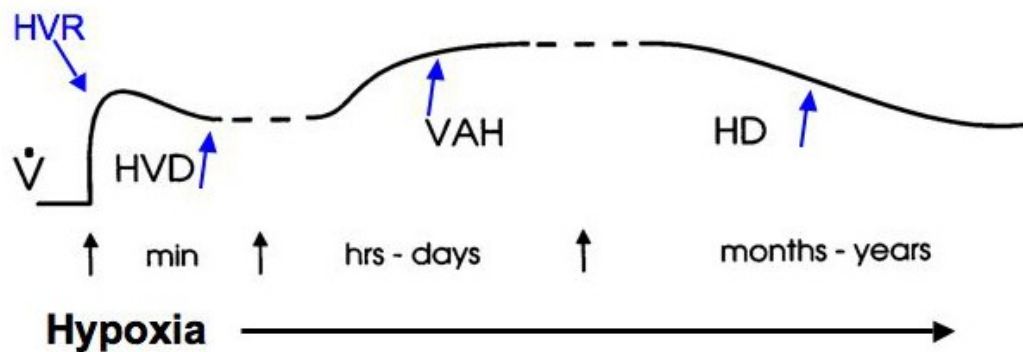


Fig.1.1. Ventilation vs. Time in sustained hypoxia. The initial increase in ventilation in acute hypoxia is termed the hypoxic ventilatory response (HVR). After several minutes, ventilation decreases slightly, termed hypoxic ventilatory decline (HVD), and plateaus over several hours. As hours become days, ventilation rises again in ventilatory acclimatization to hypoxia (VAH) and a new plateau that will persist for weeks. As weeks become months and years, ventilation may decrease slightly, termed hypoxic desensitization (HD).

The time course for the development of VAH depends both on hypoxic severity and the mammalian species studied. Goats demonstrate VAH in as few as 6 hrs (Forster *et al.*, 1981) while ponies require 24hr (Forster *et al.*, 1976). Meanwhile, humans have been shown to require 4 days at 2900m of altitude (Rahn & Otis, 1949)

and 10 days at 4300m (Forster *et al.*, 1975) with higher elevations paradoxically requiring longer acclimatization times. Altitudes over 8000m may require more than 30 days (West, 1988). Rats have a similar VAH time course as humans and, hence, are widely used as an animal model for VAH (Olson & Dempsey, 1978).

VAH is characterized by an increased slope of the *isocapnic* HVR and also a persistent hyperventilation upon return to normoxia (Fig.1.2). Allowing poikilocapnia can mask the increased slope seen in VAH.

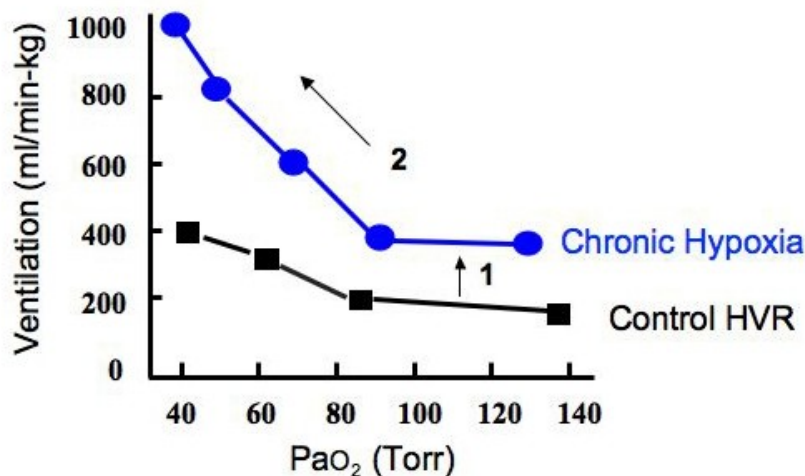


Fig.1.2. Two Components of Ventilatory Acclimatization to Hypoxia (VAH). VAH is characterized by an increased drive to breathe in normoxia (1) and an increased slope of the isocapnic hypoxic ventilatory response (HVR, 2). Modified from (Aaron & Powell, 1993) by Dr. Katie Wilkinson.

Upon return to normoxia, ventilation decreases to baseline with a time course similar to VAH in a process termed ventilatory deacclimatization to hypoxia (VDH) (Dempsey *et al.*, 1979).

1.3.2 Theories of VAH

Severinghaus and *et al.* (1963) were among the first investigators to propose a theory to explain VAH. They hypothesized that active transport of bicarbonate in

cerebrospinal fluid corrects the alkalotic cerebrospinal pH and restores the CSF H^+ -sensitive drive to breathe from central chemoreceptor cells. This effect along with the hypoxic drive to breathe from the chemoreceptors would then lead to the increased ventilation seen in VAH versus HVR, where decreased P_{CO_2} and alkalotic cerebrospinal fluid would inhibit breathing. They further hypothesized that cerebrospinal pH compensation would be more complete with the previously observed renal compensatory mechanism of increased bicarbonate excretion to bring arterial pH levels back down from the respiratory alkalemia seen with hyperventilation (Severinghaus *et al.*, 1963). Early data showed normal pH in the cerebrospinal fluid of subjects who spent a week at altitude and of high altitude natives (Severinghaus *et al.*, 1963; Severinghaus & Carcelen, 1964), however subsequent data showed that cerebrospinal pH was indeed alkaline in chronically hypoxic subjects (Forster *et al.*, 1975).

The remaining sources of stimulus for the chemosensors normalize with time in chronic hypoxia. Pa_{O_2} increases and Pa_{CO_2} decreases as ventilation increases (Rahn & Otis, 1949). Arterial pH does not show a difference between acute and chronic hypoxia, however, due to compensatory mechanisms (Dempsey & Forster, 1982). These observations, in turn, suggest that either chemoreceptors' sensitivity or the processing of their stimulation, and not the chemical stimulation itself, explains VAH.

1.3.3 Changes in the Carotid Body During Chronic Hypoxia

The carotid body is essential to VAH and undergoes plasticity during chronic hypoxia. Denervating the carotid body prevents VAH development in cats, dogs, goats, ponies, rats, and sheep (Bouverot *et al.*, 1973; Smith *et al.*, 1986; Vizek *et al.*, 1987; Olson *et al.*, 1988). With exposure to chronic hypoxia, the carotid body displays increased O_2 -sensitivity such that the frequency of carotid sinus nerve firing for a

given Pa_{O_2} increases relative to chronically normoxic animals. With 1 hour of sustained hypoxic exposure, single chemoreceptor fiber activity in goats steadily increases for at least 4 hours of hypoxia (Nielsen *et al.*, 1988). Cats, on the other hand, do not show the increase in firing rate in response to an acute hypoxic bout of 3 hours as do goats; however cats do display increased firing rates with exposure to 4 weeks of chronic sustained hypoxia (Barnard *et al.*, 1987). Increased peripheral chemoreceptor sensitivity is also observed in whole carotid sinus nerve activity of cats exposed to 48 hours of chronic hypoxia and of rats exposed to 3-16 days of hypoxia (Barnard *et al.*, 1987; Vizek *et al.*, 1987; Chen *et al.*, 2002b).

This carotid body plasticity is responsible for the augmented HVR seen in the initial hours of chronic hypoxia. In goats whose carotid body blood supply was isolated, 6 hours of carotid body hypoxia in the absence of systemic hypoxia resulted in a time dependent increase in the HVR (Busch *et al.*, 1985). However, this HVR effect was absent after 6 hours of carotid body hypercapnia (Bisgard *et al.*, 1986) and after systemic hypoxia with the carotid body alone perfused with normoxic blood (Weizhen *et al.*, 1992). Within the initial few hours of chronic hypoxia, carotid bodies are necessary and sufficient to elicit VAH.

Although the pathway by which carotid body sensitivity changes during chronic hypoxia remains unknown, structural and functional changes have been described and provide clues to the pathway underlying the process. The carotid body morphology changes dramatically with acclimatization to chronic hypoxia, primarily through Type I glomus cell enlargement (Mills & Nurse, 1993). This change decreases the covering of glomus cells by sustentacular cells (Kusakabe *et al.*, 1993), which increases the potential area available for gap junction connections. This, in turn, has been shown to enhance glomus cell sensitivity (Eyzaguirre & Abudara, 1999).

However, such morphological changes occur with days of hypoxic exposure and cannot explain the increased carotid body sensitivity seen with only a few hours of hypoxia.

As the O₂-sensor in the carotid body remains unknown, the changes in the carotid body with chronic hypoxia that explain the increased sensitivity remain equally unknown. Changes in ion channel distribution in glomus cells that may increase excitability have been observed, including a lower density of K⁺ channels and a higher density of Na⁺ and L-type Ca²⁺ channels (Stea *et al.*, 1992; Hempleman, 1995, 1996). Endothelin 1 (ET-1) and its receptor ET_A have been shown to increase hypoxic sensitivity. Both proteins are up-regulated in the glomus cells during chronic hypoxia (Chen *et al.*, 2000), and blocking the ET_A receptor can reverse the increased carotid body sensitivity observed with chronic hypoxia (Chen *et al.*, 2002a; Chen *et al.*, 2007). Changes in neurotransmitter systems including dopamine (Tatsumi *et al.*, 1995; Huey *et al.*, 2000) and acetylcholine (He *et al.*, 2006) during chronic hypoxia have been observed, however the significance of the changes remains unknown.

Regardless of the mechanism, the carotid body undergoes plasticity that increases its O₂-sensitivity during chronic hypoxia and this is necessary for the development of VAH.

1.3.4 Changes in the Central Nervous System During Chronic Hypoxia

While the carotid body is the principle sight of plasticity underlying the increased HVR seen within the first few hours of chronic hypoxia, the central nervous system also displays plasticity but over a different time course. Following the observation that doxapram, a chemoreceptor activator, increased the HVR in chronically hypoxic subjects; brain stem respiratory control centers were hypothesized to show different responsiveness with chronic hypoxic exposure (Forster *et al.*, 1974;

Dempsey & Forster, 1982). The increased responsiveness of the CNS respiratory control centers to chronic hypoxia while afferent signals remain unchanged is also known as increased CNS gain of the HVR.

Evidence of this increased CNS gain of HVR was demonstrated in a comparison of recordings of phrenic motor output in response to electrical stimulation of the carotid sinus nerve in normoxic and chronically hypoxic rats under anesthesia. Rats acclimatized to 7 days of chronic sustained hypoxia showed increased phrenic nerve output for a set carotid sinus nerve stimulation relative to normoxic, unacclimatized controls. Different acclimatization periods were studied, and 2 days of chronic hypoxia showed differences between normoxic and chronically hypoxic animals that approach significance. Such findings are consistent with the hypothesis that CNS plasticity is chiefly responsible for the differences apparent with longer hypoxic exposures especially, VAH (Dwinell & Powell, 1999). Through what mechanism this plasticity occurs remains unknown, although the activation of O₂-sensitive transcription factors appears to play a significant role (Powell & Fu, 2008). In VAH, the mechanism is undoubtedly complicated by the continuous interplay between the carotid body, which undergoes its own plasticity, and the CNS, which partially relies on afferent input from the carotid body.

1.4 Mechanisms of Plasticity in Chronic Sustained Hypoxia

1.4.1 Inflammation

Chronic inflammation has been well established at inducing chronic pain states with hyperalgesia and allodynia. However, only more recently has the role of uniquely *neuroinflammatory* mechanisms in pain receptor hyperexcitability been recognized (Watkins & Maier, 2002). The activation of and invasion by immune cells such as macrophages, neutrophils, and mast cells have been shown to induce remodeling and

increase sensitivity in pain receptors, or nociceptors. Mechanoreceptors that normally do not signal pain have been shown to release neurotransmitters unique to pain pathways in response to inflammatory mediators (Neumann 1996). Several studies of chronic inflammation and neuropathic pain, reviewed by (Watkins & Maier, 2002), have consistently observed significant up-regulation in the following cytokines: interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF α). A recent study also demonstrated increased mRNA expression of these three cytokines as well as a chemokine, MCP-1, in isolated carotid bodies of rats acclimatized to chronic hypoxia (Liu *et al.*, 2009). The cytokines were not just expressed by immune cells but were shown to be produced by other cells morphologically consistent with Type I and Type II cells. Prior work has shown that Type I cells in culture display increased cytokine expression in response to 1 week of chronic hypoxic exposure (Lam *et al.*, 2008). In addition, they demonstrated immune cell invasion of the carotid body with leukocytes. Both cytokine expression and leukocyte invasion in the rat carotid bodies were significantly decreased in chronically hypoxic rats if the rats were given ibuprofen (4mg/kg/day) or dexamethasone (0.1mg/kg/day) while acclimatizing. Ibuprofen is a well established non-steroidal anti-inflammatory medication, blocking cyclooxygenases 1 and 2, while dexamethasone is a glucocorticoid steroid with strong anti-inflammatory and immunosuppressant effects. Furthermore, the study also examined isolated carotid bodies and their sensitivity by recording from the carotid sinus nerve while bathed in a hypoxic solution (Fig.1.3). Normoxic carotid bodies displayed a hypoxic response that persisted with ibuprofen and dexamethasone administration. Chronically hypoxic carotid bodies displayed a hypoxic “super-response” that was extinguished to a baseline hypoxic response with administration of either anti-inflammatory drug.

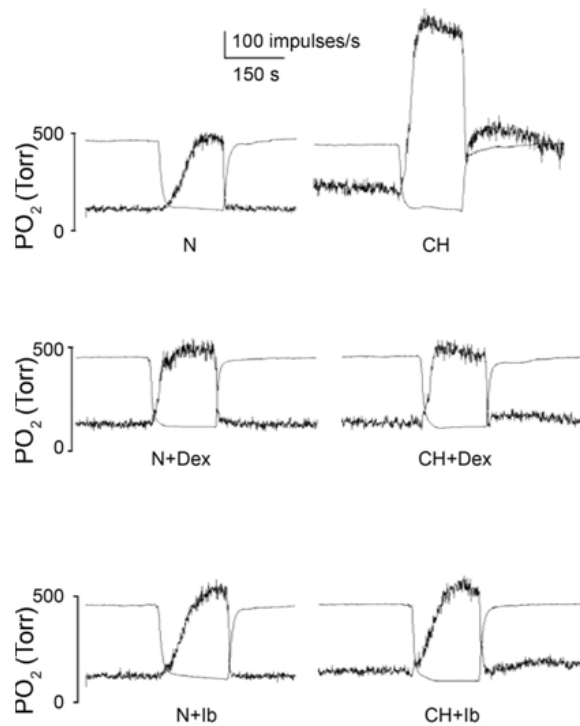


Fig.1.3. Recordings from isolated superfused carotid bodies vs. time. In normoxic rats (N), the carotid body displays increased firing when P_{O_2} is decreased in superfusate (top left). This response is not affected by administration of dexamethasone (Dex, middle left) or ibuprofen (Ib, bottom left). Chronically hypoxic rats (CH) display a carotid body super response to hypoxia (top right) that diminishes to the control (N) response with Dex (middle right) or Ib (bottom right).

These studies were confined to isolated carotid bodies and did not examine inflammatory marker expression in the respiratory control centers of the medulla or how those expression levels and how ventilation change with administration of anti-inflammatory drugs such as ibuprofen. The first of the studies detailed below focused on examining these specific issues.

Recent studies by a group in Hong Kong replicated the isolated carotid body experiment only instead of using chronic sustained hypoxia, the researchers exposed rats to chronic intermittent hypoxia (CIH) where for 7 days the rats were exposed to an O_2 concentration alternating between 21% and $5 \pm 0.5\%$ at 60 cycles/h, 8 h per day

diurnally (Lam *et al.*, 2012). They observed a similar pattern of chronic inflammation in the carotid body with increased levels of IL-1 β , IL-6, and TNF α as well as increased cytokine receptors and increased inflammatory cell migration. Like the chronic sustained hypoxic rats, rats undergoing CIH but treated with dexamethasone (0.1 mg/kg/day) or ibuprofen (4 mg/kg/day) showed decreased macrophage infiltration. Markers of oxidative stress also increased but showed attenuated levels when anti-inflammatory drugs were used to treat the CIH rats.

1.5 Mechanisms of Plasticity in Intermittent Hypoxia (IH)

1.5.1 Long Term Facilitation (LTF) in IH

Intermittent hypoxia (IH) consists of short bouts of hypoxia followed by a return to room air. Since the 1940s, exposure to IH in anesthetized animals has been known to produce persistent hyperventilation with increased respiratory motor neuron activity that persists for hours after several bouts IH (MacFarlane & Mitchell, 2008). Only in recent years has this phenomenon been labeled as long-term facilitation (LTF, Fig. 1.4 and 1.5) and become an active area of intensive research using animal models. LTF

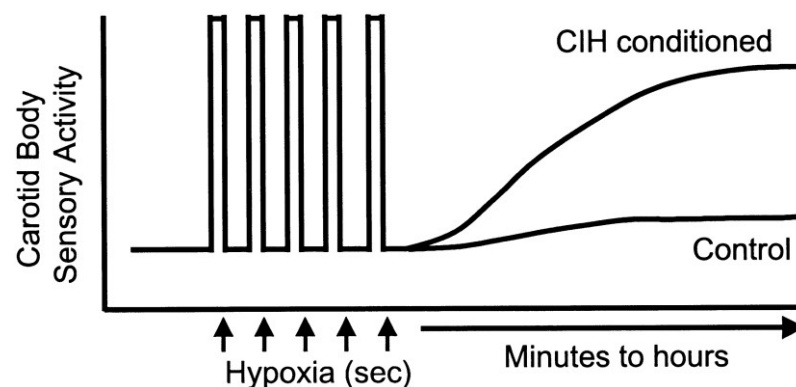


Fig.1.4. Schematic illustration of induction of LTF of sensory discharge in the rodent carotid body by CIH. (Prabhakar, 2001).

has been observed even in asleep human subjects (Babcock & Badr, 1998). Hypothesized roles of LTF include stabilizing breathing during sleep,

counterbalancing respiratory depression during hypoxia, and reflecting general adaptive mechanisms in the motor control of breathing with clinical applications in amyotrophic lateral sclerosis (ALS) and spinal cord injury (Wilkerson *et al.*, 2007). Ultimately, LTF is a current model for learning and memory in the respiratory control system, though its full significance remains unclear.

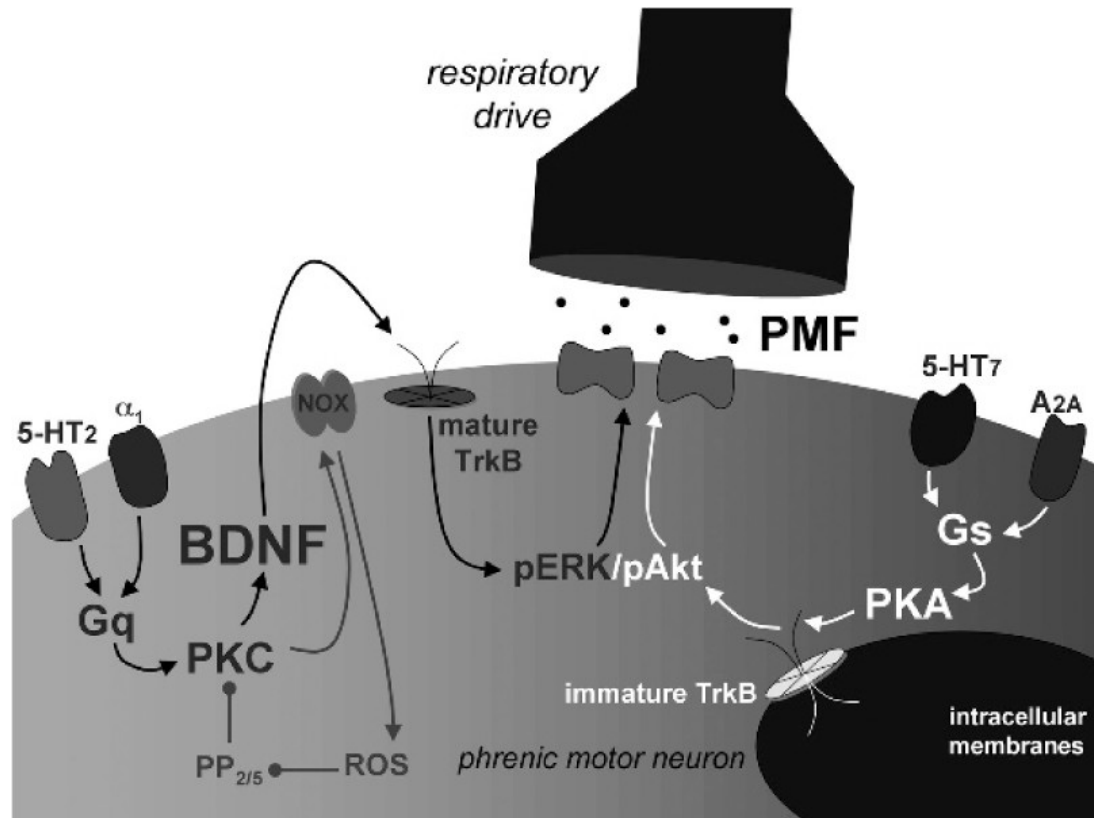


Fig.1.5. Current model of convergent pathways to LTF. The “Q” pathway (*left, black arrows*) undergoes intermittent activation of Gq-coupled receptors (5-HT₂; α₁). Subsequent activation of protein kinase C (PKC) initiates new brain-derived neurotrophic factor BDNF synthesis and increases NADPH oxidase (NOX) activity producing ROS. Protein phosphatases (PP_{2/5}) normally inhibit LTF, but are themselves inhibited by NADPH oxidase (NOX) dependent ROS formation. The “S” pathway (*right, white arrows*) represents another pathway to LTF. (Dale-Nagle *et al.*, 2010).

ROS in Learning and Memory

Not only are ROS potential signals for hypoxia but data show that ROS may play an important role in learning and memory. Previous work has implicated ROS in long-term potentiation (LTP), a leading model of learning and memory that involves N-methyl-D-aspartate (NMDA) receptors. LTP and LTF have some similarities but also have some important differences (Klann & Dever, 2004). ROS scavenging by superoxide dismutase (SOD) was actually shown to prevent LTP in hippocampal CA1 neurons, a region in the brain where spatial learning and memory are believed to occur; however, once LTP had been established, scavenging ROS was ineffective at reversing LTP (Klann, 1998). In addition, ROS may assist in regulating the activity of protein kinase C (PKC), a wide-ranging enzyme activated by bound NMDA receptor, during LTP (Klann *et al.*, 1998). SOD overexpressing mice have shown decreased spatial memory in a water maze task along with impaired LTP in the hippocampus (Gahtan *et al.*, 1998). Interestingly, in older mice used to study the effects of aging, which increases ROS and decreases the ability to scavenge ROS, treatment with a SOD mimetic led to better performance at the water maze task and longer life-span (Quick *et al.*, 2008). These results imply a range of ROS necessary for normal functions where ROS levels outside of the range contribute to decreased spatial learning and shorter life. Thus, with ROS having demonstrated neural signaling roles in a model of learning and memory, perhaps they may have a role in the increased HVR seen during VAH.

1.5.2 ROS and Intermittent Hypoxia

IH, or repeated short bursts of hypoxia (10% O₂), has been studied as a model for sleep apneas (Wilkerson *et al.*, 2007). ROS have been shown to be increased in both the carotid bodies and cerebral cortex of mice exposed to IH (Peng *et al.*, 2006).

Superoxide from NOX has also recently been implicated as a critical signal for LTF in the hypoglossal and phrenic motor nuclei (MacFarlane *et al.*, 2008; Macfarlane *et al.*, 2009). Indeed, the group responsible for these studies hypothesizes an essential role for ROS in sustained hypoxia in those nuclei as well. They postulate that ROS modulate the balance between protein kinases and phosphatases, favoring kinases and, thus, phosphorylation, see Fig.1.6 (Wilkerson *et al.*, 2007). However, they have evidence that in acute sustained hypoxia (25 minutes of 10% O₂) ROS formation is insufficient to elicit enough phosphorylation to induce LTF.

A recent study by Khan *et al.* (2011) has shed some light on the mechanisms of increased ROS levels in the carotid body with IH exposure. IH has been shown to transcriptionally upregulate NOX-2 activity in the carotid body (Peng *et al.*, 2009; Yuan *et al.*, 2011) and to inhibit Complex I activity of mitochondria (Khan *et al.*, 2011). The IH mediated NOX activation was demonstrated to be short-lasting and, through Ca⁺², to inhibit mitochondrial Complex I activity, producing long-lasting mitochondrial ROS. In addition to increased production, the same group has shown that IH down-regulates ROS scavenging by SOD-2, further increasing ROS levels (Nanduri *et al.*, 2009).

1.5.3 ROS: Production, Scavenging, and Detection

Reactive oxygen species (ROS) refer to a wide array of chemical substances, including but not limited to superoxide (O₂⁻), hydrogen peroxide (H₂O₂), peroxynitrite (ONOO⁻), and hydroxyl radical (OH⁻) all of which have an unpaired electron on an oxygen atom. However, *superoxide* will be the focus of the studies described in Chapter 3.

In 1956 Harman hypothesized a causative role for ROS in aging, and ROS have been implicated in toxicity and neurodegeneration ever since (Harman, 1956).

Through the work of Mittal and Murad (1977), however, O_2^- was shown to increase production of cGMP, a second messenger, by stimulating guanylate cyclase. These experiments marked the beginning of investigation into the beneficial cellular signaling effects of ROS.

ROS are produced through a variety of cellular processes which include synthesis by NADPH oxidase (NOX) and mitochondria (Bedard & Krause, 2007). Other mechanisms include Ca^{+2} release, indirect synthesis by monoamine oxidase (MAO) or cyclooxygenase (COX), and synthesis by nitric oxide synthase (NOS) at an

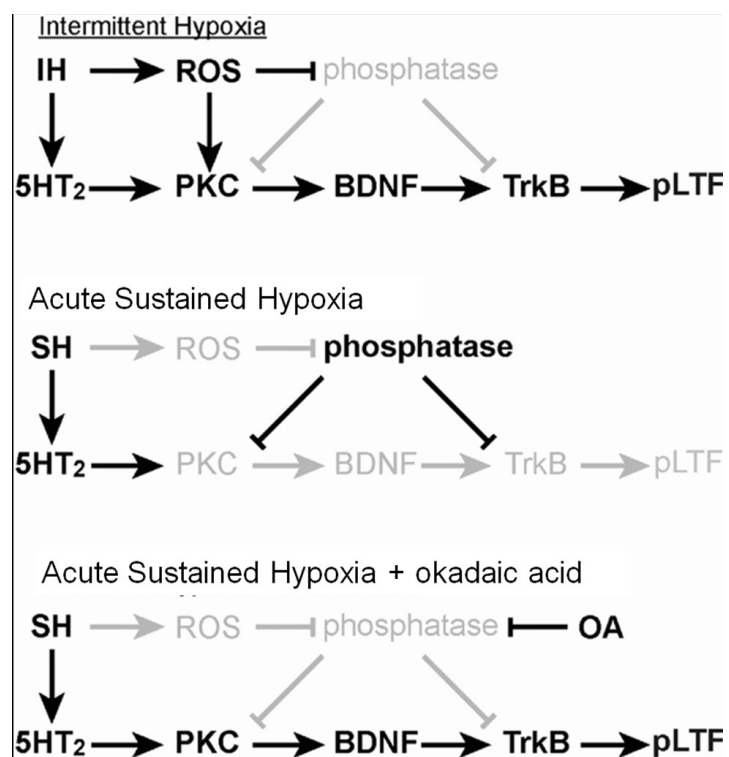


Fig.1.6. LTF Model adapted from (Wilkerson *et al.*, 2007) shows ROS inhibiting phosphatases that, in turn, act to inhibit protein kinase C (PKS) and TrkB. In intermittent hypoxia, ROS also stimulates PKC allowing the 5HT₂ signal to proceed to produce LTF. Acute hypoxia, however, decreases ROS, removing the inhibition on phosphatase, blocking LTF. Okadaic acid can be used as a substitute for ROS and block the phosphatase allowing LTF.

active site different from that used to produce NO. Ascertaining the relative significance of each mechanism in the nervous system has proven difficult, though NOX is believed to be chiefly responsible for O_2^- generation in non-neuronal cells especially in immune cells such as phagocytes (Bedard & Krause, 2007; Kishida & Klann, 2007). Multiple NOX isotypes with different subunits and differential tissue expression are known to exist (Bedard & Krause, 2007).

ROS in too high amounts can lead to toxicity and neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease, so the scavenging of potentially toxic ROS is a critical component of normal, physiologic ROS signaling. Superoxide is typically processed by superoxide dismutase (SOD), although it can spontaneously dismutate to H_2O_2 as well. H_2O_2 , in turn, is degraded into H_2O and O_2 by catalase. Three isoforms of SOD have been described based upon where they localize: cytosolic SOD (CuZnSOD), mitochondrial SOD (MnSOD), and extracellular SOD (ECSOD) (Thiels & Klann, 2002). Scavengers are also important when considering changes in ROS levels since increased ROS can occur either through increased production or decreased scavenging.

Numerous *in vivo* and *in vitro* ROS detection methods exist, yet none is without its problems. One method involves the use of a fluorescent dye, dihydroxyethidium (DHE), which can be injected into an animal and will provide an indirect measure of cumulative ROS production from the time of injection. DHE can be injected intraperitoneally in excess amounts and will distribute throughout the whole animal, even crossing the blood brain barrier and entering neurons. Within a neuron, DHE will bind superoxide releasing the fluorescent ethidium ion which binds, in turn, to nucleic acids creating a robust fluorescent signal capable of visualization with laser excitation with a confocal microscope. This method is limited, however, by

the time course required to allow DHE to distribute throughout the tissues and produce a measurable signal.

Another ROS detection method is the aconitase assay. Aconitase catalyzes the isomerization of citrate to isocitrate in the tricarboxylic acid cycle and is inactivated by superoxide (Gardner & Fridovich, 1991; Flint *et al.*, 1993). The level of O_2^- can be determined based on how much activated aconitase is present in a tissue. The enzymatic activity can be measured spectrophotometrically at 340nm, corresponding to the formation of NADPH a product downstream of the isocitrate. This method is limited *in vivo* by the inability to provide a measure over time in the whole animal; it can only provide a snapshot in time and requires tissue harvesting and, thus, sacrificing of the animal.

Another method of detection is using electron paramagnetic resonance spectroscopy (EPR). EPR is based on the spin properties of the unpaired electron in a magnetic field. It represents the only way to truly and directly measure ROS production. However, the procedure and reagents necessary to carry out the spectroscopy preclude the use of the technique for ROS detection in brain tissue of unanesthetized, live animals; it has been used mainly for measurements in blood.

1.5.4 ROS and O_2 Sensing

According to an early hypothesis by Helmut Acker (1981) for sensing O_2 by carotid bodies, ROS were proposed to be products of a membrane-bound NOX that would shift the cellular redox balance and, thus, act as a second messenger in oxygen sensing. ROS were believed to be produced proportionally to the Pa_{O_2} of the surrounding tissue environment so that ROS decreased in hypoxia. However, there is also evidence to support an increase in ROS during hypoxia, i.e. decreased P_{O_2} (Duranteau *et al.*, 1998; Guzy *et al.*, 2005; Bedard & Krause, 2007).

Elevated ROS mimics hypoxia by inhibiting redox sensitive K^+ channels, and the overexpression of the NOX4 isotype augments the inhibitory effect of hypoxia on TWIK (Tandem of Pore Domains in a Weak Inward Rectifying K Channel)-related acid sensitive K⁺-1 (TASK-1) channels (Chamulitrat *et al.*, 2003; Lee *et al.*, 2006). In addition, the source of ROS in hypoxia may be different in different tissues (Marshall *et al.*, 1996; Guzy *et al.*, 2005; He *et al.*, 2005). Mitochondrial cytochrome-c oxidase and NOX represent the leading candidates for ROS production for the hypoxic phenomenon with both molecules having demonstrated their importance in hypoxia sensing (Guzy *et al.*, 2005; He *et al.*, 2005). Although the focus of the experiments described above is the source of increased ROS production, ROS levels may increase also by reduced scavenging or quenching ability.

In brain stem areas sensitive to P_{aO_2} (the rostral ventrolateral medulla and pre-Bötzinger complex), heme oxygenases appear to have differential expression based on hypoxic exposure (D'Agostino *et al.*, 2009). Heme oxygenase-2 increases bilirubin levels which can in turn quench ROS and regulate ion channels (Na^+ , K^+ , and TASK) as described above.

Affects of chronic hypoxia on ROS in O_2 sensitive chemoreceptors are relatively unstudied. One study (Maiti *et al.* 2006) measured ROS production in the brains of rats exposed to a simulated altitude of 6100m with a rate of ascent of 300m/min for 20min. Areas of the brain examined were the cortex, striatum, and hippocampus with the hippocampus being most susceptible to hypoxia. Each area showed increased levels of free radical formation, primarily H_2O_2 , using a spectrofluorimetric assay with 2',7'-dichlorofluorescein-diacetate (DCFHDA).

1.6 Specific Aims

The goals of this dissertation are to investigate the roles of inflammation and ROS in the medullary respiratory control centers during acclimatization to hypoxia.

The first set of experiments tests the hypothesis that inflammation is part of the essential brainstem signaling mechanism that produces VAH. In order to test this hypothesis, I proposed experiments measuring IL-1 β , IL-6, and TNF α in the NTS in rats being treated with the anti-inflammatory drug ibuprofen or saline during the acclimatization process as well as the HVRs of rats under those conditions to determine the physiologic consequence of inflammatory blockade. The methods and results of these experiments were published recently (Popa *et al.*, 2011), and the article is included in its entirety in Chapter 2.

The second set of experiments tests the hypothesis that ROS decrease with hypoxic exposure and that they contribute to VAH in mice. In order to test this hypothesis, I proposed experiments measuring ROS in normoxia, acute hypoxia, chronic sustained hypoxia, and acute intermittent hypoxia under control conditions and under chronic anti-oxidant treatment. I used chronic anti-oxidant treatment to attempt to disrupt VAH and, thus, tested the HVRs of mice undergoing blockade of ROS production.

Chapter 2: Ibuprofen blocks time-dependent increases in hypoxic ventilation in rats.

2.1 Introduction

Ventilatory acclimatization to hypoxia is a time-dependent increase in ventilation during chronic sustained hypoxia (Powell *et al.*, 1998). Several laboratories have demonstrated that this involves increased O₂-sensitivity of carotid body chemoreceptors resulting in increased afferent input to ventilatory chemoreflexes for a given arterial P_{O₂} after chronic hypoxia (reviewed by (Powell, 2007). Additionally, we have shown time-dependent changes in the central nervous system (CNS) processing of carotid body chemoreceptor input resulting in a greater respiratory motor output for a given afferent input (Dwinell & Powell, 1999). Plasticity in the carotid body-ventilatory chemoreflex with chronic hypoxia involves changes in neurotransmitters and ion channels in the carotid bodies and CNS (reviewed by Powell, 2007), and changes in gene expression controlled by Hypoxia Inducible Factor 1, HIF-1 (Kline *et al.*, 2002; Powell & Fu, 2008).

Recently, inflammatory processes have been shown to be important for the increased O₂-sensitivity of carotid bodies with chronic sustained hypoxia (Liu *et al.*, 2009). Using an *in vitro* rat carotid body preparation, these investigators showed that chronic sustained hypoxia increases the frequency of action potentials recorded in the carotid sinus nerve when P_{O₂} is lowered in a solution superfusing the carotid body. They also found increased mRNA expression for inflammatory cytokines in the carotid body with chronic hypoxia. The increased cytokine expression, as well as the

increased carotid body neural response to acute hypoxia, was blocked by ibuprofen and dexamethasone treatment during the chronic hypoxia.

To test the physiological significance of these inflammatory signals for plasticity in chronically hypoxic *in vitro* carotid bodies, we studied the effects of ibuprofen on ventilatory acclimatization to hypoxia in conscious rats. Also, we measured the effects of chronic hypoxia and ibuprofen on cytokine expression in the central nervous system (CNS) and present evidence that inflammatory signaling in the CNS contributes to ventilatory acclimatization to hypoxia.

2.2 Methods

2.2.1 Experimental Animals

Adult, male rats (Sprague-Dawley, Charles River) were housed in standard rat cages in a vivarium, with a 12:12-h light-dark cycle and fed a standard rat diet *ad libitum*. All experiments were approved by the University of California, San Diego, Animal Care and Use Committee. The experiments conformed to national standards for the care and use of experimental animals as well as the American Physiological Society's "Guiding Principles in the Care and Use of Animals." At the end of an experiment, rats were euthanized with an overdose of sodium pentobarbital (Sleep Away, 150mg/kg IV) and death was confirmed by open chest exsanguination via cardiac ventricular incision.

2.2.2 Experimental Groups

Animals were housed in individual cages in either normoxia (N) or chronic hypoxia (CH). Rats acclimatizing to CH were placed in a hypobaric chamber for 7 days at 0.5 atm ($P_{I_{O_2}} \approx 70$ Torr); N rats were housed in the same room outside the

chamber. CH rats were significantly lighter than N rats at the time of experiments ($268 \pm 8\text{g}$ vs. $349 \pm 7\text{g}$). The chamber was opened once daily for ~20 min for regular cage maintenance and drug injection or for removing animals for experimentation. Four experimental groups were studied: (1) NS = chronic normoxia with saline injections, (2) NI = normoxia with ibuprofen injections, (3) HS = CH injected with saline, and (4) HI = CH injected with ibuprofen.

2.2.3 Experimental Drugs

Ibuprofen solution (2 mg/ml) was prepared from commercially available syrup (20mg/mL, McNeil Children's Motrin Berry-Flavored, Lot SHM046) diluted with sterile normal saline (0.9% NaCl). Ibuprofen injections (4mg/kg IP, ≈ 0.6 ml) and saline injections of a similar volume (0.6 ml) were made daily.

2.2.4 Surgical Procedures

After 5 days in designated experimental conditions, we catheterized animals to sample arterial blood gases. Anesthesia was induced with 5% isoflurane in 100% O₂ and isoflurane was decreased to 2.5-3% for maintenance. A customized catheter (polyethylene PE-50 drawn out under heat to an end diameter approximately equivalent to PE-10 tubing) was inserted in the femoral artery through a skin incision. The catheter was advanced into the abdominal aorta, secured to the artery with suture (5.0 silk) tied around tubing glued to the outside of the catheter, filled with heparin (10,000 Units/mL) and heat-sealed at distal end. The distal end of the catheter was led subcutaneously along the back to emerge from a metal button (Instech) sutured to underlying muscle at the base of the neck. A spring sheath secured to the button protected the distal end of the catheter.

Finally, a telemetric thermometer (G-2 Emitter, Respironics) was implanted in the abdominal cavity through a midline incision. Muscle and skin were sutured together separately and antibiotic ointment (Fura-Zone, Squire) was placed over every incision site.

2.2.5 Physiological Measurements

After 1 week of acclimatization, a whole body plethysmograph (7L, plexiglass) was used to measure ventilatory responses to hypoxia and hypercapnia measurements as previously described (Reid & Powell, 2005) Briefly, flow (3 L/min) was maintained through the chamber during ventilatory measurements and pressure changes due to warming and humidification of inhaled gases were measured (MP45 with 2 cm H₂O diaphragm, Validyne) and used to calculate tidal volume by the method of Drorbaugh and Fenn (1955). Arterial blood was sampled through polyethylene tubing (PE 50) leading out the top of the chamber and attached to the arterial catheter with 25 gauge hypodermic tubing. O₂ and CO₂ concentrations in the chamber were changed with a mass flow controller (Sable Systems MFC-4) and monitored with a mass spectrometer (Marquette 1100). Ventilatory data was digitized (Labview, National Instruments, v2.5.0) and analyzed using a custom Matlab-based program for V_T, f_R and their product, \dot{V}_I .

Ventilatory measurements were performed with baseline O₂ levels equal to the environment in which the animals were housed the previous week (NS and NI =21%; HS and HI=10%). The animals acclimated to the box for 30min and were then given a 5-10min challenge (10% O₂ for NS and NI; 21 or 30% O₂ for HS and HI) to test responsiveness. Then the protocol began to measure ventilation in normoxia (21% O₂), hypoxia (10% O₂) and hypercapnia (7% CO₂, 30% O₂) with a return to baseline conditions (e.g. 21% O₂ for NS and NI) for 15min between conditions. Different gas

levels were maintained for 15min and ventilation was measured in a stable sample with at least 20 breaths between 10 and 15min after changing gas concentrations. Arterial blood was sampled (0.2mL) during ventilatory measurements for P_{O_2} , P_{CO_2} , pH and hematocrit analysis (GEM Premier 3000, Instrumentation Laboratory). Blood gas values were corrected to body temperature as measured with the telemeter.

In a separate cohort of rats ($n = 3-5$ per experimental group), we measured metabolic rates in normoxia and hypoxia (10% O_2). The protocol was similar to that for the ventilatory measurements described above. O_2 and CO_2 concentrations were measured in the plethysmograph under steady state conditions and then gas flow into the chamber was blocked and the chamber was sealed for 3min. O_2 and CO_2 concentrations were measured at the end of this period, then flow through the chamber was restored and \dot{V}_{O_2} and \dot{V}_{CO_2} were calculated using mass balance.

2.2.6 Cytokine Measurements

In a third cohort of rats exposed to the same experimental conditions ($n = 5$ per group) we measured expression of mRNA for IL-1 β , IL-6, and TNF α in biopsies of the nucleus tractus solitarius (NTS) using quantitative PCR (qPCR). Rats were euthanized and their brainstems were removed and immediately frozen in liquid nitrogen. Samples were allowed to thaw just enough to make a transverse section between the *calamus scriptorius* and 2mm rostral. This section was refrozen in liquid nitrogen and then allowed to thaw just enough to allow the dorsal region, containing the nucleus tractus solitarius (NTS), to be cut away from the remainder of the section. The NTS biopsies were placed in a 1.5mL Eppendorf tube and weighed. We extracted mRNA in 150 μ L buffer in the Eppendorf tubes (RNeasy Minikit, Qiagen, and rapid homogenization), adjusted the final volume to 600 μ L with buffer and performed an

added step of on-column DNase digestion (Qiagen). Concentration of mRNA was determined with a micro-volume UV-vis spectrophotometer (Nanodrop 2000). A cDNA synthesizing kit (Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR) was used to reverse transcribe the mRNA and prepare the samples for qPCR using Power SYBR Green (Applied Biosystems). qPCR reactions were performed in duplicate on the same day with reaction mix (SYBR green, primers, RNase free H₂O, and cDNA) for each reaction made together to reduce pipetting errors. Primer sequences were as follows (FW, forward; RW, reverse):

Rat IL-1 β : FW CACCTCTCAAGCAGAGCACAG

RW GGGTTCCATGGTGAAGTCAAC

Rat IL-6: FW TCCTACCCCAACTTCCAATGCTC

RW TTGGATGGTCTTGGTCCTTAGCC

Rat TNF α : FW AAATGGGCTCCCTCTCATCAGTTC

RW TCTGCTTGGTGGTTTGCTACGAC

Housekeeping genes used to normalize the above cytokines were Rat β -2 microglobulin (B2M) and ribosomal protein L13a (RPL13A), which are unchanged by hypoxia in the central nervous system (Tang *et al.*, 2010). Primer sequences were as follows (FW, forward; RW, reverse):

Rat B2M: FW CGTCGTGCTTGCCATTCAGA

RW GACGGTTTTGGGCTCCTTCA

Rat RPL13A: FW CCATTGTGGCCAAGCAGGTA

RW GCTTTCGGAGAAAGGCCAGA

2.2.7 Data Analysis

Ventilatory (V_T , f_R , \dot{V}_I), blood gas (P_{aO_2} , P_{aCO_2} , pH_a) and metabolic (\dot{V}_{O_2} , \dot{V}_{CO_2}) data were analyzed with mixed-factorial ANOVA: 2 inspired O_2 levels (21 and 10%) X 4 experimental groups (NS, NI, HS, and HI). When significant interactions ($P < 0.05$) were found, the LSD post-hoc test was used to determine which means were significantly different ($P < 0.05$).

Cytokine qPCR data was analyzed with 1-way ANOVA and when significant interactions were detected ($P < 0.05$), a post-hoc Tukey's test was used to determine which means were significantly different ($P < 0.05$) from each other (Keppel & Wickens, 2004).

All results are reported as mean \pm standard error of the mean (SEM). Statistical analysis was performed using SPSS software (PASW Statistics, v17.0) and 2-tailed tests were used unless stated otherwise.

2.3 Results

2.3.1 Ventilatory Response to Hypoxia

In normoxic rats, ibuprofen had no effect on \dot{V}_I or its components, f_R and V_T , breathing normoxic or hypoxic gas (Fig.2.1). Chronic hypoxia increased \dot{V}_I in saline treated rats breathing normoxic and hypoxic gas, as expected for ventilatory acclimatization to sustained hypoxia. Ibuprofen had no effect on the persistent hyperventilation in chronically hypoxic rats breathing normoxic gas but ibuprofen blocked the increase in \dot{V}_I breathing hypoxic gas (Fig.2.1). The increase in f_R with chronic hypoxia was smaller with ibuprofen treatment although there were no significant interactions between experimental groups and O_2 level being breathed for either f_R or V_T (Fig.2.1).

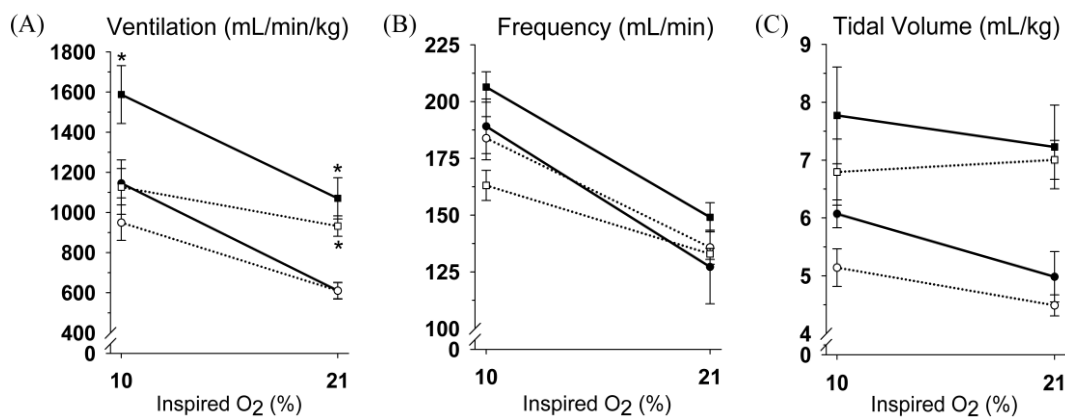


Fig.2.1. Ventilatory responses to hypoxia in normoxic (circles) and chronically hypoxic rats (squares) treated with saline (filled symbols) or ibuprofen (open symbols). Ibuprofen significantly reduces V_I in chronically hypoxic rats breathing 10% but not 21% O₂ (* $p \leq 0.02$ vs. NS at a given O₂%, $n = 6$ except NS $n = 5$).

2.3.2 Arterial Blood Gases and Metabolism

Table 2.1 shows that P_{aO_2} decreased with inspired O₂ but was not significantly affected by chronic hypoxia or ibuprofen. However, P_{aCO_2} was significantly lower with chronic hypoxia versus normoxia breathing 21% or 10% O₂ with no differences between ibuprofen or saline treatment. Arterial pH was lower also in all conditions with chronic hypoxia versus normoxia, as expected for the metabolic compensation for the chronic respiratory alkalosis with chronic hypoxia. Hematocrit increased significantly with chronic hypoxia (44.1 ± 1.1 versus $35.6 \pm 0.8\%$ for controls, $p < 0.05$).

Table 2.1. Arterial blood gases and pH of normoxic and chronically hypoxic rats treated with saline (NS and HS, respectively) or ibuprofen (NI and HI, respectively). For given inspired O₂, Pa_{CO₂} and pH_a decreased with chronic hypoxia but there was no effect of ibuprofen vs. saline and Pa_{O₂} was not different. †p < 0.01 vs. NS, *p < 0.03 vs. NI.

Group	O ₂ (%)	Pa _{O₂}	Pa _{CO₂}	pH _a
NS (n=5)	21	86.8±1.9	38.0±0.6*	7.44±0.01
NI (n=6)		86.5±3.9	34.2±1.0†	7.45±0.01
HS (n=2)		86.5±4.5	18.0±1.0†*	7.39±0.02*
HI (n=3)		83.3±1.5	19.3±0.9†*	7.41±0.00
<hr/>				
NS (n=5)	10	40.6±2.2	22.6±0.2	7.55±0.01
NI (n=6)		39.0±1.3	22.5±0.6	7.56±0.01
HS (n=2)		39.0±3.0	14.5±0.5†*	7.48±0.02†*
HI (n=3)		38.3±1.7	16.7±1.8†*	7.50±0.01†*

To determine if decreased metabolic rate could explain the similar Pa_{CO₂} with decreased \dot{V}_I in HI versus HS groups, we measured \dot{V}_{O_2} and \dot{V}_{CO_2} in a new cohort of rats using the same experimental treatments. Table 2.2 shows that acute hypoxia (10% O₂) decreased metabolic rate in all groups but there were no differences between groups. Acute hypoxia tended to increase R and it exceeded 1.0, indicating a non-steady state as CO₂ is washed out of the body during acute hypoxic hyperventilation. \dot{V}_I and Pa_{CO₂} were not significantly different in this small cohort of chronically hypoxic rats with ibuprofen (n=3) versus saline (n=3) treatment either: $\dot{V}_I = 1076 \pm 116$ vs. 1160 ± 282 mL/(min•kg) and Pa_{CO₂} = 17.7 ± 0.7 vs. 17.0 ± 2.0 Torr, respectively.

However, we did not observe a difference in \dot{V}_{O_2} or \dot{V}_{CO_2} with ibuprofen treatment before or after chronic hypoxia.

Table 2.2. O₂ consumption, CO₂ production and respiratory exchange ratio (V_{O_2} , V_{CO_2} , and R) of normoxic and chronically hypoxic rats treated with ibuprofen (NI and HI, respectively) or saline (NS and HS, respectively). Acute hypoxia (10% O₂) significantly decreased V_{O_2} and V_{CO_2} (* $p < 0.02$) but there were not differences between experimental groups. R tended to increase with acute hypoxia ($p = 0.06$).

Group	O ₂ (%)	\dot{V}_{O_2}	\dot{V}_{CO_2}	R
NS (n=4)	21	2.59±0.08	2.29±0.05	0.89±0.04
NI (n=5)		2.69±0.16	2.41±0.20	0.89±0.03
HS (n=3)		2.94±0.05	2.49±0.08	0.85±0.01
HI (n=3)		2.42±0.35	2.43±0.27	1.04±0.20
NS (n=4)	10	1.98±0.11*	2.09±0.10*	1.05±0.03
NI (n=5)		1.92±0.12*	2.08±0.10*	1.09±0.03
HS (n=3)		2.00±0.14*	2.23±0.11*	1.12±0.03
HI (n=3)		2.19±0.14*	2.30±0.12*	1.05±0.02

2.3.3 Responses to CO₂

The effect of ibuprofen to blunt the ventilatory response in chronically hypoxic rats was unique to hypoxic stimulation. There were no significant effects of ibuprofen on the ventilatory response to hypercapnia (7% CO₂ in 30% O₂) in normoxic rats (\dot{V}_I for NS (n=5) 1675±153 vs. NI (n=6) 1312±78 mL/(min•kg)) or chronically hypoxic rats (\dot{V}_I for HS (n=6) 2162±103 vs. HI (n=5) 1858±171 mL/(min•kg)). Also, there were no significant differences in arterial blood gases or pH_a with ibuprofen vs. saline in normoxic rats ($P_{aO_2} = 145 \pm 7.9$ vs. 137.5 ± 6.0 Torr, $P_{aCO_2} = 47.4 \pm 2.6$ vs. 45.0 ± 1.3 Torr, pH_a = 7.32 ± 0.01 vs. 7.33 ± 0.01 , n = 5 vs. 6, respectively) or chronically hypoxic rats ($P_{aO_2} = 159.0 \pm 1.0$ vs. 140.5 ± 12.5 Torr, $P_{aCO_2} = 39.5 \pm 1.5$ vs. 33.5 ± 1.5 Torr, pH_a = 7.12 ± 0.02 vs. 7.23 ± 0.01 , n = 2 vs. 2, respectively).

2.3.4 Inflammatory Cytokines in the NTS

Fig.2.2 shows cytokine expression (mRNA) levels in the NTS for the four experimental groups. The data is normalized to two housekeeping genes previously shown to be unaffected by hypoxia (Tang *et al.*, 2010). IL-6 and IL-1 β behaved similarly to \dot{V}_I with increases in chronic hypoxia that were blocked by ibuprofen, while ibuprofen had no effect in normoxic rats. TNF α was not affected by this duration of chronic hypoxia or ibuprofen.

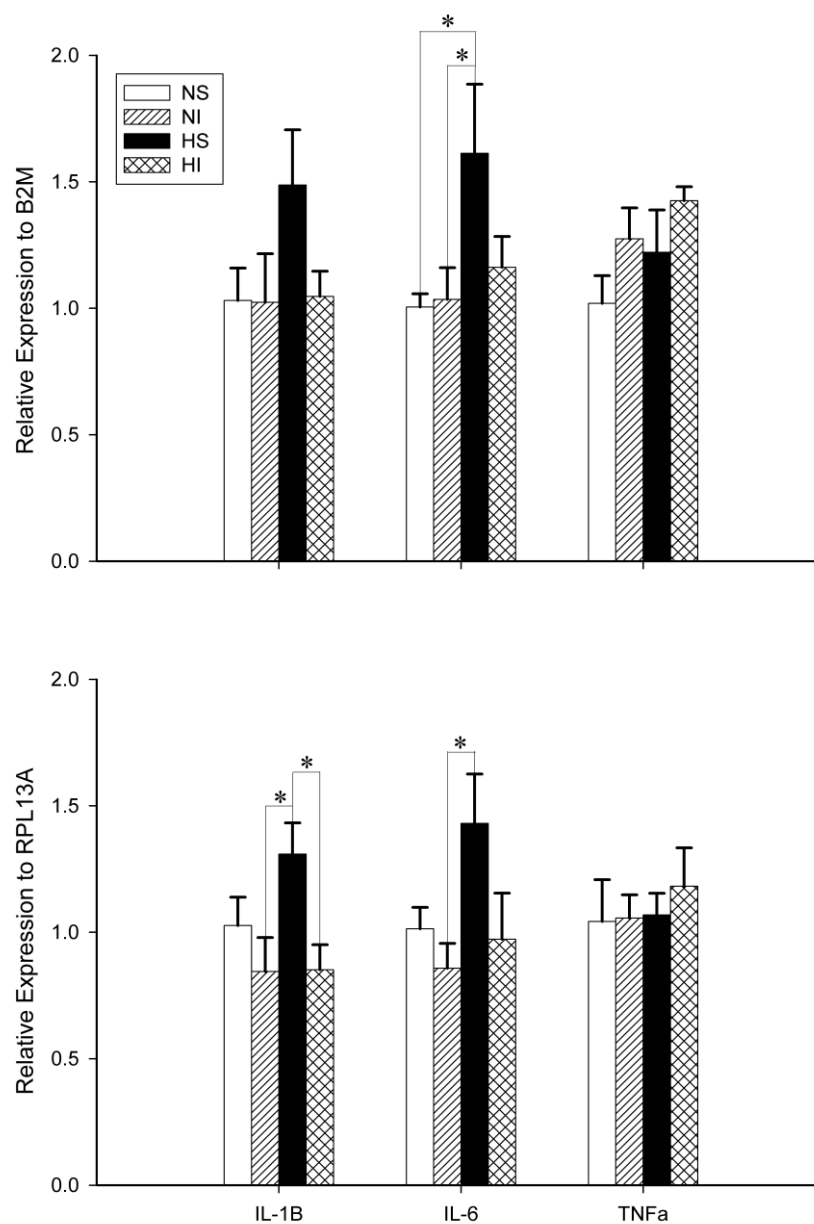


Fig.2.2. mRNA levels for IL-1 β , IL-6 and TNF α in control (N) rats and after 7 days of chronic hypoxia (H). Cytokine expression is normalized to B2M in the top panel and RPL13A in the lower panel. TNF α was not affected significantly by any treatment. Chronic hypoxia significantly increased IL-1 β and IL-6 (HS vs. NS) and this was blocked by ibuprofen (HI vs. HS). * $p < 0.05$, 1-tailed, $n = 5$ except $n = 4$ for IL-1 β in HS.

2.4 Discussion

The results showed an effect of ibuprofen on ventilation during hypoxia in chronically hypoxic rats but not normoxic rats (Fig.2.1). However, there were no significant effects of ibuprofen on ventilation in rats breathing normoxic or hypercapnic gas before or after acclimatization to chronic hypoxia. Hence, the data indicate that the previously demonstrated effect of ibuprofen to block the time-dependent increase in carotid body discharge with chronic hypoxia (Liu *et al.*, 2009) is physiologically significant in terms of blocking the time-dependent increase in hypoxic ventilation during chronic hypoxia.

2.4.1 Critique of Methods

Although ibuprofen blocked the increase in \dot{V}_I in acute hypoxia after chronic hypoxia, Pa_{CO_2} was not significantly increased by ibuprofen treatment (Table 2.1) as predicted if only ventilation changed. One possible explanation is that ibuprofen decreased CO_2 production but we did not measure any significant differences when we measured \dot{V}_{CO_2} in a separate group of rats (Table 2.2). There were only three rats in each chronically hypoxic group for these measurements, so we do not have strong statistical power to indicate that \dot{V}_{CO_2} is not changing with ibuprofen. Also, we did not observe a significant effect of ibuprofen on \dot{V}_I in this small cohort. Hence, further experiments will be necessary to resolve the role of metabolic responses in the effects of ibuprofen in ventilatory acclimatization to hypoxia.

Another possible explanation for decreased ventilation without increased Pa_{CO_2} is a change in physiological dead space with ibuprofen treatment. Olson (1994) reported increased physiological dead space, or decreased alveolar ventilation, in rats during acute hypoxia. Physiological dead space returned to normal values after 4 to 7

days of chronic hypoxia so we would not expect to see differences in our chronically hypoxic rats. However, if such an increase in physiological dead space involved inflammatory responses, then the time course and resolution of such changes could differ between experiments depending upon the immune status of the animals being studied. If ibuprofen blocked an increase in physiological dead space, then Pa_{CO_2} would be lower for any overall \dot{V}_I , which is in the direction of our results. Hence, this is another problem warranting further investigation.

Although not significant, Pa_{CO_2} tended to be higher in the chronically hypoxic rats treated with ibuprofen versus saline breathing 10% O_2 (16.7 ± 1.8 vs. 14.5 ± 0.5 Torr, respectively) or 21% O_2 (19.3 ± 0.9 vs 18.0 ± 1.0 Torr, respectively). Similar trends were not observed in normoxic rats, and in fact, Pa_{CO_2} was significantly lower with ibuprofen in normoxic rats breathing room air for unexplained reasons (Table 2.1). Summarizing, the most conservative explanation of the results is that ibuprofen blunts the time-dependent increase in hypoxic ventilation. However, these experiments do not have the statistical power to distinguish potential effects of the initial immune status of the animals or ibuprofen on changes in metabolic rate or gas exchange efficiency with chronic hypoxia.

The changes in cytokine gene expression we measured may not necessarily reflect changes in protein levels but they agree well with previous observations for carotid bodies (Lam *et al.*, 2008; Liu *et al.*, 2009). Similar to Liu and co-workers, we referenced cytokine gene expression to house-keeping genes shown to be stable in chronic hypoxia (Tang *et al.*, 2010). We also selected house-keeping genes (B2M and RPL13A) with expression levels similar to that of the cytokines being studied, to reduce errors from normalizing to extremely large values.

2.4.2 Physiological Mechanisms

Liu and co-workers (2011) propose that chronic hypoxia evokes some of the same mechanisms in the carotid body that are responsible for hyperalgesia, i.e. increased pain sensation to a noxious stimulus. This is based on several observations, including their own (Liu *et al.*, 2009). Hyperalgesia depends on cytokines that are released from activated immune cells and glia, such as IL-1 β , IL-6 and TNF- α , which increase excitability in sensory nerve endings (Watkins & Maier, 2002). These cytokines increase in the carotid bodies with chronic hypoxia (Liu *et al.*, 2009) and in sensory neurons they can up-regulate genes coding for specialized transduction molecules (e.g. acid sensitive ion channels, ASICs) and voltage gated ion channels (e.g. Nav1.7) that are involved in carotid body chemoreception (reviewed by Liu *et al.*, 2011). Also, the time course of changes in inflammatory cytokines and increased O₂-sensitivity in the carotid body with chronic hypoxia are similar and both are blocked with ibuprofen (Liu *et al.*, 2009). Finally, the dose of ibuprofen used in the isolated carotid body studies (Liu *et al.*, 2009) was the same as we used, and has been shown to suppress phenotypic changes in rat primary sensory neurons induced by inflammation (Voilley *et al.*, 2001). Considered together, the results support the idea that the effects of ibuprofen to block inflammatory-induced increases in carotid body O₂-sensitivity, which share common mechanisms with peripheral nervous system plasticity during chronic inflammatory pain, contribute to the changes in ventilatory acclimatization to hypoxia that we observed with ibuprofen.

Inflammatory cytokines also enhance synaptic transmission between primary afferents and neurons in the CNS in neuropathic pain, which involves both presynaptic and postsynaptic changes (Tsuda *et al.*, 2005). We hypothesize that inflammatory signaling contributes to the increased ventilatory sensitivity to carotid body

chemoreceptor afferent input with chronic hypoxia we observed previously (Dwinell & Powell, 1999; Wilkinson *et al.*, 2010). Fig.2.3 shows how we measured an “increased CNS gain of the hypoxic ventilatory response (HVR)” in anesthetized rats (Dwinell & Powell, 1999). Minute phrenic activity (the neural analog of ventilation), was greater in chronically hypoxic rats compared to normoxic controls for any given stimulation frequency of the carotid sinus nerve (the neural analog of hypoxic stimulation). In our current experiments, chronically hypoxic rats treated with ibuprofen would be predicted to have the same frequency of carotid body discharge as normoxic controls at any PaO_2 (cf. Liu *et al.*, 2009). Hence, the increased CNS gain of the HVR should have increased hypoxic ventilation in our chronically hypoxic rats treated with ibuprofen but we observed no difference from normoxic controls (Fig. 2.1). This implies that ibuprofen blocked the increased CNS gain of the HVR, in addition to carotid body sensitization. Given the effect of ibuprofen to block increases in IL-1 β and IL-6 with chronic hypoxia in the brainstem (Fig. 2.2), we propose that inflammatory signals are necessary for CNS plasticity contributing to ventilatory acclimatization to hypoxia.

Although chronic hypoxia increases inflammatory mediators in both the peripheral and central nervous system, the sources may differ in the carotid bodies and CNS respiratory centers. In carotid bodies, there is evidence for cytokines from invading immune cells, type I glomus cells and glial-like type II cells (Lam *et al.*, 2008; Liu *et al.*, 2009). We did not isolate the cellular source of cytokines with chronic hypoxia in our NTS biopsies but both neurons and glia are known to produce cytokines in the CNS (Watkins & Maier, 2002; Tsuda *et al.*, 2005). In the carotid body, the role of resident versus migrating macrophages with chronic hypoxia (cf. Liu *et al.*, 2009) remains to be determined. The source of migrating immune cells in the

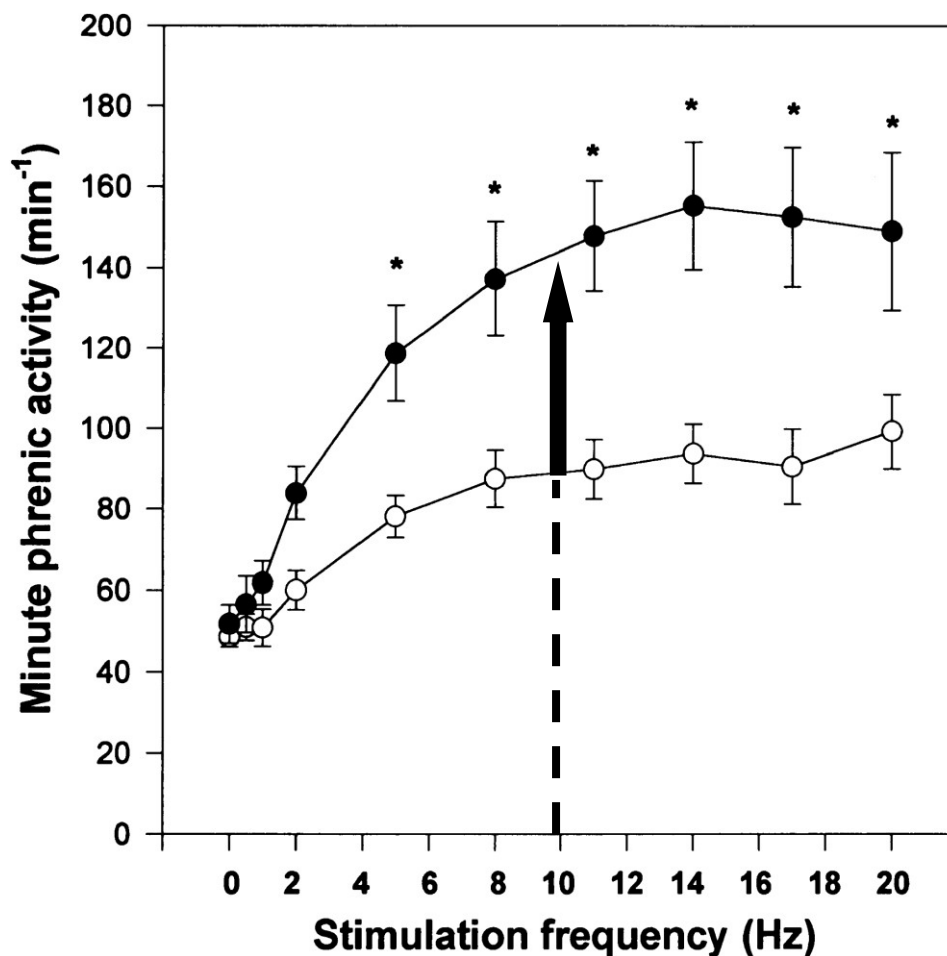


Fig.2.3. Effects of chronic hypoxia on the phrenic response to carotid sinus nerve stimulation in anesthetized rats (after Dwinell & Powell, 1999). Minute phrenic ventilation (the product of phrenic burst frequency and rectified, integrated phrenic amplitude) increases significantly more with increasing frequency of electrical stimulation of the carotid sinus nerve in chronically hypoxic rats (filled symbols) than in normoxic controls (open symbols). Vertical arrow at carotid sinus nerve stimulation frequency = 10 Hz shows how ventilation is predicted to increase in chronically hypoxic rats when the increase in carotid body O₂-sensitivity with chronic hypoxia is blocked by ibuprofen. By contrast, we observed no increase in hypoxic ventilation with ibuprofen in chronic hypoxia (cf. Fig. 2.1) implying ibuprofen blocks plasticity in the CNS with chronic hypoxia too.

carotid body is unknown but the highly vascularized nature of carotid bodies and their fenestrated endothelium (Fidone & Gonzalez, 1986) may render them more susceptible to migrating immune cells than other sites. Differences in the observed

time-course of cytokine changes with chronic hypoxia between carotid bodies and the NTS also suggest different sources, stimuli or responses. After 7 days of chronic hypoxia, IL-1 β returned to control levels in carotid bodies (Liu *et al.*, 2009) but it was elevated in the NTS (Fig.2.2). Finally, differential inflammatory responses to chronic hypoxia are suggested also by the different effect of ibuprofen on hypoxic versus normoxic ventilation after chronic hypoxia (Fig.2.1). Ventilatory acclimatization to hypoxia involves both an increased hypoxic ventilatory response, and persistent hyperventilation when normoxia is restored (Bisgard and Neubauer, 1995). The increased normoxic ventilatory drive involves altered regulation of arterial P_{CO₂} (Weil, 1986) and potentially different sites of respiratory plasticity (Nichols *et al.*, 2009). Further studies are needed to determine spatially and temporally specific inflammatory responses to chronic hypoxia in cardiorespiratory control pathways.

The most fundamental question raised by these results is, how does chronic hypoxia signal an inflammatory response? One candidate is HIF-1 α , which increases with chronic hypoxia in both carotid bodies and CNS respiratory centers (reviewed by Powell & Fu, 2008). Recently, HIF-1 α has been shown to regulate innate immunity and it is proposed that this relationship evolved to allow phagocytic cells to operate efficiently in the hypoxic microenvironments of infected tissues (Zinkernagel *et al.*, 2007). Conditional deletion of HIF-1 α in myeloid cells blocks the normal inflammatory response (Cramer *et al.*, 2003) and HIF-1 α increases TNF- α by a nitric oxide-dependent mechanism (Peyssonnaud *et al.*, 2005). Hypoxia also indirectly activates NF- κ B, which promotes transcription of TNF- α and other cytokines. In its inactive state in the cytosol, NF- κ B is bound to the inhibitory protein I κ B. When I κ B is phosphorylated by I κ B kinase (IKK- β), it separates from NF- κ B and is degraded. Then NF- κ B translocates to the nucleus and binds to DNA response elements to

promote transcription. In normoxia, IKK- β is inhibited by prolyl hydroxylases that depend on O₂ but in hypoxia, IKK- β is disinhibited, thereby activating NF- κ B (Rius *et al.*, 2008).

Conversely, NF- κ B promotes expression of HIF-1 α , linking the evolutionarily ancient stress responses of hypoxia and innate immunity (Rius *et al.*, 2008). Ibuprofen blocks nuclear translocation of NF- κ B, which would block NF- κ B transcription of IL-6, IL-1 β and TNF- α (Stuhlmeier *et al.*, 1999). In other words, ibuprofen has anti-inflammatory effects independent of its classical effect to inhibit cyclooxygenase 1 and 2, which control the production of inflammatory prostanoids that can sensitize nociceptors. However, a role for changes in prostaglandin modulation of the carotid bodies with ibuprofen treatment has not been ruled out (reviewed by Liu *et al.*, 2011).

2.4.3 Significance and Future Directions

The dose of ibuprofen used in this study is equivalent to the therapeutic dose used for pain in humans so it is interesting to consider how NSAIDs could affect people experiencing sustained hypoxia. Acute Mountain Sickness (AMS) is very common in otherwise healthy people who travel rapidly to altitudes above 2000m (West *et al.*, 2007). Headache is a universal feature of AMS, which is commonly treated with nonsteroidal anti-inflammatory drugs (NSAID) such as ibuprofen. AMS resolves as acclimatization to high altitude improves arterial oxygenation but our results predict that NSAID could block acclimatization. Hence, ibuprofen as a common treatment for AMS has the potential to prolong the problem by blocking or delaying the mechanisms of ventilatory acclimatization to hypoxia.

It is not known if patients with chronic hypoxemia, for example with chronic obstructive pulmonary disease, exhibit ventilatory acclimatization to hypoxia and have neural plasticity in ventilatory chemoreceptor chemoreflexes. Furthermore, it is not

known if ibuprofen can reverse ventilatory acclimatization to hypoxia or if it just blocks its induction. Hence, further study is necessary to determine if NSAIDs may also be contraindicated for patients with chronic hypoxemia.

Chapter 2, in full, is a reprint of the material as it appears in *Respiratory Physiology and Neurobiology* Volume 178, Issue 3, 30 September 2011, Pages 381–386. Popa D, Fu Z, Go A, Powell FL. The dissertation/thesis author was the primary investigator and author of this paper.

Chapter 3: Reactive oxygen species decrease in chronic sustained hypoxia but increase in acute intermittent hypoxia

3.1 Introduction

Intermittent hypoxia (IH), or repeated short bursts of hypoxia (eg. 10% O₂) has been studied as a model for changes in ventilatory control with sleep apneas (Wilkerson *et al.*, 2007). IH causes long term facilitation of ventilation (LTF), manifested as increased respiratory motor neuron activity that persists for hours after several bouts of IH, which is hypothesized to stabilize breathing during sleep apnea (Mahamed & Mitchell, 2007). However, LTF is also studied as a model for learning and memory in the respiratory control system (MacFarlane & Mitchell, 2008).

Recently, superoxide (O₂⁻) from NADPH oxidase (NOX) has been implicated as a critical signal for LTF in the hypoglossal and phrenic motor nuclei (MacFarlane *et al.*, 2008; Macfarlane *et al.*, 2009). ROS have been hypothesized to modulate the balance between protein kinases and phosphatases, favoring kinases and, thus, phosphorylation in sustained hypoxia (Wilkerson *et al.*, 2007). However, in acute sustained hypoxia (25 minutes of 10% O₂) ROS formation is insufficient to elicit enough phosphorylation to induce LTF.

Plasticity in respiratory control has also been described in chronic sustained hypoxia (i.e. days to weeks). Ventilatory acclimatization to hypoxia (VAH) is an increase in the acute hypoxic ventilatory response (HVR) and a persistent increase in normoxic ventilation after chronic sustained hypoxia (Powell *et al.*, 1998). Hence, LTF and VAH have properties in common but generally they have been considered as different mechanisms and LTF, but not VAH, depends on serotonin receptor activation

(Baker-Herman & Mitchell, 2002). While ROS are necessary for LTF (see above), they are not expected to increase with chronic sustained hypoxia. However, here we show changes in ROS with chronic sustained hypoxia and hypothesize that ROS serve as a modulator of respiratory centers in the brainstem and contribute to VAH with chronic sustained hypoxia.

3.2 Methods

3.2.1 Experimental Animals

C57BL/6 male mice aged 8-12 weeks were housed in standard mouse cages in a vivarium, with a 12:12-h light-dark cycle and fed a standard mouse diet *ad libitum*. All experiments were approved by the University of California, San Diego, Animal Care and Use Committee. The experiments conformed to national standards for the care and use of experimental animals as well as the American Physiological Society's "Guiding Principles in the Care and Use of Animals." At the end of an experiment, mice were euthanized with an overdose of sodium pentobarbital (Sleep Away, 150mg/kg IV) and death was confirmed by open chest exsanguination via cardiac ventricular incision.

3.2.2 Experimental Groups

Mice were randomly assigned to 8 experimental groups of 10-12 mice each based on the oxygen concentration breathed and on whether or not they received daily anti-oxidant treatment for 7-10 days prior to experimentation. Experiments consisted of either physiologic measurement or ROS level measurement. 4 different oxygen environments were used: normoxia (N, 21% O₂), acute sustained hypoxia (AH, 10% O₂ for 2 hours prior to measuring), chronic sustained hypoxia (H, 10% O₂ for 7-10days), and acute intermittent hypoxia (IH, 10% O₂ for 5 min followed by 21% O₂

for 5 min for 12 cycles/hour for 2 hours on the experimental day). Mice acclimatizing to chronic hypoxia were placed in a hypobaric chamber for 7-10 days at $P_{\text{barometric}} = 380\text{mmHg}$ ($P_{\text{I}_2\text{O}_2} \approx 70\text{ Torr}$) or in a flow-through box containing their standard cages during ROS measurements. In the flow through box, compressed air (21% O_2) and N_2 were mixed to give 10% O_2 that flowed in at 3L/min; this maintained $\text{CO}_2 \leq 0.3\%$ in the box. Normoxic mice were housed in the same room outside the chamber in standard cages. The chamber was opened once daily for ~20 min for regular cage maintenance and drug injection or for removing animals for experimentation.

Mice within each different O_2 environment were further divided by whether they received daily intraperitoneal (IP) injections of saline vehicle (**NS** = normoxic saline mice; **AHS** = acute hypoxic saline mice; **HS** = chronic hypoxic saline mice; **IHS** = acute intermittent hypoxic saline mice) or daily MnTMPyP (drug) injections (**NM** = normoxic MnTMPyP mice; **AHM** = acute hypoxic MnTMPyP mice; **HM** = chronic hypoxic MnTMPyP mice; **IHM** = acute intermittent hypoxic MnTMPyP mice).

In addition, 2 groups were used as controls in the detection of ROS. Three mice were assigned to a control group that received an IP injection of DMSO (the vehicle for ROS measurement), and another 3 mice were assigned to a group that received no IP injection. Both of these negative control groups were housed before and after DHE injection in room air.

3.2.3 Experimental Drugs

Manganese (III) Tetrakis(1-Methyl-4-Pyridyl)Porphyrin (MnTMPyP, Enzo Pharmaceuticals), a true scavenger of superoxide able to cross the blood brain barrier was prepared for injection by dilution with sterile normal saline (0.9% NaCl). MnTMPyP injections (5mg/kg IP, $\approx 0.1\text{ ml}$) and saline injections of a similar volume

(0.1 ml) were made daily for 7-10 days according to group assignment for both physiologic and ROS level measurements.

3.2.4 ROS Measurement with DHE and Fluorescent Microscopy

Dihydroethidium (DHE) was used as a fluorescent marker for O_2^- in mice of all groups according to an established protocol (Quick & Dugan, 2001; Quick *et al.*, 2008). DHE powder (Invitrogen, 0.25mg vial) was dissolved in 1.25mL of dimethyl sulfoxide (DMSO) to give a 20mg/mL stock solution. To protect DHE from reacting with air and light, the vial was sparged with N_2 after each use and stored at in a dark vial at $-20^\circ C$. Each mouse received 2 IP injections of 27mg/kg DHE, 30 minutes apart, for a total dose of 54mg/kg DHE. The dose per animal was determined and DHE was pipetted into Eppendorf tubes and protected from light. Each dose was diluted further 1:1 in sterile saline due to the toxicity of DMSO. A 20g mouse, therefore, received in each IP injection the following dose: $(0.020kg \times 27mg/kg) / (20mg/mL) = 27\mu L$ of the stock DHE solution which was diluted with 27 μL of sterile saline immediately prior to that injection for a total volume/injection of 54 μL . After 30 minutes, the mouse received a second, identical injection. The DHE was allowed to distribute throughout the mouse for 2 hours from the time of the first injection, after which the mouse was euthanized and its brain stem harvested.

HS and HM mice remained in a box with 10% O_2 flowing through for 2 hours after the first DHE injection. Meanwhile, the remaining mice were housed for 7 days in normoxia (21% room air). After 7 days, the mice are further divided into chronic normoxic (N) and acute hypoxic (AH) experimental groups. Once injected with DHE, N animals remain in room air for 2 hours while AH animals are placed into a box with 10% O_2 similar to that used for housing the mice in chronic hypoxia for 2 hours as well.

After 2 hours from the first injection, we anesthetized (sodium pentobarbital 210mg/kg body weight) each mouse and perfused them with heparinized saline (10mL) then 4% paraformaldehyde in PBS (≥ 40 mL) using left ventricular/aortic catheterization. Once fixation was complete, the brain stem was removed and post-fixed in 2% paraformaldehyde in PBS for 24-48 hours. After post-fixation was complete, the brainstem was soaked in 30% sucrose in PBS for 24-48 hours after which it was frozen and cryopreserved. Isopentane chilled in liquid N₂ was used to freeze the brainstem sitting on a cork and surrounded by tissue freezing medium. Once frozen, the brainstem was stored at -80°C and then sectioned using a cryostat at -18°C to produce 30 μ m thick sections. Sections were collected in deionized water for floated mounting on a slide. We mounted serial sections sequentially on 3 slides. One served as a primary slide for analysis while 2 served as backups. The mounted medullary sections were treated with anti-fade that contains DAPI staining for nuclei (ProLong Gold), cover-slipped, dried overnight, and sealed along the edges using clear nail polish.

An Olympus Fluoview 1000 spectral deconvolution confocal microscope was used to image the sections at 10x and 40x magnification. Two sections from each mouse medulla were used for analysis of intensity of DHE fluorescence within the nucleus tractus solitarius (NTS), a chemosensitive respiratory control nucleus in the dorsal medulla (Nattie & Li, 2002; Feldman *et al.*, 2003), DMV, and XII. Both sections contained portions of all 3 nuclei; the rostral section was at the level of the obex (\sim Bregma – 7.20mm), where the Fourth Ventricle begins closing off, while the other section was 180 μ m more caudal (\sim Bregma – 7.40mm) and under the area postrema (AP). Each nuclei was imaged bilaterally per section, resulting in a total of 6 images per animal. Laser settings to image DHE were excitation at $\lambda = 543$ nm and

emission at $\lambda \geq 590\text{nm}$ and were not changed between slides to ensure intensities remained comparable. Emission settings were 425-475nm for DAPI and 590-690nm for DHE.

Captured images were analyzed for fluorescent intensity in neurons using Image J. In ImageJ, the images were split into 2 channels (DAPI and DHE) and formatted in 16 bit gray scale. Regions of interest (ROI) in the image were uniformly drawn using a customized macro for each nucleus to ensure comparability. Essentially, the macro applied a grid to each image and then a rectangular region of interest was drawn onto the image (Fig.3.2). For the NTS, rectangular ROIs (25.1mm X 15.75mm) were used for the rostral and caudal sections (respectively) of the brainstem. The rostral NTS ROI was placed 30-45 μm from the dorsal edge of the brainstem and 30-45 μm lateral from the fourth ventricle. The caudal NTS ROI was placed 60 μm from the dorsal edge of the brainstem and 45 μm diagonal from the AP. For the DMV, a ROI with an area of 15.2mm² was used on the medial portion of the DMV. For the XII, the entire 40x image (~101mm²) of the medial portion of the XII was utilized as the ROI.

Each ROI was thresholded for intensity starting with the automatic value provided ImageJ and was adjusted, if necessary, until any background pinpoint staining was excluded. In order to determine the amount of staining in a cell, the “Analyze Particles” tool in ImageJ was used with an area threshold of 30 μm^2 -infinity and no limit on circularity. Results were output to a table from which the “Summarize” tool was used to provide per cell intensity averages and standard deviations. Outlines of the measured cells were also drawn in a separate image, displaying a map of the measured intensities. These drawings were used to qualitatively determine if the thresholding operation was appropriately identifying

individual cells within the ROI. The average fluorescent intensities of the neurons were multiplied with the number of neurons measured per image and then averaged per brainstem (i.e. individual animal). This weighting of the average was done to account for small discrepancies in the number of neurons detected per image and to ensure that each cell detected had an equal weight in the average intensity for the animal subject in question. The resulting average was normalized to the average value from the entire NS group. The normalized values were then averaged across the group in which each mouse was assigned and used to compare the intensities between groups using ANOVA.

Glial fibrillary acidic protein (GFAP) was used to stain glia in two NS mice to attempt to further localize DHE staining (Fig. 3.1). The brainstems were dissected, prepared, and sectioned as described above. The immunostaining was performed according to the free-floating IHC method (Bernstein *et al.*, 2004). Briefly, the sections were rinsed in PBS, treated with 3% H₂O₂ in methanol for 30 min at room temperature, blocked in 5% goat serum in phosphate buffered saline (PBS), incubated for 48hrs in chicken anti-GFAP antibody 1:1000 (Millipore, Billerica MA) in blocking solution at 4°C, then treated with biotinylated goat anti-chicken antibody 1:1000 (Promega Corp, Madison WI). An Avidin-Biotin complex staining kit (Vectastain, Vector Labs, Burlingame CA) allowed for visualization of glial cells expressing GFAP. Imaging was performed as before with the addition of GFAP to produce a triple stained image.

3.2.5 ROS Measurement with the Aconitase Activity Assay

In order to confirm the results of the experiment using DHE for the NTS region, the aconitase assay, an enzyme activity assay that indirectly measures ROS, was used as a second method of ROS measurement. Saline control groups (NS, AHS,

HS) were used, although AHS mice received 1 hour of hypoxia prior to sacrifice (vs. 2hr AH for those given DHE) in an attempt to better describe the time course of ROS changes in AH. These mice were anesthetized with 40 mg/kg of ketamine/xylazine, after which brainstems were dissected out and immediately frozen in liquid N₂. Using brainstem landmarks, the NTS was removed from the frozen brainstem and weighed.

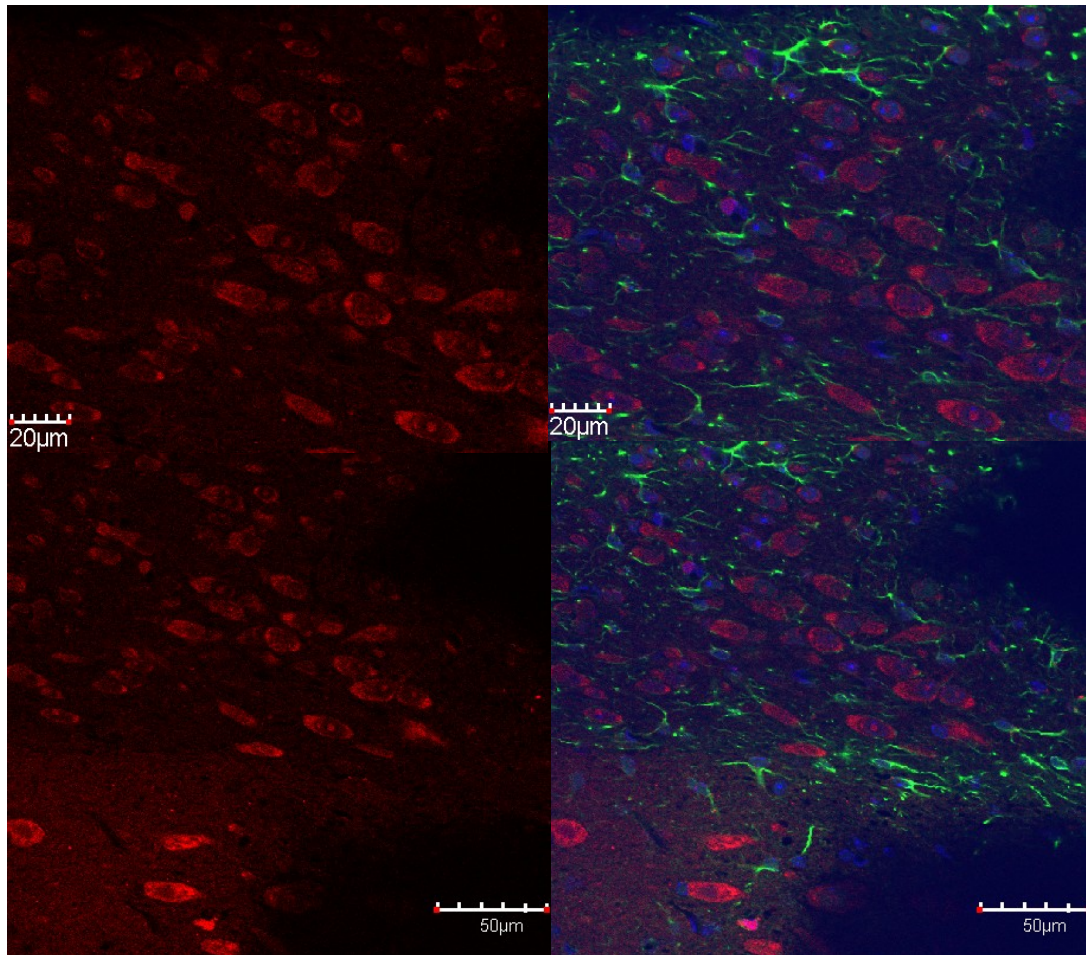


Fig.3.1. ROS are produced in neurons and not glia. A section of the NTS-DMV border region are shown in the top panels, and a section of DMV-XII border is shown in the bottom panels. DHE (red) staining is shown on the left panels, while DHE, GFAP(green), and DAPI (blue) staining in the right panels reveals that glia are stained with GFAP and DAPI but not DHE. Neurons, detected morphologically, show strong ROS signaling in the cytoplasm and nucleolus with no GFAP staining.

The NTS tissue was rapidly homogenized on ice in stabilizing buffer (50mM Tris-HCl (pH 7.4), 2mM Na Citrate, 0.6 mM MnCl₂) at a ratio <5:1. The homogenate was stored at -80°C for less than 2 weeks. To run the assay, the homogenate was thawed at 25°C, chilled on ice, and centrifuged at 2000 rpm for 3 minutes followed by 12,500 rpm for 8 min all at 4°C. The supernatant was collected and vortexed. A portion of this extract ($\leq 30\mu\text{L}$, 10-100 μg of protein) was added to 1.0 mL of freshly prepared reaction mix containing 0.2mM NADP⁺, 5mM Na Citrate, and 1 unit/mL Isocitrate dehydrogenase in 50mM Tris-Hcl + 0.6 mM MnCl₂ [pH 7.4] pre-equilibrated to 25°C in a 1cm cuvet. Absorbance at 340nm was monitored at intervals of 2 min for 60 min using a spectrophotometer (Beckman DU-640B). The reaction reagents were used as a blank solution, with absorbance measured every 20sec for 3 min to determine if the blank solution led to any absorbance changes. A dilution factor was calculated by dividing 1000 μL (the reaction volume in the cuvet) by the volume of sample extract in μL added to the 1mL cuvet. Aconitase activity was calculated from the linear increase in absorbance during the assay according to the following formula:

$$\frac{[(\text{Slope of the sample absorbance} - \text{Slope of the blank during 3 min}) * \text{Dilution Factor}]}{0.00622}$$

The 0.00622 term represents the extraction coefficient for NADPH in units of mL/nmol for a light path of 1cm (determined by the cuvet), assuming that isocitrate dehydrogenase converts one molecule of citrate to one molecule of NADPH. This formula ultimately calculates mUnits of aconitase activity since mU=nmol/mL. The enzymatic activity was then normalized to the protein concentration of the remaining extract from each sample, measured using the BioRad protein quantification kit. This normalization also eliminated the need to include the original dilution of the freshly

harvested and frozen brain tissue in isolation buffer. Final results were expressed in mUnits aconitase activity/ mg sample protein.

3.2.6 Physiological Measurements

Ventilatory responses to hypoxia and hypercapnia were measured in unrestrained 12-week old male mice in the NS, NM, HS, and HM groups using a whole body barometric plethysmograph (500ml) modified for continuous flow (Reid & Powell, 2005). Flow was maintained through the chamber while a pressure transducer (mp45 with 2cm H₂O diaphragm, Validyne, Northridge CA) recorded the pressure changes due to the warming and expansion of inhaled gases.

On the experimental day, the mice were weighed and sealed into the plethysmograph chamber along with a temperature and humidity probe (Thermalert TH5, Physitemp, Clifton NJ). A constant gas flow (330ml/min) was delivered with a rotameter (603, Matheson, Montgomeryville PA) and measured with a flow meter (S-110, McMillan Co., Georgetown TX) upstream of the chamber. Gases exited the chamber through a valve and into a vacuum pump (Model 25, Precision Scientific Co, Chicago IL) in order to isolate pressure changes from breathing in the chamber during constant flow with high input and output impedances. This also allowed chamber pressure to be maintained near atmospheric pressure and to reference pressure measurements in the chamber to atmospheric pressure. Inspired and expired oxygen and carbon dioxide fractions were measured by a mass spectrometer (MGA1100, Marquette Electronics, Milwaukee WI) sampling from the chamber.

All ventilatory parameters were recorded on a digital acquisition program (Labview v2.5.0, National Instruments, Austin TX), sampling at a rate of 200Hz. A customized Matlab-based program (Mathworks, Natick MA) was used to analyze a 20 second minimum region of interest between 10-15min after changing gas

concentrations. The program measured frequency (fR breaths/min) from peaks in the plethysmograph pressure pulses and calculates tidal volume (V_T ml/breath) using 0.2ml calibration pulses and equations previously described (Drorbaugh & Fenn, 1955). The product of fR and V_T is inspired ventilation, which was normalized to produce body mass ventilation (\dot{V}_I ml/min*kg). $\dot{V}O_2$ and $\dot{V}CO_2$ were also calculated for the corresponding region of interest by measuring inspired and expired O_2 and CO_2 fractions, chamber flow rate and correcting for evaporative water loss according to a previous method (Withers, 1977).

Ventilatory measurements were performed after the animals acclimated to the plethysmograph chamber for 30 min at their chronic inspired O_2 level (10% O_2 and 21% O_2). Animals were then exposed to a 5 min challenge of experimental gas (21% O_2 and 10% O_2) to test responsiveness. Then we measured ventilatory responses to 15 min bouts of normoxia (21% O_2), hypoxia (10% O_2), and hypercapnia (21% O_2 and 7% CO_2). Mice were returned to chronic baseline conditions (10% O_2 or 21% O_2) between ventilatory measurements.

3.2.7 Data Analysis

Statistical analysis was performed using SPSS (v19.0, IBM, Armonk NY). Ventilatory (\dot{V}_I , fR, V_T) and metabolic ($\dot{V}O_2$, $\dot{V}CO_2$) data were analyzed with a two-way mixed-factorial ANOVA: 2 inspired O_2 levels (10% and 21%) x 4 experimental groups (NS, NM, HS, and HM). When significant interactions ($p < 0.05$) were found, a LSD post hoc test was used to determine which means were significantly different ($p < 0.05$) by pair-wise comparisons. All results are reported as mean \pm standard error of the mean (SEM).

3.3 Results

3.3.1 ROS Measurements

Among saline injected control mice, acute exposure to 2 hours of hypoxia (AH) showed a trend of decreased DHE staining intensity when compared to N mice, examples shown in Fig. 3.2. Mice acclimatized to chronic sustained hypoxic exposure (H) demonstrated DHE staining levels that tended back toward baseline, normoxic levels; H was greater than AH but still less than N.

MnTMPyP showed a trend of decreasing DHE staining in normoxia (N) and H but no effect in AH in all 6 regions of the medulla studied (Fig. 3.3). A significant group effect ($*=p\leq 0.05$) of hypoxia was noted for N vs AH and N vs H mice in cXII.

MnTMPyP had a different effect in mice with an acute exposure to intermittent hypoxia (IHM). IHM mice showed a trend of increasing DHE intensity versus IH controls (IHS) or NS mice. IHS mice showed a trend of decreased DHE staining when compared to NS mice, similar to acute hypoxic controls (AHS, Fig.3.4). Hence, IH had opposing effects on mice depending on whether or not they were treated with MnTMPyP: drug injection showed a trend of increased superoxide while saline injection showed a trend of decreased superoxide.

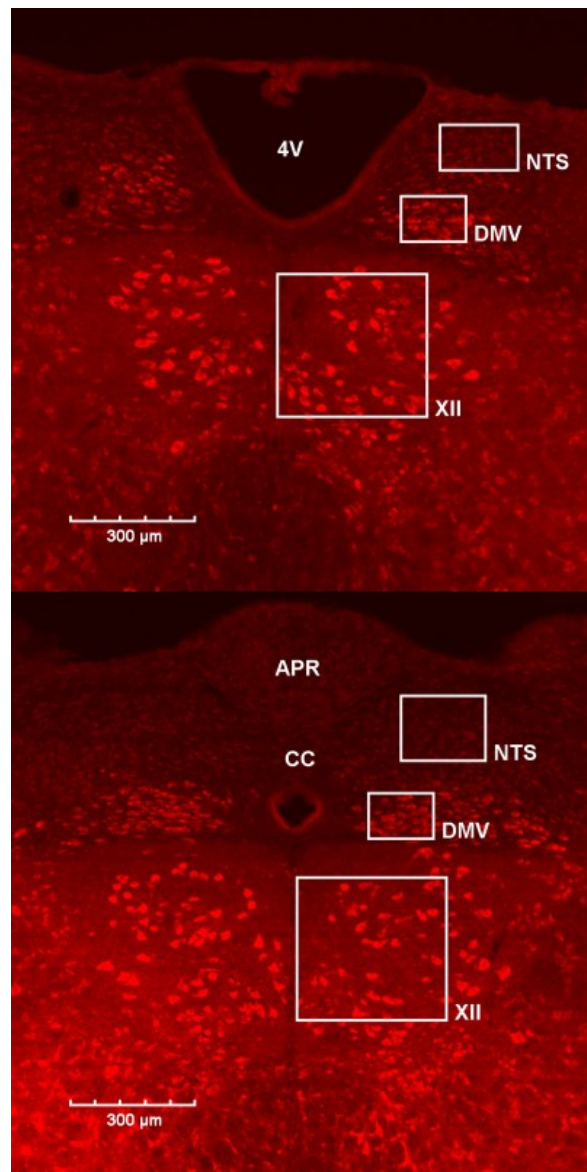


Fig.3.2. Rostral (Top, at obex) and Caudal (Above, 60μm caudal to obex) sections of the medulla stained with DHE. Boxes depict the regions of interest analyzed. 4V, Fourth Ventricle; APR, area postrema; CC, central canal.

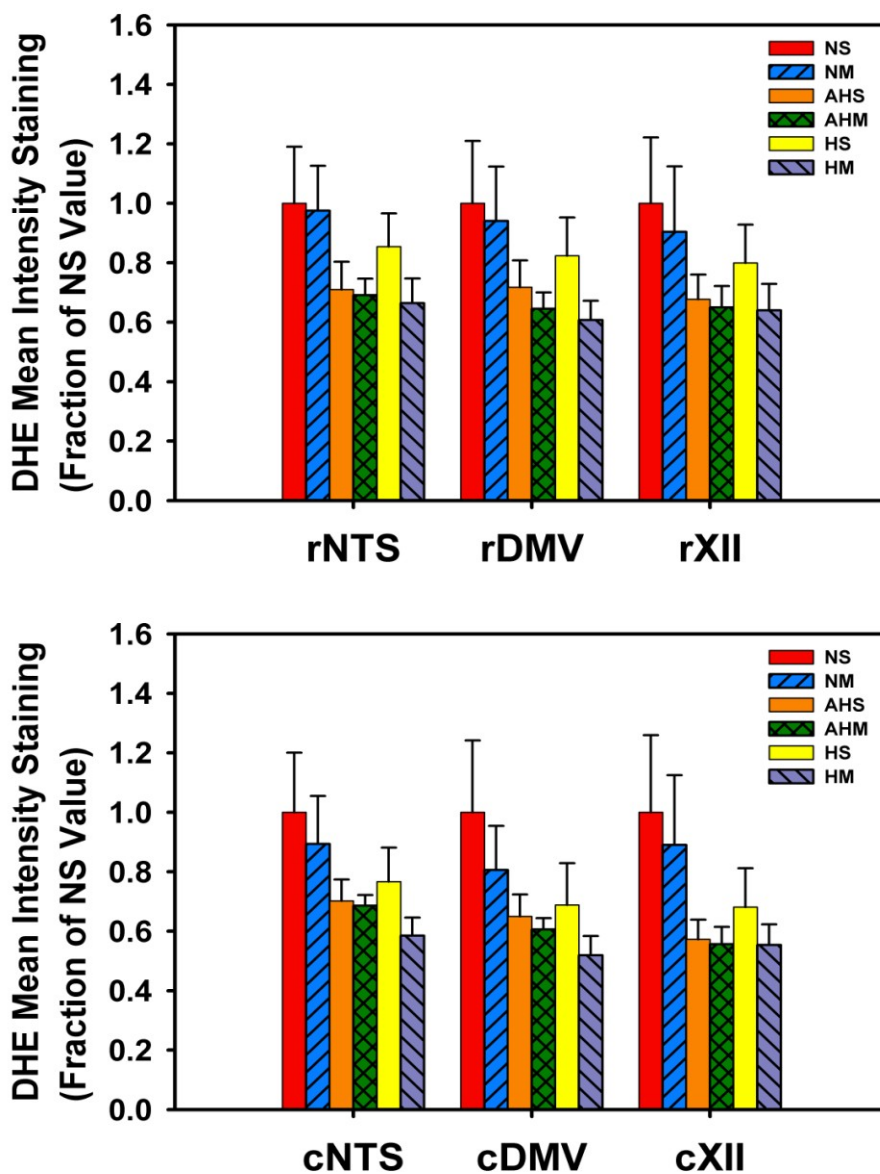


Fig.3.3. DHE staining shows a trend of a decrease with AH and then of a small increase with H. The effect of MnTMPyP on ROS in the rostral medulla in N, AH, and H (top) and in the caudal medulla (bottom) is a trend of decreased DHE staining among N and H mice with no effect in AH (n=4/group except AHS n=5). A significant group effect ($*=p\leq 0.05$) of hypoxia was noted for N vs AH and N vs H mice in cXII.

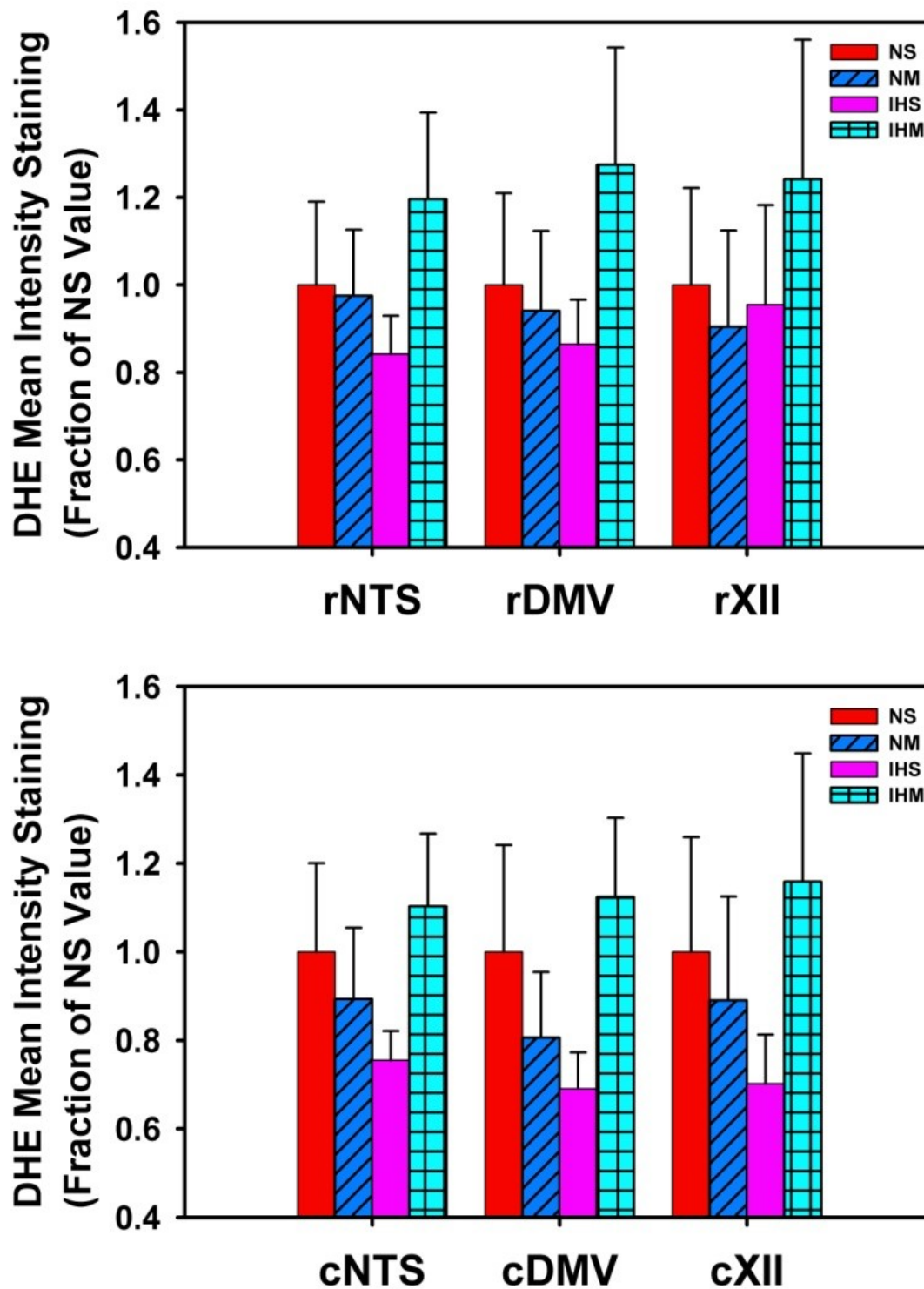


Fig.3.4. The effect of MnTMPyP on ROS in the rostral medulla in N and IH(top) and in the caudal medulla (bottom) is a trend of decreased DHE staining among IHS but increased staining among IHM (n=4/group except IHM n=5).

Aconitase enzyme activity assay results show the same trend as the results obtained using DHE. Aconitase activity, inversely correlated to ROS concentration, increased with 1 hour of acute sustained hypoxia exposure (ROS decreased) and then returned to baseline levels with chronic sustained exposure (Fig.3.5).

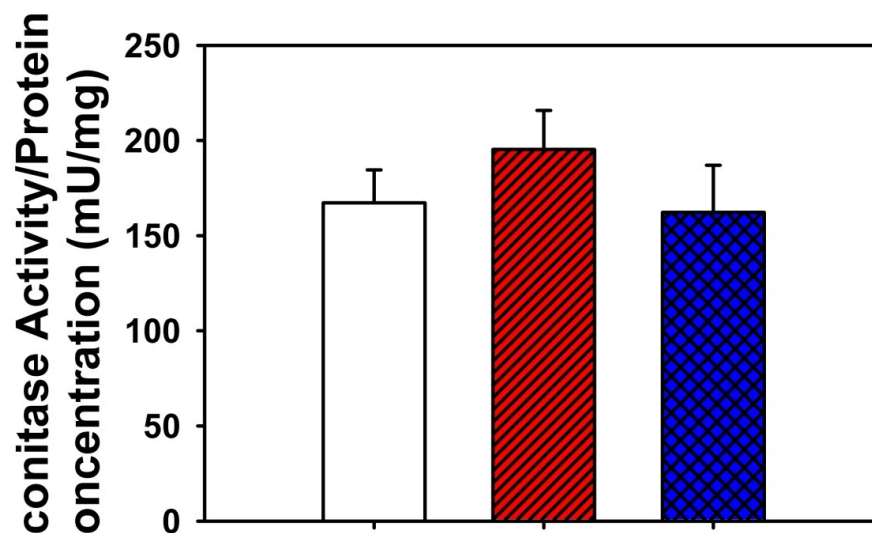


Fig.3.5. Aconitase activity enzyme assay results indicate a trend of decreased ROS in AH by greater enzyme activity whereas N and AH show similar activities. (n=6/group).

3.3.2 Ventilatory Responses

In order to test the hypothesis that ROS play a physiologic role in VAH, we measured ventilatory responses in mice given the superoxide scavenger MnTMPyP daily during exposure to chronic (7days) hypoxia or normoxia. HM mice showed no significant effect of daily MnTMPyP on \dot{V}_I compared with HS controls while breathing 10%O₂ and 21%O₂ (Fig.3.6A). NM mice treated with daily MnTMPyP showed significantly increased \dot{V}_I ($p \leq 0.05$) compared with NS controls while breathing 21% O₂ but showed no drug effect on \dot{V}_I compared with NS controls while

breathing 10%O₂ (Fig.3.6A). Both normoxic and chronically hypoxic saline injected control mice displayed HVRs and ventilations consistent with well established values. Hypercapnic hyperventilation in chronically hypoxic (H) and normoxic (N) mice showed no effect on ventilation with MnTMPyP administration (Fig.3.6D). When \dot{V}_I was divided into its component parts, f_R and V_T , no significant effect of drug treatment was noted (Fig.3.6B,C,E,F).

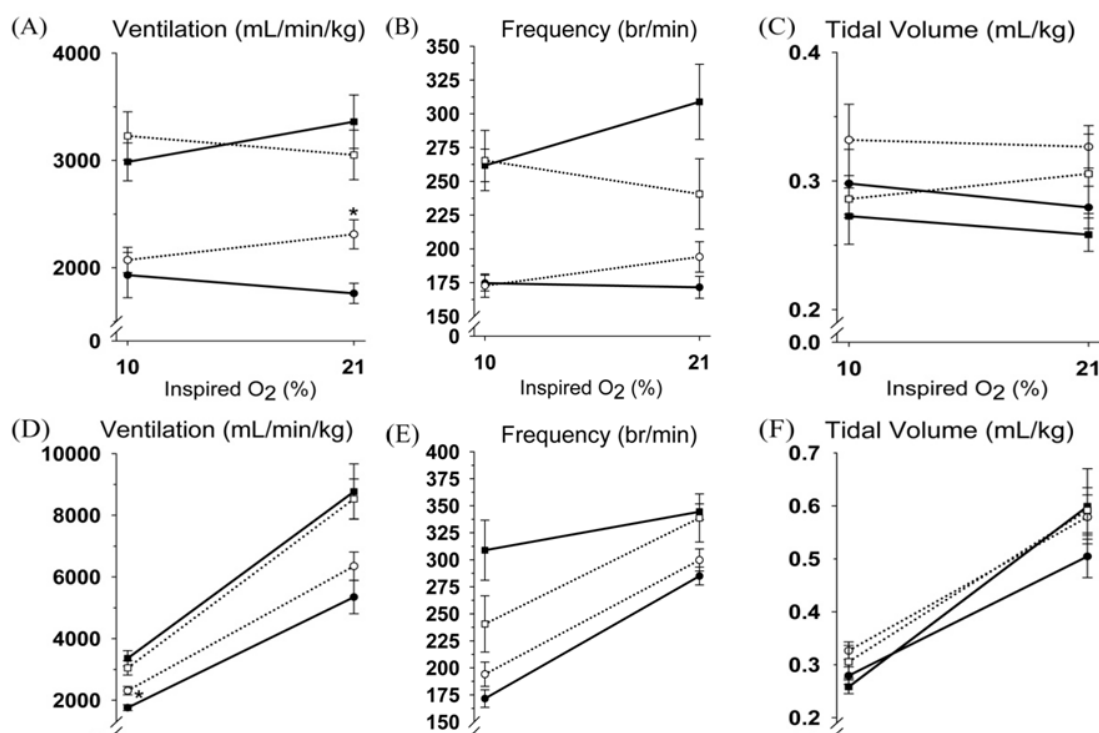


Fig.3.6. The effect of daily MnTMPyP on the poikilocapnic HVR (Panels A-C) and hypercapnic ventilatory response (HCVR, Panels D-F). Groups: NS (n=6), closed circles; NM (n=7), open circles; HS (n=6), closed squares; HM (n=6), open squares. A significant effect ($p \leq 0.05$) of MnTMPyP was seen among N mice breathing 21% O₂. The difference resulted from a mixed response of frequency and tidal volume.

O₂ and CO₂ consumption and body weight were also measured in these groups of mice. Daily MnTMPyP injection led to no changes in O₂ consumption or CO₂

production (\dot{V}_{O_2} and \dot{V}_{CO_2} , respectively) or the respiratory exchange ratio (R), although acclimatization did significantly increase \dot{V}_{O_2} and \dot{V}_{CO_2} . The metabolic data are summarized in Table 3.1. Chronically hypoxic mice weighed significantly less than normoxic mice at the time of experiments (23.7 ± 0.7 g for HS vs. 27.1 ± 0.7 g for NS, mean \pm SEM, $p=0.01$). MnTMPyP injection had no significant effect on body weight in normoxic mice (27.1 ± 0.7 g for NS vs. 27.3 ± 1.1 g for NM, mean \pm SEM) and chronically hypoxic mice (23.7 ± 0.7 g for HS vs. 23.3 ± 0.9 g for HM, mean \pm SEM).

Table 3.1. Metabolic measurements show a significant increase with acclimatization and no effect of daily MnTMPyP.

Group	O ₂ (%)	\dot{V}_{O_2} (ml/min*kg)	\dot{V}_{CO_2} (ml/min*kg)	R
NS (n=5)	21	35.38 \pm 4.28	27.15 \pm 3.31	0.77 \pm 0.01
NM(n=6)		42.74 \pm 5.07	33.59 \pm 3.97	0.79 \pm 0.01
HS (n=6)		52.21 \pm 2.33*	43.01 \pm 1.74*	0.83 \pm 0.01*
HM(n=6)		57.20 \pm 5.49*	48.09 \pm 5.70*	0.83 \pm 0.02*
NS (n=5)	10	23.34 \pm 2.64	20.22 \pm 1.69	0.88 \pm 0.03
NM(n=6)		21.47 \pm 2.26	19.07 \pm 1.25	0.91 \pm 0.04
HS (n=6)		40.53 \pm 2.22*	36.54 \pm 1.53*	0.91 \pm 0.03
HM(n=6)		39.34 \pm 4.10*	35.11 \pm 4.25*	0.89 \pm 0.02

* $p \leq 0.02$ when compared to NS for a given O₂%

3.4 Discussion

The results show trends of decreased ROS production with acute hypoxia and a return towards control with chronic hypoxia. MnTMPyP treatment of normoxic mice. MnTMPyP treatment tends to decrease ROS in chronically normoxic or hypoxic mice but not in acutely hypoxic mice. Intermittent hypoxia also decreased ROS like acute hypoxia but MnTMPyP increased ROS in intermittent hypoxia. Changes in ROS did

not correlate with changes in ventilation during acute and chronic hypoxia with chronic, systemic administration of MnTMPyP. Hence, the results did not support our hypothesis. However, they represent the first comparative measurements, to our knowledge, of ROS levels in the brainstem respiratory centers during acute, chronic, or acute intermittent hypoxia. Future experiments are necessary to rigorously disprove our original hypothesis.

3.4.1 Critique of Methods

Both DHE fluorescence and the aconitase activity assay represent indirect measures of ROS concentrations. However, direct ROS measurement technology (i.e. electron paramagnetic resonance, EPR) remains limited in applicability and feasibility at this time. The small size of the respiratory control centers in the brain of mice limits the utility of EPR, and the tissue must be dissected or biopsied before analysis much like in the aconitase enzyme assay. Comparisons of multiple nuclei within a single animal would be difficult given their proximity to one another. In addition, the utility of EPR is dependent on the spin trapping molecule used to stabilize the often short lived ROS. These technical challenges represent significant barriers for the widespread use of EPR especially in light of the excellent detection capabilities of DHE.

The IH protocol used to treat the study mice was assumed to elicit LTF. Most previous studies used anesthetized rats with phrenic nerve recording to observe LTF, although ventilatory LTF has been observed in conscious rats (Olson *et al.*, 2001) and mice (Terada *et al.*, 2008). However, we did not observe LTF in our conscious mice.

3.4.2 Physiological Mechanisms

Chronic sustained hypoxia appears to decrease ROS, which subsequently increases to approach baseline levels. Two potential mechanisms for the initial decrease exist: decreased production of ROS or increased scavenging. In the

production of ROS, mitochondrial dysfunction and NOX production appear to play the most significant roles in hypoxia (Bedard & Krause, 2007). With AH exposure, mitochondria might be expected to increase ROS levels due to low O₂ availability and increased dysfunction. However, ROS levels decrease in AH suggesting that mitochondria are likely not the significant source in changing ROS levels we measured with hypoxic exposure. NOX production of ROS, on the other hand, may decrease as O₂ decreases the substrate for superoxide. NOX isotypes have been shown to be targets of HIF-1 α , with increased mRNA and protein expression in mouse pulmonary artery smooth-muscle cells (Diebold *et al.*, 2010). Increased NOX expression may explain the trend for increase in ROS levels from acute to chronic hypoxia. With hypoxia decreasing O₂ substrate, increasing NOX expression could serve as a compensatory mechanism to reestablish ROS levels near to baseline. NOX-4 has also been previously shown to have hypoxic sensitivity via increased ROS production and decreasing K(v) channel currents in rats and isolated rat pulmonary arterial smooth muscle cells (Mittal *et al.*, 2012).

Increased scavenging by SOD may also play a role in the decreased ROS levels seen in acute hypoxia. While cytoplasmic SOD (CuZnSOD) and extracellular SOD (EcSOD) show constitutive expression, mitochondrial SOD (MnSOD) has been shown to be inducible by pro-inflammatory cytokines such as TNF- α and lymphotoxin (Wong & Goeddel, 1988) as well as IL-1 and lipopolysaccharide (Visner *et al.*, 1990). Our earlier work (Popa *et al.*, 2011) along with previous work (Liu *et al.*, 2009) demonstrated the increased expression of cytokines in both the CNS and glomus cells of the carotid body, establishing the possibility that MnSOD may be induced with AH and then regress as hypoxia proceeds from acute to chronic. Whether impaired NOX mediated production ROS or induced MnSOD scavenging or a combination of both

explains the pattern of ROS production in chronic hypoxia observed here remains unanswered. A first step in answering this question will be localizing where within a neuron the ROS levels change with hypoxic exposure.

When MnTMPyP was administered, AH mice showed ROS levels similar to those chronically hypoxic mice treated with vehicle control indicating that the effects of the hypoxic exposure and MnTMPyP administration were not additive and that a basal level of ROS was maintained. This suggests that MnTMPyP does not access ROS produced in acute hypoxia.

ROS levels and minute ventilation did not appear to correlate with one another. While ROS levels decrease in AH and approach N levels in H, ventilation increases in AH and then increases further with H. The effect of reducing ROS using MnTMPyP appears to be best illustrated in the significant hyperventilation seen among normoxic mice breathing room air. While daily MnTMPyP injection did not block VAH, ROS clearly can modulate ventilation. One limitation of the study is that IP MnTMPyP does not allow for distinctions between carotid body and CNS effects. Hence, the mechanism by which ROS do so remains unclear.

Intermittent hypoxia produced unexpected results. The effect of IH on DHE staining was dependent on whether or not MnTMPyP had been given in the 7-10 days prior to IH exposure and DHE measurement. In MnTMPyP treated mice, IH increased DHE signaling whereas IH decreased DHE signaling in saline treated control mice. One interpretation of the data is that chronic MnTMPyP administration downregulates the innate antioxidant capabilities of the mice. IH has been well established as a pro-oxidative environmental stressor with mitochondria producing the largest portion of the increase in ROS in mouse cortical neurons (Shan *et al.*, 2007). In the setting of an acute increase in ROS as expected in IH, the administration of MnTMPyP daily prior

to IH exposure may have caused a downregulation of the mice's physiologic scavenging ability and produced greater DHE staining as a result. Such downregulation may occur through the downregulation of MnSOD so that when exposed to IH, the MnSOD available for induction was insufficient to quench the ROS produced from IH.

The effect of IH among saline injected normoxic control mice, meanwhile, was a trend toward decreased ROS to a level between N and AH mice injected with saline. While ROS levels were expected to increase in these IH mice, the trend of decreased ROS via DHE measurement may indicate an under-reporting of the true ROS concentration. Alternatively, the net production of ROS in the medulla over 2 hours may decrease from decreases in ROS with bouts of acute hypoxia. The staining seen among IHM mice, therefore, is more likely a measure of the true ROS levels observed in IH. Finally, with a pro-oxidative stimulus such as IH, MnSOD may be induced and dismutate superoxide more quickly than DHE is able to diffuse into a neuron following IP injection. One way to investigate this possibility would be to more directly and rapidly administer DHE to neurons either through an injection into the CNS, an *in situ* CNS slice preparation with a DHE bath, or even cultured neurons.

3.4.3 Significance

The results of this study are the first to attempt to determine the role of ROS in VAH in those medullary centers. Measurements revealed trends for changes in ROS production with chronic hypoxia in the medullary respiratory control centers, although they were not statistically significant. Attempts to determine physiological significance of these potential changes in ROS for VAH using MnTMPyP were inconclusive. We speculate this is because of compensatory changes in ROS

metabolism with chronic administration of the anti-oxidant. This is based on the unexpected results with intermittent hypoxia, which decreased ROS levels measured by DHE in control mice but increased it in ROS in MnTMPyP-treated animals.

Our original hypothesis was that ROS could contribute to VAH by a mechanism similar to the role of ROS in LTF (Wilkerson *et al.*, 2007). If the trend for ROS levels to increase with chronic relative to acute hypoxia persists, then ROS may inhibit kinases and promote phosphorylation of NMDA receptors to increase ventilatory drive and/or the hypoxic ventilatory response in VAH as described for LTF. Increasing the number of animals studied should answer the questions about ROS in the medullary respiratory centers. However, different experimental designs will be necessary to demonstrate the physiological significance of such changes in ROS for VAH.

Chapter 4: Conclusions and Future Directions

4.1 Inflammation

We were able to show that the previously demonstrated effect of ibuprofen to block the time-dependent increase in carotid body discharge with chronic hypoxia (Liu *et al.*, 2009) is physiologically significant in terms of blocking the time-dependent increase in hypoxic ventilation during chronic hypoxia and that ibuprofen appears to act both in the carotid body per Liu *et al.* and the CNS. However, the mechanism and site of ibuprofen action within the CNS remain unclear.

In the study performed by Liu *et al.*, they showed monocyte invasion into the carotid body in response to cytokines and the chemokine, monocyte chemoattractant protein-1 (MCP-1), which were secreted by the chemosensitive type 1 glomus cells. However, the CNS is an immunologically privileged site that excludes immune cells via the blood brain barrier (Fabry *et al.*, 1994). Immune cell invasion of the CNS would only occur when the injury was sufficient to break down the blood brain barrier which we would not expect to occur at our level of hypoxia (10%O₂). Instead, I expect the immune response to occur primarily through the activation of microglia, the resident macrophages of the brain. Activated microglia have been shown to secrete the cytokines we measured in our experiment, i.e. IL-1, IL-6, and TNF- α (reviewed by Gehrmann *et al.*, 1995). Antibodies against biomarkers of microglial activation such as CD68, CD11b, and Iba-1 exist to assess the amount of activated microglia using immunohistochemistry (Choi *et al.*, 2012; Gallagher *et al.*, 2012). Future experiments

using immunohistochemistry to examine the expression of these microglial activation biomarkers could help define the immune response of the CNS during chronic hypoxic exposure.

In addition to microglia, endothelial cells lining the cerebral vasculature may also be a source of the inflammatory cytokines we measured in chronically hypoxic rats. Brain endothelial cells compose the blood brain barrier, yet they are capable of both secreting and responding to cytokines such as IL-6 and TNF- α (Verma *et al.*, 2006). Thus, brain endothelial cells may secrete the increased cytokines measured after acclimatization to hypoxia.

Immunohistochemistry can be used to localize cytokines produced by hypoxia within the brainstem (Amsen *et al.*, 2009) and could quantitatively distinguish between the production of cytokines by microglia, endothelial cells, or even possibly neurons. Varying times of hypoxic exposure (1 day, 3 days, 5 days) would need to be studied in addition to our standard 7 day exposure both with immunohistochemistry and also qPCR to better understand the time course of cytokine expression since the study by Liu *et al* established differential time courses of increased cytokines in the carotid body that may also occur in the NTS.

In addition to localizing the immune response to hypoxia, describing the downstream targets of this hypoxic response represents another important step for understanding the significance of this response. I propose that follow-up experiments using immunohistochemistry and gel electrophoresis to determine the location and amount of mediators downstream of cytokines. TNF- α and IL-1 β each transduces its

inflammatory signal through NF- κ B, a transcription factor with wide-ranging effects that include signaling through the JAK/STAT pathway, an alternative to the second messenger system (Devin *et al.*, 2000; Martin & Wesche, 2002; Gloire *et al.*, 2006). IL-6 also uses the well studied JAK/STAT pathway that eventually signals through Ras, a well studied family of small GTPases that modulate intracellular signaling networks (Heinrich *et al.*, 1998; Heinrich *et al.*, 2003). This suggests that well-known effects of Ras on the kinase cascade that includes mitogen-activated protein kinase (MAPK), capable of regulating mRNA translation, for example might occur with chronic hypoxia as well.

4.2 *Reactive Oxygen Species*

The results of our measurements of ROS show trends of decreased ROS production with acute exposure to hypoxia and with MnTMPyP treatment of normoxic mice. In contrast, IH increased ROS after MnTMPyP treatment. I speculate that ROS metabolism may change with chronic administration of the anti-oxidant, contributing to the unexpected effect observed during intermittent hypoxia where MnTMPyP treatment increased ROS relative to controls. Hence, I was not able to determine the physiological significance of the potential changes in ROS for VAH using MnTMPyP.

Further experiments are necessary to determine the species of ROS, their source and the location of their effects. In Figure 4.1, the ROS pathways and known scavengers and inhibitors are depicted as well as another detection method for superoxide exclusive to the mitochondria (MitoSox).

In addition, variations of MnTMPyP administration represent a logical next step. The current study used chronic MnTMPyP. However a one time, acute administration of the anti-oxidant in each of the conditions would prove useful in determining if chronic

MnTMPyP administration impacted ROS production or scavenging. CNS administration of MnTMPyP could also provide valuable insight about the ROS production in the CNS isolated from systemic changes in oxidative states (e.g. phrenic nucleus of the cervical spinal cord versus carotid body effects).

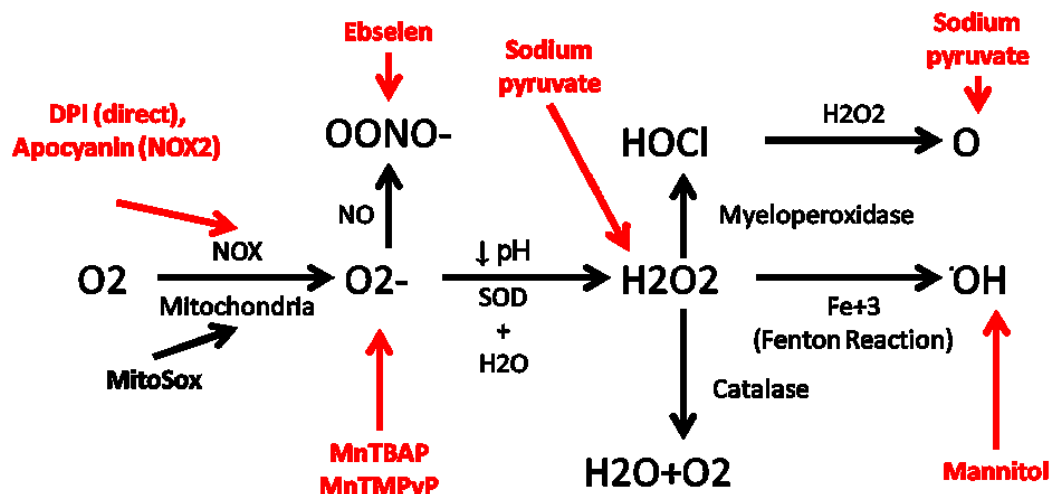


Fig.4.1. Reactive oxygen species production pathways (black) and scavengers/inhibitors (red) represent future experimental targets to hone in on the physiological significance of ROS on VAH. MitoSox is a detection molecule similar to DHE but is exclusive for detecting mitochondrial superoxide. O₂⁻, superoxide; NO, nitric oxide; OONO⁻, peroxynitrite; H₂O₂, hydrogen peroxide; HOCl, hypochlorous acid; O, singlet oxygen; ·OH, hydroxyl radical; DPI, diphenylene iodonium; MnTBAP, Mn (III) tetrakis (4-benzoic acid) porphyrin.

Localization of the ROS produced throughout the conditions of N, AH, and H is another important question to answer. Determining if the ROS changes occur through mitochondrial production changes using MitoSox or through NOX mechanisms using NOX inhibitors like DPI and apocyanin would have a significant impact in establishing a refined model of how ROS modulate VAH.

A future experiment with administration of the antioxidant and DHE simultaneously will be useful in quantifying the amount of decrease in ROS signaling

since the effect of acute treatment with MnTMPyP in hypoxia remains unknown. Other anti-oxidants also exist such as Mn(III)tetrakis(4-Benzoic acid)porphyrin chloride (MnTBAP), a compound similar to MnTMPyP but different in how it interacts with superoxide. MnTMPyP quenches superoxide and prevents superoxide's conversion to other ROS while MnTBAP acts like SOD and converts superoxide to H₂O₂ (personal communication with Dr. Ganesh Kumar). These anti-oxidants may prove good candidates for future studies in order to compare superoxide with other types of ROS in the brainstem respiratory control centers.

4.3 Interactions between ROS and Inflammation

Multiple studies have established that ROS and inflammation are tightly linked, each interacting with the other often via the transcription factor NF- κ B (Haddad, 2002; Bubici *et al.*, 2006; Gloire *et al.*, 2006; Pantano *et al.*, 2006; Morgan & Liu, 2011). With this well established link, the next logical step in investigating the interaction between inflammation, ROS, and VAH would be to combine the two studies above by treating rodents with ibuprofen and then measuring superoxide with DHE. Based on our findings that ibuprofen decreases ventilation among chronically hypoxic rats breathing 10%O₂ and ROS show a trend of returning to baseline levels in chronically hypoxic mice, I predict that ibuprofen would blunt the increase in ROS among chronically hypoxic animals (relative to acutely hypoxic subjects) but that ibuprofen would have no effect among normoxic or acutely hypoxic animals. While each of the previous studies used a different animal model, I believe a switch to just mice would be preferable to rats. Mice have the advantage of transgenic knockout of molecular targets that open the door for future experiments that rats do not possess. Mice are also easier to house and cheaper in cost (housing and experimental

manipulations). The smaller size of mice, however, presents potential difficulties with procedures such as surgeries and harvesting tissues such as brainstem regions that are larger in rats. Rats present an advantage for our laboratory since we have well-established protocols and equipment to perform physiological measurements on the animals. Recently, however, our laboratory has undertaken more experiments with mice and established similar protocols and the necessary technical tools for employing physiologic mouse models. Based on this reasoning, I would chose to begin combining inflammation and ROS experiments in mice but, certainly, leave the door open to working with rats should the mice prove more difficult to use.

With a link established between inflammation and ROS production in chronic hypoxia, the next step would be to begin deciphering the mechanism by which the two pathways interact. Given the fact that a number of pathways that converge on the transcription factor NF- κ B and the well established role it plays in linking the two processes, investigating NF- κ B expression during the acclimatization process and during administration of ibuprofen while measuring ROS could be a fruitful starting point.

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