"Obstructive Sleep Apnea:
daytime assessment and treatment of a nighttime disorder"

by

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SIGNED: Jennifer R. Vranish
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DEDICATION

This dissertation is dedicated in memory of my dad, Michael Cloonan, who is my ultimate inspiration for all that I do and who taught me the importance of education and hard work.
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ABSTRACT

Obstructive sleep apnea (OSA) is a disease characterized by nighttime airflow limitation, hypoxemia, arousal from sleep, and elevated sympathetic activity and blood pressure. With time, this nighttime dysfunction gives rise to daytime hypertension and a heightened risk for cardiovascular disease. Current treatment options for OSA are not always effective for all patients and the gold-standard intervention, continuous positive airway pressure, has discouraging compliance rates. The work set forth in this dissertation has as its focus a novel intervention for sleep apnea known as inspiratory muscle training (IMT). IMT improves respiratory function and cardiovascular health but has not been implemented previously as a treatment for OSA. As such, Study 1 implements IMT in individuals with mild and moderate OSA, with the objective of assessing the effects of training on the cardiorespiratory parameters of this disease. We randomly assigned 24 individuals with mild-moderate OSA into one of two groups: training vs. placebo, to assess the effects of 6 weeks of training on overnight polysomnography, subjective sleep quality, blood pressure, circulating inflammatory T cells, and plasma catecholamine content. Our results show IMT-related improvements in sleep quality, reduction in the number of arousals from sleep and in periodic limb movements following 6 weeks of training. Most important, IMT was associated with a significant reduction in systolic (~12 mmHg) and diastolic (~5 mmHg) blood pressure, relative to sleep apneics who undertook 6 weeks of placebo training. Additionally, individuals in the training group exhibited ~30% lower levels of sympathetic activity, as measured by plasma catecholamines, relative to placebo trained peers.
The mechanism(s) that underlie the IMT-related reductions in blood pressure and sympathetic activity remain to be determined. However, in an effort to determine the precise respiratory stimulus that contributes to the results obtained in Study 1, we subsequently assessed the specific respiratory components of IMT to determine which component (large intrathoracic pressures and/or large lung volumes) likely contributes to the reduction in blood pressure in Study 1. The results of this study conducted in normotensive adults show that respiratory training that entails either large negative or positive intrathoracic pressures reduces systolic and diastolic blood pressure in healthy young adults. Importantly, neither the generation of large lung volumes alone nor performance of daily paced breathing is sufficient to lower blood pressure.

Study 3 is a methodologic study that has as its focus upper airway electromyography (EMG) and the utility of assessing EMG activity across a range of conditions and breathing tasks in wakefulness. Because OSA traditionally has been viewed as the result of neuromuscular dysfunction of the upper airway that occurs during sleep, the aim of this work was to develop a “fingerprint” of healthy electromyographic activities during the day in healthy adults across a range of breathing tasks, body positions, and from two different muscle compartments of the upper airway. The findings from this study demonstrate regional differences in muscle activity that vary as a function of body position and task. These data from healthy subjects provide the basis of comparison for subsequent studies in individuals with obstructive sleep apnea.
INTRODUCTION

Obstructive sleep apnea

In the United States an estimated 2-4% of middle-aged men and women (Young et al., 1993; Punjabi, 2008) and 1-4% of children suffer from obstructive sleep apnea (OSA) (Young et al., 2002). OSA is characterized by partial or complete obstruction of the upper airway during sleep, referred to as an apnea or hypopnea, respectively. An apnea is defined as a cessation in airflow for at least 10 seconds and a hypopnea as a reduction in airflow (≥ 30%) that results in an arousal from sleep or ≥ 4% reduction in arterial oxygen saturation (Patil et al., 2007). The apnea hypopnea index (AHI) is the number of these events per hour of sleep and provides a rating of disease severity as follows: <5, normal; 5-15, mild OSA; 16-30, moderate OSA; >30, severe OSA. Current estimates indicate 1 in 5 adults has mild OSA and 1 in 15 adults has moderate-severe OSA (Young et al., 2004).

OSA arises from anatomic and/or neuromuscular dysfunction (Patil et al., 2007). Anatomical features such as large tonsils, recessed chin, and other variations in craniofacial morphology contribute to a narrowing of the upper airway. Additionally, neuromuscular abnormalities including diminution of inspiration-related upper airway muscle activity, render the airway more susceptible to collapse (Sauerland, E.K. and Harper, 1976; Remmers et al., 1978; Mezzanotte et al., 1996; Katz & White, 2004; Fogel et al., 2005).

Risk factors for OSA include sex (males > females), ethnicity (African American, Hispanic, and Asian > Caucasian) and age (40-70) (Young et al., 2004; Punjabi, 2008). The incidence of OSA is greater in those who are overweight or obese due to the additional anatomical burden that fat and fatty deposits place on the upper airway and respiratory pump (Young
et al., 2004). Smoking and alcohol consumption (Young et al., 2004; Punjabi, 2008) along with familial history of sleep apnea (Redline & Tishler, 2000; Young et al., 2002) also increase the risk of OSA.

Sleep and OSA

A hypnogram showing the typical distribution of sleep stages in a healthy adult is presented in Figure 1A. In general, adults enter sleep via Stage 1 non-rapid eye movement (NREM) sleep and progress from Stage 1 to Stage 2 and then into Stages 3-4 NREM sleep. NREM sleep is characterized by synchronous low frequency (Stage 1: 4-8 Hz, Stage 2: 10-15 Hz, Stage 3: 2-4 Hz, Stage 4: 0.5-2 Hz), high amplitude (50-200 µV) EEG activity and generalized muscle atonia (Dement & Kleitman, 1957; Purves et al., 2001; Kryger et al., 2005). The first REM sleep period is reached within ~90 minutes of sleep onset and is characterized by desynchronous high frequency (20-50 Hz), low amplitude (10-50 µV) EEG activity similar to wakefulness, with periods of increased muscle tone associated with dreaming. In general, healthy sleep comprises uninterrupted cycles of NREM and REM sleep repeated at 90 minute intervals over a period of 6-8 hours (Kryger et al., 2005). In a healthy individual, ~5% of total sleep time is spent in Stage 1, 45-55% in Stage 2, 15-20% in Stage 3-4 and 20-25% in REM sleep (Kryger et al., 2005).

Sleep-related breathing disorders disrupt normal sleep architecture. Apneas and hypopneas result in periodic reductions in blood oxygen levels that arouse the individual from sleep to re-establish breathing and restore blood oxygenation. Subsequently, the individual returns to sleep, their airway obstructs, and they arouse once again. While arousals preserve blood oxygenation, they also prevent the individual from attaining stable
sleep and as a result individuals with sleep apnea spend proportionally more time in Stage 1 and 2 NREM sleep and less time in Stage 3-4 NREM sleep (Figure 1B)(Kryger et al., 2005). Sleep disturbances give rise to daytime somnolence, impaired cognitive function, poor work performance, impaired driving ability, and an overall reduction in quality of life (Bédard et al., 1991; NHLBI, 1995; NHTSA, 2000; Parthasarathy, 2005).

**Figure 1.** Representative hypnograms highlighting the sleep architecture of a healthy adult (A) and an adult with OSA (B). A. The healthy adult cycles through NREM and REM sleep at 60-90 minute intervals. This individual arouses from sleep on only two occasions. The healthy adult attains Stages 3-4 NREM early in the night and successively longer periods of REM sleep (30-40 minutes) over the course of 8 hours sleep. B. In contrast, the individual with OSA attains Stage 3-4 sleep within 30 minutes of sleep onset but is unable to attain that level again due to repeated arousal from sleep (15 times in 8 hours). The adult with OSA remains in Stage 2 sleep with frequent brief (10-20 minute) REM sleep periods distributed throughout the night.

**Assessment of sleep.** A diagnosis of sleep apnea requires overnight polysomnography (PSG) performed by an approved sleep laboratory. Standard PSG entails electrooculography (EOG), electroencephalography (EEG), electromyography (EMG) of the submental and limb
musculatures, electrocardiography (ECG), pulse oximetry, and tracking of chest and abdominal wall motions and nasal airflow. The resultant physiologic signals are used to diagnose OSA, determine disease severity, and can be used to evaluate treatment efficacy.

In addition to objective measures of sleep, evaluation also includes subjective measures of sleep quality using standardized psychological tests such as the Epworth Sleepiness Scale (ESS) and Pittsburgh Sleep Quality Index (PSQI) (Mondal et al., 2013). The ESS assesses daytime somnolence and the PSQI yields information about perceptions of sleep quality, sleep duration, percentage of sleep time relative to time spent in bed (sleep efficiency), amount of time in bed before falling asleep (sleep latency), number of sleep disturbances, use of sleep medications, and the extent to which sleep disturbance impacts daytime function (Buysse et al., 1989). The PSQI is validated as a test that assesses the psychological aspects of sleep and has high test-retest reliability. PSQI sleep quality scores correlate nicely with objective measures of sleep reported in individuals with cancer, insomnia, and sleep apnea (Carpenter & Andrykowski, 1998; Backhaus et al., 2002; Buysse et al., 2008).

**Autonomic function and OSA**

Sleep has a marked effect on nervous system function and its effect on autonomic nervous system activity is particularly important in OSA. In health, sympathetic nervous system activity is lower and parasympathetic activity is higher in NREM sleep relative to levels in wakefulness. Thus, in the progression from Stage 1 through to Stage 3-4 sleep, sympathetic activity diminishes (Hornyak et al., 1991), parasympathetic activity increases (Baharav et al., 1995), and blood pressure and heart rate decline (Kryger et al., 2005). In general, NREM
sleep is characterized by a 10-20% reduction in blood pressure (Calhoun & Harding, 2010) and a 15-20% reduction in heart rate (Snyder et al., 1964). By comparison, REM sleep is characterized by cyclic increases in sympathetic activity and reductions in parasympathetic activity that result in acute fluctuations in blood pressure and heart rate comparable to those in wakefulness (Kryger et al., 2005).

Normal autonomic nervous system balance is disrupted when sleep is disturbed. Studies of sleep deprivation and insomnia show that poor sleep quality and/or alterations in sleep architecture can contribute to the development of hypertension (Calhoun & Harding, 2010) secondary to arousals and reductions in Stage 3-4 slow wave sleep. The effects of insomnia on autonomic balance are similar to the effect that arousals and poor sleep architecture have in OSA, as both are characterized by surges in sympathetic activity which cause blood pressure and heart rate to rise (Hornyak et al., 1991; Bradley & Floras, 2000; Yoon & Jeong, 2001; Leuenberger et al., 2005; Calhoun & Harding, 2010). However OSA is also characterized by repeated airway obstruction and hypoxemia, which also acutely increase sympathetic activity and blood pressure, making it difficult to separate respiratory-related changes in autonomic activity from the alterations which result from poor sleep quality. Although sympathetic activity declines and parasympathetic activity increases as the individual returns once again to sleep, a subsequent apnea or hypopnea results in hypoxemia and arousal from sleep that causes another sympathetic surge and blood pressure and heart rate to rise once again. Depending on disease severity, this sequence of events can occur 5-15 times per hour of sleep in mild OSA and up to 50-60 times per hour in severe OSA.
Importantly in OSA, sympathetic activation persists beyond the acute hypoxemia and arousal to remain elevated throughout the night (Somers et al., 1995). Such persistent sympathetic activation and resultant increased blood pressure provides no nighttime relief for baroreceptors. And, when the cycle of hypoxemia and arousal is repeated each night for weeks, months and years, the result is chronic sympathetic activation (Narkiewicz & Somers, 2003) and hypertension evident in some 50% of sleep apneics (Fletcher, 1995; Nieto et al., 2000; Strollo et al., 2014) (Figure 2).

**Figure 2.** Schematic representation of how sleep apnea can contribute to cardiovascular co-morbidities. Sleep apnea is characterized by intermittent hypoxia and arousal from sleep that lead to increased sympathetic nervous system activity, oxidative stress, and systemic inflammation, all of which are risk factors for cardiovascular disease. (adapted from: Kent et al., 2011)

*Mechanisms that contribute to chronic hypertension*

Two mechanisms are believed to promote the progression from an acute surge in blood pressure in response to hypoxemia and arousal to chronic high blood pressure. The first mechanism is an alteration in baroreflex sensitivity and the second is an alteration in the regulation of the renin-angiotensin-aldosterone system (Bradley & Floras, 2000).

Under normal circumstances, mean arterial blood pressure is a function of total peripheral vascular resistance and cardiac output (i.e. stroke volume x heart rate). Blood pressure
homeostasis is accomplished by regulation of vascular resistance and/or cardiac output via the baroreflex loop (Figure 3). In this loop, increases in blood pressure are sensed by aortic and carotid baroreceptors from which primary afferents convey impulses to neurons in the caudal nucleus of the tractus solitarius (NTS). Baroreceptor-sensitive NTS neurons make monosynaptic connections onto sympathetic nuclei in the rostral and caudal ventrolateral medulla (RVLM and CVLM) and onto parasympathetic nuclei in the dorsal motor nucleus of the vagus (DMV) and nucleus ambiguus (NA). Together these pathways regulate autonomic balance (Guyenet, 2006; La Rovere et al., 2008). Baroreceptor-sensitive NTS neurons inhibit RVLM neurons directly and/or indirectly via the CVLM. Inhibition of sympathetic premotor neurons of the RVLM reduces activation of sympathetic pre- and postganglionic neurons to decrease cardiac contractility and reduce vasoconstriction, peripheral vascular resistance and therefore blood pressure. Baroreflex activation of NTS neurons simultaneously excites parasympathetic preganglionic neurons in the DMV and NA to reduce heart rate, cardiac output, and blood pressure. In this way, the baroreflex loop achieves moment-to-moment regulation of blood pressure.

**Figure 3. Baroreflex control of blood pressure.** An increase in blood pressure results in inhibition of sympathetic activity and excitation of parasympathetic activity, to lower blood pressure and heart rate. [BaroR = baroreceptors, NTS = nucleus tractus solitarius, DMV = dorsal motor nucleus of the vagus, NA= nucleus ambiguus, RVLM & CVLM = rostral & caudal ventrolateral medulla.] (adapted from: Guyenet, 2006; La Rovere et al., 2008)
The baroreflex is equally important in the regulation of blood pressure over the long term as evidenced by carotid body denervation that gives rise to chronically elevated mean arterial blood pressure (~22 mmHg) (Smit et al., 2002). By the same token the inappropriate blood pressure regulation seen in cardiovascular disease is attributed to the same circuit and to decreased baroreflex sensitivity (La Rovere et al., 2008). ¹

Although individuals with OSA are hypertensive they also exhibit reduced baroreflex sensitivity (Ziegler et al., 1995; Carlson et al., 1996). Thus, larger changes in blood pressure are required to elicit the baroreflex response in this population (La Rovere et al., 2008). Although an upward shift or resetting of baroreflex sensitivity has been correlated with hypertension (Ziegler et al., 1995; Carlson et al., 1996; Grassi et al., 1998) and cardiovascular disease (Bristow et al., 1969; Heusser et al., 2010), it is unclear which is cause and which is effect – does hypertension and cardiovascular disease diminish baroreflex sensitivity or do alterations in the baroreflex sensitivity cause hypertension?

Evidence that hypertension diminishes baroreflex sensitivity is supported by reported reductions in vascular compliance (McVeigh et al., 1991) that lessen distention of aortic and carotid mechanoreceptors and attenuate the baroreflex response (Bristow et al., 1969). Conversely, evidence that dysfunction in the baroreflex loop gives rise to hypertension (Bristow et al., 1969) is supported by studies in the hypertensive rat that demonstrate a loss of aortic baroreceptor function (Judy & Farrell, 1979) as well as increased inhibition of NTS and CVLM neurons and increased excitation of RVLM neurons (i.e. overall elevated

¹ Baroreflex sensitivity is estimated by the magnitude of the change in heart rate, blood pressure, and/or sympathetic activity for a given increase in blood pressure. In health, baroreflex sensitivity ensures detection of small changes in blood pressure that give rise to marked reductions in heart rate and peripheral vascular resistance.
sympathetic activity), which contribute to neurogenic hypertension (Colombari et al., 2001).

**Renin-angiotensin-aldosterone system (RAAS).** A second potential contributor to the development of chronic hypertension lies within the RAAS (Bradley & Floras, 2000; Fung, 2014). The RAAS regulates blood pressure through its effects on systemic vascular resistance via vasoconstriction and water reabsorption at the kidney, secondary to activation of angiotensin II (AngII) and aldosterone secretion. Hypertension is associated with chronic activation of the RAAS that excites RVLM neurons (Li & Guyenet, 1996) and increases splenic sympathetic nerve discharge (Ganta et al., 2005) via AngII. AngII receptors also are present on baroreceptor-sensitive neurons in the NTS, and microinjection of AngII into the caudal NTS attenuates the baroreflex (Matsumura et al., 1998; Boscan et al., 2001).

Elevated plasma AngII and aldosterone have been documented in people with untreated sleep apnea (Møller et al., 2003) and CPAP administration in the short term (4 weeks) (Nicholl et al., 2014) and long term (14 months) (Møller et al., 2003) reduces plasma renin, AngII, and aldosterone by ~25% and lowers daytime mean arterial blood pressure by 5-7 mmHg. These findings indicate that OSA-related hypertension may in part be attributed to RAAS over-activity.

**Assessment of autonomic function**

To determine whether a given intervention is effective in treating the cardiovascular sequelae of OSA requires assessment of autonomic function. In the clinical realm such assessments typically have relied upon noninvasive measures, the most common of which
is heart rate variability (HRV). HRV uses standard electrocardiography to detect beat-to-beat changes in heart rate (R-R intervals), and spectral analysis to identify the frequencies at which heart rate is modulated by the autonomic nervous system. R-R intervals vary as a function of fluctuations in blood pressure, body temperature, and circadian rhythm (Stein et al., 1994). Heart rate also varies in phase with the respiratory cycle, such that pulse rate increases during inspiration and decreases during expiration – a phenomenon referred to as respiratory sinus arrhythmia (RSA). High frequency components (0.15-0.4 Hz) of HRV reflect respiratory modulation of R-R intervals (10-15 cycles per minute) mediated by pulmonary stretch receptor afferents carried in the vagus nerve (CN X) and are widely used to assess parasympathetic drive (Hirsch & Bishop, 1981; Baharav et al., 1995; Bernardi et al., 2001). However, because the low frequency components of HRV (0.05-0.15 Hz) represent variation in R-R intervals due to the baroreflex loop (6 cycles per minute) and are correlated both with parasympathetic and sympathetic modulation of heart rate (Malik & Camm, 1993; Jarrin et al., 2012) the reliability of HRV for evaluating sympathetic activity remains controversial (Malik & Camm, 1993).

An alternative gauge of sympathetic activity is provided by measures of circulating plasma catecholamines – norepinephrine released from postganglionic neurons, epinephrine released from the adrenal medulla, and dopamine released from central neurons and the adrenal glands. Plasma norepinephrine and epinephrine regulate blood pressure by effects on cardiac contractility and peripheral vascular resistance (Pfeifer et al., 1983; Guyenet, 2006). Dopamine makes up ~2-5% of plasma catecholamine content as it is primarily converted to norepinephrine, yet is also known to be elevated with stress (Kvetnansky et al., 2013). Importantly, a strong positive correlation exists between plasma catecholamine
levels and mean arterial blood pressure (Louis et al., 1973; Shimada et al., 1985), and between plasma catecholamines and sympathetic nerve activity recorded at the peroneal nerve (Seals et al., 1988; Grossman et al., 1991; Ng et al., 1993). Thus, plasma catecholamine content can be used to gauge the efficacy of a given treatment on sympathetic nervous activity (Duncan et al., 1985; Arida et al., 1996; Pinto et al., 2013).

Plasma catecholamines reflect norepinephrine and epinephrine release minus re-uptake, and can be determined from venous blood samples and quantified via high performance liquid chromatography (HPLC) (Hjemdahl, 1984). This technique is used to quantify system-wide elevations in sympathetic activity associated with aging, cardiovascular disease, diabetes, hypothyroidism, sleep disorders, and psychological illness (Wyatt et al., 1971; Coulombe et al., 1976; Cryer et al., 1978; Pfeifer et al., 1983; Shimada et al., 1985; Zoccali et al., 2002) as well as in OSA (Eisenberg et al., 1990; Pinto et al., 2013), insomnia (Cortelli et al., 1991), and hypertension (Goldstein, 2015).

**Immune function and OSA**

Immune system dysfunction also is implicated in the development of hypertension (Dzielak, 1992) via the overproduction of inflammatory cytokines by T lymphocytes. As shown in Table 1, helper and cytotoxic T cells perform “pro-inflammatory” functions as their activation results in the production of cytokines that increase inflammatory processes and result in the proliferation and activation of other pro-inflammatory cell types. In contrast, regulatory T cells are viewed as “anti-inflammatory” as they down-regulate immune responses and suppress the proliferation of pro-inflammatory cells.
In OSA the balance between pro- and anti-inflammatory processes is reportedly dysfunctional and there is evidence of increased activation of CD8+ T cells to a phenotype with greater cytotoxic capacity (i.e. overexpression of perforin and TNF-α) (Dyugovskaya et al., 2005a, 2005b), elevated helper T cell counts, increased Th1:Th2 ratio, and reduced regulatory T cell counts (Steiropoulos et al., 2009; Tan et al., 2013). Elevated levels of circulating inflammatory cytokines including TNF-α, IL-6, and IL-8 also contribute to systemic inflammation, cellular adhesion molecule expression, and leukocyte adhesion (Schiffrin, 2014). Vascular aggregation of immune cells can result in increased aortic stiffness and endothelial dysfunction, both of which are identified as key contributors to hypertension (Zhang & Crowley, 2015). Studies in rodents underscore the importance of the immune system in this regard as RAG1 knockout mice, which lack B and T lymphocytes, have a blunted response to AngII- and deoxycorticosterone acetate (DOCA) salt-induced hypertension; and the adoptive transfer of T cells to RAG1-/- mice restores the hypertensive response (Guzik et al., 2007). However, resting blood pressure does not differ between knockout mice and control animals, indicating that T lymphocytes do not alter blood pressure independent of a pathophysiologic stimulus (Guzik et al., 2007), highlighting an important link between the immune, hormonal, and nervous mechanisms of hypertension.

The potential for hypoxemia and rapid re-oxygenation to increase production of reactive oxygen species (ROS) via NADPH-oxidase, xanthine-oxidase, and nitric oxide synthase (Lavie, 2014) is another important consideration in OSA. Overproduction of ROS leads to oxidative stress by shifting the balance between pro-oxidant and anti-oxidant molecules in favor of the former (Lavie, 2003). Oxidative stress triggers inflammation and promotes accumulation of fluid and immune cells at the site of infection or injury (Libby et al., 2002).
The reverse also is true, such that inflammation triggers oxidative stress and intensifies tissue damage. Repeated arousal from sleep also may augment inflammation as insomnia and sleep deprivation are associated with increased levels of inflammatory cytokines (IL-4, IL-6, IFN-γ, TNF-α) (Sakami et al., 2002; Motivala, 2011; Tobaldini et al., 2015). Because individuals with OSA experience chronic oxidative stress and inflammation (Kent et al., 2011; Lavie, 2014) they are at greater risk of developing atherosclerosis and hypertension (Libby et al., 2002; Jelic et al., 2008; Arnaud et al., 2009a; Kent et al., 2011; Schiffrin, 2014).

Assessment of immune system function. Assessing immune system function traditionally is performed by measuring levels of circulating cytokines obtained from plasma samples and detected using ELISA. Additionally, flow cytometry permits quantification of T lymphocyte subtypes responsible for the production and secretion of cytokines. T lymphocytes are labeled for their unique cell surface markers, transcription factors, or intracellular cytokines (Table 1) with antibodies coupled to fluorescent probes and then counted by a flow cytometer. In this way, T lymphocyte subtypes can be characterized in terms of their absolute number (as is the case for cytokine analysis), or expressed as a proportion relative to other T cells or total lymphocyte counts, giving a normalized or relative view of systemic immunity.
Table 1. Overview of cell surface markers, cytokine production, and function of lymphocytes. The list is not intended to be exhaustive but rather serves to highlight specific antigens and cytokines known to be elevated or down-regulated in OSA or hypertension (Dyugovskaya et al., 2005b; Tan et al., 2013; Zhang & Crowley, 2015).

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<tbody>
<tr>
<td>B cells</td>
<td>B</td>
<td>CD19+ or CD20+</td>
<td>IL-2, IL-4, IL-6, IL-10, IL-12, IFN-γ, TNF-α</td>
<td>Pro</td>
<td></td>
<td>Make antibodies, provide memory function</td>
</tr>
<tr>
<td>Helper T cells</td>
<td>Th1</td>
<td>CD3+CD4+</td>
<td>IL-2, IFN-γ, TNF-α &amp; β</td>
<td>Pro</td>
<td></td>
<td>Activate macrophages and cytotoxic T cells</td>
</tr>
<tr>
<td></td>
<td>Th2</td>
<td>CD3+CD4+</td>
<td>IL-4, IL-5, IL-6 IL-9, IL-10, IL-13</td>
<td>Pro</td>
<td></td>
<td>Activate B cells, mast cells, and eosinophils</td>
</tr>
<tr>
<td></td>
<td>Th17</td>
<td>CD3+CD4+</td>
<td>IL-17</td>
<td>Pro</td>
<td></td>
<td>Activate neutrophils, B cells, and T cells</td>
</tr>
<tr>
<td>Cytotoxic T cells</td>
<td>Tc</td>
<td>CD3+CD8+</td>
<td>TNF-α, perforin, granzymes, granulysin</td>
<td>Pro</td>
<td></td>
<td>Induce apoptosis</td>
</tr>
<tr>
<td>Regulatory T cells</td>
<td>Treg</td>
<td>CD3+CD4+ CD25+ FOXP3+</td>
<td>IL-9, IL-10, TGF-β</td>
<td>Anti</td>
<td></td>
<td>Suppress activation and proliferation of T cells</td>
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(adapted from: Janeway et al., 1999; Lund, 2008; Abbas & Lichtman, 2009; Harrison et al., 2010; Male et al., 2013)
Management of OSA

Although individuals with mild OSA typically will benefit from changes in lifestyle including reduced caloric intake, sleeping position, and/or use of dental or oral devices, the standard treatment for OSA is continuous positive airway pressure or CPAP, which delivers a steady stream of pressurized air to the upper airway via the mouth and/or nose, that stents the airway and prevents its collapse. CPAP effectively eliminates apneic and hypopneic events and oxygen desaturations during sleep (NHLBI, 1995), and has been shown to reduce daytime sympathetic nervous system activity (Narkiewicz et al., 1999), and to decrease CD4+ positive T cells and plasma levels of TNF-α (Steiropoulos et al., 2009). Surprisingly, the effects of CPAP on blood pressure are less clear with two studies reporting null (Narkiewicz et al., 1999) or placebo (Dimsdale et al., 2000) effects on blood pressure and five placebo-controlled trials reporting declines in blood pressure ranging from 2-10 mmHg (Mayer et al., 1991; Faccenda et al., 2001; Bazzano et al., 2007; Barbé et al., 2010; Martínez-García et al., 2013). Despite CPAP’s efficacy in treating the respiratory symptoms of OSA, compliance rates are poor and the device is not well-tolerated (Weaver & Grunstein, 2008). Noise from the CPAP pump is reported to disturb sleep of the OSA user and the bed partner, and the face mask is cumbersome and frequently removed during the night. Present estimates for CPAP adherence range between 46-83% per four hours of sleep (Weaver & Grunstein, 2008).

Respiratory training

In view of the cardiovascular risks associated with OSA and the reduced rates of compliance with CPAP use, there is an urgent need for alternative cost-effective supplemental treatment options (Randerath et al., 2011). Of the myriad of options
available, respiratory training is of particular interest given recent evidence that certain training regimens improve respiratory and cardiovascular parameters (Geddes et al., 2005; Logtenberg et al., 2007; Altena et al., 2009; Cahalin et al., 2013; Hering et al., 2013).

In Study 1 we assess inspiratory muscle training (IMT) for its suitability as an adjunct intervention for early stages of OSA. IMT is a long-time respiratory training program that was initially devised to wean ventilator-dependent individuals from their machines (Abelson & Brewer, 1987; Aldrich et al., 1989). IMT has been used subsequently to improve athletic performance (Volianitis et al., 2001; Griffiths & Mcconnell, 2007; Ray et al., 2010; Guy et al., 2014) and to treat respiratory dysfunction associated with COPD, asthma, and neuromuscular disorders (McCool & Tzelepis, 1995; Wang et al., 2002; Ramirez-Sarmiento et al., 2002; Weiner et al., 2003; Padula & Yeaw, 2007; Lima et al., 2008; Silva et al., 2013). More recently, 4-8 weeks of daily IMT improved limb blood flow in patients with congestive heart failure (Chiappa et al. (2008), improved hemodynamics and baroreceptor sensitivity in a rodent model of heart failure (Jaenisch et al. (2011), and reduced blood pressure in individuals with hypertension (Ferreira et al. 2013). Although one previous study documented improvements in nocturnal end-tidal CO₂ and arterial O₂ in tetraplegics with sleep-disordered breathing (Wang et al., 2002), the effects of IMT on respiratory, nervous, and cardiovascular function in subjects with OSA are unknown.

IMT requires the subject to inhale against a resistance and to generate repeated large inspiratory pressures (e.g. ~50-80 mmHg). In this regard, IMT challenges the respiratory musculature with a pressure load much as free weights impose a load on muscles of the limbs. IMT can be performed on a threshold loading device which requires the subject to
generate a predetermined pressure to open a valve and allow air to flow. Alternatively, training can be performed on a \textit{resistive loading device} that offers a constant resistance. Both devices typically are coupled to a computer that provides auditory or visual feedback during training, and subjects are given a target pressure to reach. Both the threshold and resistive forms of IMT require the individual to generate very large negative (inspiratory) pressures, large tidal volumes, comparable inspiratory flow rates, and result in near-identical improvements in respiratory muscle strength (Hostettler \textit{et al.}, 2011).

The magnitude of pressure generated during IMT is determined from the individual’s maximal inspiratory pressure ($\text{PI}_{\text{max}}$). Standard inspiratory muscle training protocols require subjects to perform 30 breaths per day but differ in regard to the magnitude of the inspiratory effort (e.g., 50-80\% $\text{PI}_{\text{max}}$) and duration (e.g., 5-7 days per week, for 4-12 weeks) (Tzelepis \textit{et al.}, 1994; McCool & Tzelepis, 1995; Kellerman \textit{et al.}, 2000; Volianitis \textit{et al.}, 2001; Markov \textit{et al.}, 2001; Wang \textit{et al.}, 2002; Ramirez-Sarmiento \textit{et al.}, 2002; Weiner \textit{et al.}, 2003; Griffiths & Mcconnell, 2007; Chiappa \textit{et al.}, 2008; Ray \textit{et al.}, 2010; Hostettler \textit{et al.}, 2011; Sapienza \textit{et al.}, 2011; Held & Pendergast, 2014). The IMT protocol undertaken in this study falls at the midpoint of the published range and requires subjects to complete 30 training breaths daily (75\% $\text{PI}_{\text{max}}$), for a period of 6 weeks. During training, subjects are instructed to breathe across a full range of lung volumes (i.e. total lung capacity to residual lung volume) (Hostettler \textit{et al.}, 2011).

With any intervention, false positive outcomes (placebo effects) may result from familiarity with the protocol, experimental setting, or training device. Accordingly, we included a sham-treated placebo group in which individuals perform training in a format that is
identical to the treatment group but that differs only in the magnitude of the (inspiratory) pressure generated during training (i.e. 15% $P_{\text{I} \text{max}}$ as compared with 75% $P_{\text{I} \text{max}}$). Training at 15% $P_{\text{I} \text{max}}$ has a negligible effect on respiratory or cardiovascular function, but serves to identify the effect that familiarity with the experimenter, facility, and training protocol may have on stress, blood pressure, and sleep (Volianitis et al., 2001; Laoutaris et al., 2007; Chiappa et al., 2008; Ferreira et al., 2013; Guy et al., 2014).
Study 1 – Inspiratory muscle training in obstructive sleep apnea

Introduction

CPAP is the gold standard treatment for sleep apnea but it is not tolerated by ~50% of individuals diagnosed with OSA (Weaver & Grunstein, 2008). Other treatments exist, but most are ineffective (NHLBI, 1995), and alternative therapies are in high demand (Randerath et al., 2011). In view of the complex nature of OSA, the ideal treatment would address the sleep, respiratory, and cardiovascular dysfunctions that are characteristic of this disease. In this case we focused on a respiratory training regimen known as inspiratory muscle training (IMT) shown to benefit respiratory and cardiovascular disease populations (McCool & Tzelepis, 1995; Wang et al., 2002; Ramirez-Sarmiento et al., 2002; Weiner et al., 2003; Padula & Yeaw, 2007; Lima et al., 2008; Chiappa et al., 2008; Jaenisch et al., 2011; Silva et al., 2013; Ferreira et al., 2013), but never before implemented in those with OSA.

Our primary objective was to determine the effect of IMT on standard measures of sleep, respiratory, and cardiovascular function in people diagnosed with mild-moderate OSA who were unwilling or unable to conform to traditional CPAP therapy. We randomly assigned individuals to a training or placebo group and evaluated sleep (duration, quality, architecture, and apnea hypopnea index), blood pressure (systolic and diastolic pressures), plasma catecholamine content (epinephrine, norepinephrine, and dopamine), and T lymphocyte populations (pro-inflammatory Th1, Th2, and Th17 helper T cells, cytotoxic T cells, and anti-inflammatory regulatory T cells), before and after 6 weeks of daily IMT.

Because IMT has been shown previously to improve respiratory and cardiovascular parameters, we anticipated that IMT would reduce the frequency of apneic and hypopneic events, the severity of hypoxemic events (% oxygen desaturation), and the number of
nighttime arousals. These outcomes would in-turn improve subjects’ perceptions of sleep quality, lower systolic and diastolic blood pressures, lower sympathetic nervous system activity, and reduce pro-inflammatory T cell counts but increase anti-inflammatory T cell counts.

**Methods**

**Study population.** We recruited twenty-four individuals diagnosed with OSA and randomly assigned them to the training or placebo group. Age, sex, and anthropomorphic data for each group are presented in Table 2 and individual subject data in Appendix A. Subjects were nonsmokers, without history of respiratory or neuromuscular disease, and free from CPAP treatment or sleep medications. The following assessments were performed at intake and again following 6 weeks of IMT.

**Sleep assessment**

a) Subjects were required to undergo overnight polysomnography (PSG) including electrooculography (EOG), electroencephalography (EEG), electromyography (EMG), electrocardiography (ECG), pulse-oximetry, thoracoabdominal movements, and oronasal airflow and which yielded information on sleep architecture, limb movements, frequency and duration of arousals, apneas and hypopneas and number and severity of oxygen desaturations. Each subject underwent two overnight PSGs - one at intake and a second at the end of week 6.

b) Subjects completed the Pittsburgh sleep quality index (PSQI), a sleep quality survey that documents subjective estimates of sleep duration, sleep efficiency, sleep latency, sleep disturbances, use of sleep medication, and daytime dysfunction
PSQI scores range from 0-21, with scores ≤5 considered normal, and higher scores indicative of poor sleep quality.

**Table 2.** Sex, age, anthropomorphic data, and maximal inspiratory pressure (PI\textsubscript{max}) for both groups, and pre- vs. post-training where appropriate. (*P < 0.01, pre vs. post training).

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>Age</th>
<th>Height</th>
<th>Weight (kg)</th>
<th>PI\textsubscript{max} (mmHg)</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>M/F</td>
<td>(years)</td>
<td>(cm)</td>
<td>Pre</td>
<td>Post</td>
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<td>61.5 ±3.9</td>
<td>174.9 ±2.5</td>
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<td>84.8</td>
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<td>±5.7</td>
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<tr>
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<td>8/4</td>
<td>69.1 ±3.4</td>
<td>170.8 ±1.7</td>
<td>77.4</td>
<td>77.8</td>
</tr>
<tr>
<td>(n=12)</td>
<td></td>
<td></td>
<td></td>
<td>±5.3</td>
<td>±5.3</td>
</tr>
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</table>

**Respiratory assessment**

a) Lung function was assessed by standard spirometry and included forced expiratory volume in 1.0 second (FEV\textsubscript{1.0}), forced vital capacity (FVC), forced inspiratory volume in 1.0 second (FIV\textsubscript{1.0}), forced inspiratory vital capacity (FIVC), FEV\textsubscript{1.0}/FVC, FIV\textsubscript{1.0}/FIVC, peak expiratory flow (PEF), and peak inspiratory flow (PIF) in accordance with the guidelines of The American Thoracic Society (Miller et al., 2005).

b) Respiratory muscle strength was assessed by having subjects generate a maximal inspiratory pressure (PI\textsubscript{max}) against a constant resistance (Black & Hyatt, 1969; Kellerman et al., 2000). Subjects' PI\textsubscript{max} was determined from the average of the three largest pressure values generated (Table 2).
**Cardiovascular measures**

a) Blood pressure was determined via sphygmomanometer and stethoscope at the brachial artery, in accordance with current guidelines (Pickering *et al.*, 2005; Mancia *et al.*, 2013). Subjects rested for 5 minutes with back and arms supported prior to measurement. Measures were taken in triplicate, on alternating arms and averaged to obtain each individual’s systolic and diastolic blood pressures. All measures were obtained at the same time and day each week for a given subject (Appendix A).

b) Plasma catecholamines were measured following blood draw performed between the hours of 7:00 and 10:00 am. Subjects were required to fast for 12 hours and were free from caffeine for 12 hours prior to the blood draw. Venous blood samples were collected from the antecubital region following 30 minutes of supine rest in a quiet, temperature-controlled room. Samples were placed on ice in lithium heparin coated tubes (BD Vacutainer, Franklin Lakes, NJ), immediately centrifuged (4°C, 1500 RPM, 15 minutes) and the plasma frozen at -80°C. Plasma samples were analyzed for dopamine, epinephrine, and norepinephrine content via quantitative HPLC (Associated Regional and University Pathologists - ARUP Laboratories, Salt Lake City, UT).

c) Peripheral blood mononuclear cells (PBMCs) were assessed from blood samples collected as described above and placed in cell preparation tubes (BD Vacutainer, Franklin Lakes, NJ). Within two hours of collection, samples were centrifuged (25°C, 1500 RCF/G, 15 minutes). Plasma was collected and frozen at -80°C. PBMCs were re-suspended in HBSS and centrifuged (25°C, 800 RPM, 8 minutes). Supernatant
was discarded, and cells were re-suspended in HBSS and centrifuged (25°C, 600 RPM, 6 minutes). Supernatant was discarded and cells were re-suspended in 10% DMSO/90% FBS, and viably cryopreserved using the Mr. Frosty™ freezing container which froze cells at a constant rate of 1°C/min.

All samples were rapidly thawed, washed, and rested overnight (12 hours, 37°C, 5% CO₂). The following day, cells were stimulated for cytokine production with phorbol 12-myristate 13-acetate (PMA), protein transport was blocked one hour after PMA application with Brefeldin A and Monencin, and cells were stimulated for an additional 4 hours. Following stimulation, cells were stained for cell surface markers: CD3, CD4, CD8, and CD25; and for intracellular markers IL-4, IFN-γ, TNF-α; and the transcription factor FOXP3. Flow cytometry was employed to distinguish between T cell subtypes. Helper T cells were identified by the presence of CD3 and CD4 cell surface markers, and Th1, Th2, and Th17 subtypes were distinguished by cytokine production: IFN-γ, IL-4, and IL-17, respectively; cytotoxic T cells were identified by CD3 and CD8 cell surface markers; and regulatory T cells were identified by CD4 and CD25 cell surface markers and the FOXP3 transcription factor.

Inspiratory training protocol
Training and placebo groups differed from one another only in regard to the magnitude of the inspiratory pressure generated during training. Subjects allocated to the training group performed 30 daily inspirations at 75% of their maximal inspiratory pressure (PI\textsubscript{max}) whereas individuals in the placebo group performed 30 daily inspirations at 15% PI\textsubscript{max}. All subjects were blind to their group (treatment or placebo).
All subjects trained on an inspiratory threshold training device (K3 series, POWERbreathe®, Warwickshire, UK). Training comprised 30 breaths per day, performed for 6 weeks (Kellerman et al., 2000; Volianitis et al., 2001; Griffiths & Mcconnell, 2007). The device includes an onboard computer that registers each training breath and enables individuals to train daily at-home following in-lab instruction and familiarization with the device. Subjects were instructed to first exhale to residual lung volume and then to inhale against the resistive device that was set to a previously determined pressure based on the individual’s PI\textsubscript{max}. Once the target pressure was attained the resistive valve opened to allow inspiratory airflow. Note that because respiratory training improves muscle strength, training target pressures were reset weekly to a new PI\textsubscript{max} values where appropriate. Subjects came to the laboratory once each week so that their data could be uploaded from the training device and so that blood pressure and PI\textsubscript{max} could be measured.

Statistical analyses

All statistical analyses were performed via general linear model ANOVA, testing for between-group differences in sleep, respiratory, cardiovascular, and immune parameters for the independent variables: sex, pre- vs. post-training, and treatment group. Significance was set at $P < 0.05$. Within-group comparisons were performed using paired T tests, with significance adjusted according to the Bonferroni correction.

Results

At baseline (week 1), there were no between-group differences in age ($P = 0.15$), weight ($P = 0.35$), height ($P = 0.23$), systolic BP ($P = 0.90$), diastolic BP ($P = 0.43$), heart rate ($P = 0.86$), plasma epinephrine ($P = 0.34$), norepinephrine ($P = 0.32$), AHI ($P > 0.26$), apnea duration ($P > 0.15$), oxygen desaturation ($P > 0.18$), sleep architecture ($P = 0.90$), arousals
(\(P > 0.35\)), limb movements (\(P = 0.55\)), PSQI scores (\(P = 0.49\)), or PI_{max} (\(P = 0.51\)). We report no interaction between sex and any of the independent variables (\(P > 0.2\)).

Sleep measures. Overnight polysomnography revealed no within-group differences in apnea frequency (\(P > 0.28\)), apnea duration (\(P > 0.1\)), severity of oxygen desaturations (\(P > 0.2\)), total sleep time (\(P > 0.6\)), sleep architecture (\(P > 0.1\)), sleep efficiency (\(P > 0.7\)), or latency to sleep onset (\(P > 0.6\)) between pre and post training (Appendix A). Despite these sleep and respiratory symptoms remaining unchanged, there was a 40% reduction in wake after sleep onset (\(P = 0.001\)), a 33% reduction in the number of arousals per hour of sleep (\(P < 0.001\)), and a 47% reduction in periodic limb movements (\(P < 0.001\)) pre vs. post for individuals in the training group (Figure 4). Individuals in the training group also reported improved sleep quality (PSQI scores: 9.1±0.9 vs. 5.1±0.7; \(P = 0.001\)) whereas individuals in the placebo group registered no change in sleep quality (9.8±0.9 vs. 8.8±1.0; \(P = 0.46\)) (Figure 5).
Figure 4. Average (±SEM) change (post – pre) in wake after sleep onset, arousals per hour of sleep, and periodic limb movements (PLM) per hour of sleep for training and placebo groups. (*P < 0.01, pre- vs. post)

Figure 5. Mean (±SEM) PSQI scores pre- vs. post-training. Note that PSQI scores range from 0-21, with scores ≤ 5 considered indicative of healthy sleep. (*P < 0.01, pre- vs. post; #P < 0.01, relative to placebo group)
**Respiratory measures.** Spirometry values did not change with training for either group ($P > 0.05$) (Appendix A). However, as seen in Table 2, individuals in the training group exhibited a $26.5\pm4.6$ mmHg improvement in inspiratory muscle strength ($P_{\text{max}}$) ($P < 0.001$), whereas the placebo group showed no change ($P = 0.87$).

**Cardiovascular measures.** As shown in Table 3, individuals in the training group exhibited an average reduction in systolic and diastolic blood pressure of $-12.3\pm1.6$ and $-5.0\pm1.3$ mmHg, respectively ($P < 0.01$). Figure 6 depicts the decline in blood pressure over the entire 6-week training period. The change in systolic blood pressure attained significance by Week 3 whereas the change in diastolic blood pressure did not attain significance until Week 6 ($P < 0.01$). Individuals in the placebo group showed no change in blood pressure ($P > 0.05$). Note that there was no change in resting heart rate pre versus post training in either group (training group: $73.5\pm5.7$ vs. $76.8\pm2.8$; $P = 0.35$) and (placebo group: $72.8\pm3.8$ vs. $73.9\pm3.0$; $P = 0.62$).

![Figure 6](image_url)

**Figure 6.** Average ($\pm$SEM) systolic and diastolic blood pressure for each group. Note a significant decline in systolic blood pressure for the training group that begins in Week 3. ($^*P < 0.01$, relative to Week 1)
As shown in Table 3, there was a roughly 30% reduction in plasma norepinephrine for individuals in the training group ($P = 0.01$). Although epinephrine also declined, this failed to attain significance ($P = 0.051$). There was no change in plasma epinephrine ($P = 0.93$) or norepinephrine ($P = 0.56$) in the placebo group nor was there an effect of training on plasma levels of dopamine or the proportion or absolute number of circulating T cell subtypes ($P > 0.1$) (Table 3).

Table 3. Mean (±SEM) blood pressure, plasma catecholamines, and T cell subtypes, pre- vs. post-training for each group. (*$P < 0.01$, pre vs. post; significance adjusted for multiple comparisons)
Discussion

Effects of IMT on sleep and breathing

Baseline measures of sleep and sleep quality correlate well with previous reports in individuals with moderate OSA (AHI: 15-30; sleep time: 300-400 minutes; sleep architecture: 75% NREM:25% REM sleep stages; sleep latency: 15-20 minutes; wake after sleep onset: 100-150 minutes; sleep efficiency: 65-75%; arousal index: 30 events/hour; minimum O2 saturation: 75-80%; PSQI score: 10) (Carlson et al., 1993; Meslier et al., 2003; Iber et al., 2004; Steiropoulos et al., 2009; Guimarães et al., 2009; Kline et al., 2011; Tan et al., 2013). Contrary to our initial hypothesis, we found no changes in apnea frequency or duration, or the degree of oxygen desaturation in either the training or placebo groups. However, individuals in the training group exhibited fewer periodic limb movements and arousals from sleep following 6 weeks of IMT. These individuals also reported improvements in subjective sleep quality independent of any change in AHI.

Effects of IMT on blood pressure and autonomic function

The average pre-training blood pressures (127/75 mmHg) fell within the established range for clinical pre-hypertension (120-139/80-89 mmHg) for systolic but not diastolic pressures. These values are comparable to previous reports of blood pressure in moderate OSA (AHI: 15-30, BP: 127.5/80) (Pinto et al., 2013; Strollo et al., 2014).

Of note, the magnitude of IMT-related reduction in blood pressure (i.e., ~12 mmHg systolic and ~5 mmHg diastolic) is within the range of that reported for medications currently prescribed for the management of hypertension (i.e., 2-6 mmHg over 5 years) (Collins et al., 1990), and falls within range of CPAP-related reductions in blood pressure (2-10 mmHg).
following 1-6 months of treatment (Mayer et al., 1991; Faccenda et al., 2001; Bazzano et al., 2007; Barbé et al., 2010; Martínez-García et al., 2013).

Baseline plasma catecholamine levels also were comparable to levels reported in individuals with moderate OSA (epinephrine: 30-40 pg/mL; norepinephrine: 400-500 pg/mL) (Carlson et al., 1993; Ziegler, 2001). Consistent with previous reports, dopamine levels were <10 pg/mL and within the range of healthy values (Kvetnansky et al., 2013). In our study, the training group exhibited a 30% reduction in plasma norepinephrine levels, which may have contributed to the reduction in blood pressure exhibited by this group. The lowering of plasma catecholamines is comparable to that reported following 2-4 weeks of CPAP treatment (Ziegler, 2001; Pinto et al., 2013) and similar to the reduction reported after 16 weeks of aerobic exercise (60 min/day, 3 times/week, at 70-80% maximal heart rate) (Duncan et al., 1985).

*Effects of IMT on immune function*

Baseline measures of T cell subtypes were similar to previous reports in individuals with moderate OSA (CD4+: 65-70% of total T cells; CD8+: 20-25% of total T cells; Tregs: 5-8% of CD4+ cells; Th17: 0.5-1% of CD4+ cells), (Dyugovskaya et al., 2005a; Steiropoulos et al., 2009; Tan et al., 2013). Individuals in both the training and placebo groups exhibited a higher proportion of Th1 cells and a lower proportion of Th2 cells than the only previous study that distinguished between helper T cell subtypes in individuals with OSA (Tan et al., 2013).

Our results indicate that the balance of circulating pro- and anti-inflammatory T cells was unaffected by IMT. The failure to detect a change in immune function may be due to the
relatively short time course of the training – in this case 6 weeks, and may explain why a previous study assessing the effects of CPAP on circulating T lymphocyte populations showed effects after 6 months of treatment (Steiropoulos et al., 2009). Alternatively, because the apnea hypopnea index and oxygen desaturation were unchanged by our intervention, the resultant hypoxemia may have continued as the principle source of pro-inflammatory processes (Arnaud et al., 2009b; Kent et al., 2011; Lavie, 2014).

Methodological considerations
When examining immune function, there are many cell surface markers and cytokines that can be assayed for (Table 1). As such, we aimed to look for the specific markers previously shown to be altered in OSA, and those that might reasonably be affected by treatment similar to the application of CPAP (Dyugovskaya et al., 2005a, 2005b; Steiropoulos et al., 2009; Tan et al., 2013). The T lymphocyte populations were chosen for the current study because Th1, Th2, Th17 and cytotoxic T cells are increased in OSA and sleep deprivation (Born et al., 1997; Dyugovskaya et al., 2005a, 2005b; Steiropoulos et al., 2009; Tan et al., 2013) and lead to the development of hypertension in rodent models of AngII-induced hypertension (Guzik et al., 2007; Zhang & Crowley, 2015). On the other hand, regulatory T cells are decreased in OSA relative to healthy controls (Tan et al., 2013), and are known to have an anti-hypertensive effect in rodent models of hypertension (Ait-Oufella et al., 2006; Kvakan et al., 2009). Finally, studies in OSA patients show a reduction in CD4+ T cells following 6 months of CPAP therapy (Steiropoulos et al., 2009).

Previous work using heart rate variability analyses show an increase in parasympathetic activity and a decrease in sympathetic activity following 8 weeks of IMT in hypertensive
patients (Ferreira et al., 2013). Given that resting heart rate did not change with training, it is unlikely that parasympathetic modulation can account for the observed lowering of blood pressure. However, a reduction of sympathetic output and therefore total peripheral resistance would account for the drop in blood pressure. In the present study, we obtained measures of plasma catecholamine levels using this as our indicator of sympathetic activity. The use of this measure is supported by evidence of a strong correlation between muscle sympathetic nerve recordings and plasma catecholamine analysis (Seals et al., 1988; Grossman et al., 1991; Ng et al., 1993). Additionally, plasma catecholamine content has been shown to be closely associated with resting blood pressure (Louis et al., 1973; Shimada et al., 1985), and to other intervention-related changes in blood pressure including exercise (Duncan et al., 1985) and CPAP (Pinto et al., 2013).

Although overnight polysomnography is used to determine OSA severity, it is not without its limitations including considerable night-to-night variability in AHI values (Punjabi, 2008) and “first night effects” in which familiarity with the sleep laboratory affects sleep results (Quan et al., 2002). Indeed Bittencourt et al., (2001) report a difference of ≥10 respiratory events per hour of sleep between consecutive overnight sleep studies. Discrepancies also are reported for total sleep time, sleepy efficiency, and sleep architecture between unattended at-home sleep studies and in-lab sleep studies (Iber et al., 2004). Thus, while PSGs provide useful information about sleep and breathing, variability associated with in-lab assessments continue to pose a major challenge to overnight PSG validity and limit the ability to identify significant changes in apnea duration and frequency and hypoxemia.
Implications

These studies were undertaken in human clinical subjects. As such, we cannot identify the precise mechanism by which IMT changes blood pressure, plasma catecholamines, and sleep quality. Nonetheless, our findings support IMT as a suitable adjunct therapy particularly for the cardiovascular sequelae of mild or moderate OSA. IMT also holds promise in the management of cardiovascular co-morbidities associated with many other disorders, including diabetes, insomnia, rheumatoid arthritis, lupus, and depression (Kannel & McGee, 1979; Glassman, 2007; Turesson et al., 2008; Spiegelhalder et al., 2010).

In light of the findings, it seems reasonable to exclude reductions in oxidative stress and/or immune system reactivity as key contributors to IMT-related reductions in blood pressure. Rather, the findings indicate that improved sleep quality and/or reductions in plasma catecholamines are the key contributors to the improvements in blood pressure. Studies in individuals with insomnia secondary to restless leg syndrome or cancer treatment exhibit similar improvements in sleep quality with application of behavioral interventions (such as yoga or relaxation training), and demonstrate a 60% improvement in PSQI scores and ~7 mmHg reduction in blood pressure (Yang et al., 2010; Innes & Selfe, 2012). Similarly, the magnitude of reduction in plasma catecholamine levels and blood pressure is similar to that seen in CPAP administration in OSA (Pinto et al., 2013) and exercise intervention in essential hypertension (Duncan et al., 1985).
Study 2 – Respiratory stimulus contributing to IMT-related reduction in BP

Our purpose in this study was to determine the respiratory stimulus underlying IMT-related reductions in blood pressure (Ferreira et al., 2013). IMT entails the generation of large, negative intrathoracic pressures and large lung volumes. To determine the stimulus necessary for a decline in BP, we developed 5 training protocols that differed from one another in regard to the magnitude and direction of intrathoracic pressure and lung volumes generated in each training breath.

Fifty healthy adults (25 women and 25 men, age 21.0±0.3 years; height 172.5±1.4 cm; weight 68.9±2.0 kg; BMI 22.9±0.4) were randomly allocated to one of five training groups (Figure 7), and performed daily respiratory training for 6 weeks. All assessment variables were measured pre- and post-training, and monitored weekly. We assessed lung function with spirometry, and respiratory muscle strength by determining maximal inspiratory (PImax) and expiratory (PEmax) pressures. In addition, after 5 minutes of quiet rest, arterial blood pressure was measured in triplicate, on alternating arms, by an emergency medical technician blind to treatment group.

Figure 7. Training groups. Schematic depiction of the respiratory parameters manipulated for each training group. Groups differed in regard to the magnitude and/or direction of intrathoracic pressures and lung volumes generated during training. Individuals in Group 1 generated large end-inspiratory lung volumes and large negative (inspiratory) intrathoracic pressures. Individuals in Group 2 generated large end-inspiratory lung volumes and large positive (expiratory) intrathoracic pressures. Individuals in Group 3 generated large negative intrathoracic pressures in the absence of lung volume expansion. Individuals in Group 4 generated large end-inspiratory volumes alone, and those in the placebo trained Group 5 generated modest volumes and pressures typical of rest breathing.
The individuals in Groups 1-3 whose training entailed large positive or negative intrathoracic pressures exhibited a decline in systolic, diastolic, and mean arterial blood pressure following training. Because we included Group 3, which entailed generation of large inspiratory pressures in the absence of lung inflation, and Group 4 which entailed lung inflation alone, we were able to verify that the effect was independent of large lung volumes. Furthermore, inclusion of a placebo-trained group confirmed that the lowering of blood pressure was not attributable to our subjects becoming familiar with the experimental environment, and that paced breathing alone did not affect favorable changes in blood pressure (Landman et al., 2013, 2014; van Hateren et al., 2014). Last, the results also demonstrated training specificity because subjects who undertook inspiratory training improved inspiratory muscle strength ($P_{\text{Imax}}$), and similarly subjects that undertook expiratory training exhibited marked improvements in expiratory muscle strength ($P_{\text{Emax}}$). These findings provide novel insight into the respiratory modulation of blood pressure and specifically which stimulus is responsible for the IMT-related improvements in respiratory muscle strength and blood pressure.
Study 3 – Regional genioglossus muscle electromyographic activities

As outlined in the literature review, standard overnight PSG includes electromyography (EMG) that documents upper airway muscle activity. Historically, EMG has been recorded on the skin surface under the chin, or via hookwire electrodes inserted via the mouth into the genioglossus (GG) muscle. These recording sites provide estimates of regional muscle activity in the progression from quiet wakefulness to NREM sleep. GG EMG activity provides a useful index of this muscle’s activity during the night and can be used to gauge the magnitude of phasic, inspiratory activity which serves to maintain airway patency during inspiration.

Although the current literature documents GG EMG activity across a range of body positions (upright vs. supine), electrode recording locations (anterior vs. posterior regions of the muscle), and breathing tasks (rest breathing vs. hyperventilation vs. tongue protrusion vs. exercise), none of the studies assesses activity across all three manipulations. In this study, we performed a comprehensive assessment of GG EMG activity in healthy adults with the objective of subsequently applying that approach to the study of GG EMG in obstructive sleep apneics.

Our results in healthy young adults highlight distinctions in EMG activity depending on electrode recording location, such that the posterior region of the muscle displayed greater activation than the anterior GG. Additionally, the supine posture was associated with greater GG EMG activity than in upright, yet this feature was only characteristic of the anterior region of the muscle and not the posterior GG. Finally, volitionally-modulated breathing such as voluntary hyperventilation and deep breathing was associated with
greater activation of the GG than was rest breathing through the nose or mouth. Volitionally-modulated breathing was also associated with greater phasic (inspiratory) activity, whereas rest breathing was characterized by a tonic pattern of activation.

This study provides evidence that body position and breathing task play important roles in the regulation of GG motoneuron output, and highlight distinctions in GG EMG activity between the anterior and posterior compartments of the muscle. By imposing such experimental manipulations and recording from two separate muscle regions, we were able to obtain a comprehensive “fingerprint” of healthy GG EMG in the daytime. Future studies might compare these findings to GG EMG activity in OSA, with the goal of understanding how nighttime respiratory dysfunction impacts daytime upper airway activity.
CONCLUSIONS AND FUTURE DIRECTIONS

Without doubt, the most significant finding arising from the studies is the evident effects of 6 weeks of IMT on plasma norepinephrine and blood pressure. In the section that follows I review the findings from Studies 1 and 2 and discuss the acute and long term effects of this form of training (Figure 8). In the section that follows, I refer to “acute changes in blood pressure” which are changes that occurred during or immediately following a training session (i.e. within minutes to hours) and to “long-term changes in blood pressure” which are changes that persisted beyond that day's training and which influenced blood pressure over days and weeks.

Acute effects of training on BP. There is an extensive literature that documents the acute effects of inspiratory and expiratory resistance and Mueller maneuvers on blood pressure. Previous studies show that when subjects inspire against even very modest inspiratory resistances (~7cmH₂O) that this results in an increase in stroke volume, cardiac output, and systolic blood pressure secondary to increased venous return (Convertino et al., 2004, 2007, 2011). Such inspiratory efforts against a resistance also evoke acute increases in arterial blood pressure of ~5-10 mmHg (McConnell & Griffiths, 2010) an effect that is attributed to the inspiratory muscle metaboreflex via an increase in sympathetic output (St Croix et al., 2000; Sheel et al., 2001). Similarly, expiration against a resistance evokes a ~20 mmHg increase in blood pressure (Derchak et al., 2002) and there is a ~10 mmHg increase in blood pressure reported upon release of a sustained Mueller maneuver (Somers et al., 1993; Morgan et al., 1993).
Long term effects of training on BP. Repeated acute surges in blood pressure followed by long-term lowering of blood pressure are features of traditional dynamic or aerobic exercise (Arakawa, 1993; Halliwill, 2001) that typically are accompanied by declines in heart rate (Duncan et al., 1985). Traditional aerobic exercise training protocols performed by normotensive adults reportedly lower heart rate but do not affect blood pressure (Duncan et al., 1985; Arida et al., 1996). In this case, we report the converse finding - a significant decline in blood pressure in the absence of any change in heart rate. Thus, there is an important, but as yet unidentified distinction, between traditional forms of aerobic training and the respiratory training protocols implemented in this study.

Respiration greatly influences moment-to-moment blood pressure regulation (Bernardi et al., 2001; Convertino et al., 2004), and we hypothesized that IMT may act through a variety of afferent feedback mechanisms to alter central nervous control of blood pressure over the long-term. One possibility is that the large intrathoracic pressures and/or large lung volumes generated in the course of IMT increase afferent feedback to brainstem autonomic centers and modulate the balance of autonomic activity. For example, IMT-related large lung volumes attained at end-inspiration are of sufficient magnitude to stimulate pulmonary stretch receptors, whose threshold is estimated between 40-60% of inspiratory capacity (Iber et al., 1995). Pulmonary stretch receptors respond to lung inflation, and the afferent projections from PSRs terminate in the NTS where they suppress sympathetic activity and lower arterial blood pressure (Shepherd, 1981). However, based on Study 2 which showed that subjects performing deep breathing (and thus attained large lung volumes alone) showed no evidence of a reduction in blood pressure, PSR feedback is likely not sufficient to explain IMT-related reductions in resting blood pressure.
An alternative explanation may lie in the stimulation of laryngeal mechanoreceptors that detect and respond to large negative intrathoracic pressures. Like PSRs, laryngeal mechanoreceptor afferents also terminate in the NTS however, in this case activation is documented to elicit reflex *activation* of sympathetic efferents and acute *increases* blood pressure (Nadel & Widdicombe, 1962; Boushey *et al*., 1974). Nonetheless because individuals in Group 2 that trained with large positive intrathoracic pressures also exhibited reduced blood pressure, laryngeal mechanoreceptor stimulation cannot account for the observed effect.

In study 2, we identified large intrathoracic pressures as the stimulus required to affect a reduction in blood pressure. Thus, the large negative pressures generated during IMT may have stimulated baroreceptors directly much as negative pressure applied at the neck, or acted on baroreceptors indirectly via repeated shifts in central blood volume and increased cardiac output (Bevegard *et al*., 1977; Convertino *et al*., 2011). In either case repeated exposure to a negative pressure or blood volume stimulus experienced by subjects performing respiratory training may have altered baroreceptor sensitivity much as chronic electrical stimulation of the carotid bodies affects long-term reductions in blood pressure (Krieger *et al*., 1998; Brum *et al*., 2000; Wustmann *et al*., 2009; Heusser *et al*., 2010). An adjustment to baroreceptor reflex sensitivity in turn would alter sympathetic and parasympathetic balance (Jaenisch *et al*., 2011; Ferreira *et al*., 2013), and result in lowered systolic and diastolic blood pressures. Note however that results from Studies 1 and 2 indicate that IMT does not affect resting heart rate, and thus the effect on blood pressure is more likely mediated by a reduction in sympathetic activity than an increase in parasympathetic activity (Figure 8).
Whereas an alteration in baroreflex sensitivity and resultant chronic decline in sympathetic output may account for the decline in blood pressure, repeated IMT-related increases in central venous pressure also may stimulate low pressure sensing cardiopulmonary afferents. Cardiopulmonary receptors are mechanoreceptors located in the atria, ventricles and lungs, which respond to a reduction in blood volume. These mechanoreceptors are innervated by vagal afferents, and their stimulation results in increased efferent sympathetic activity, and activation of renin secretion from the kidney (Shepherd, 1981). In the context of IMT, large negative/positive intrathoracic pressures generated in the course of training favor repeated shifting of blood from the splanchnic circulation toward the heart and lungs. A repeated increase in central venous blood volume will increase cardiac output, inhibit cardiopulmonary mechoreceptors and thus reduce sympathetic activity. When stimulated intermittently over days and weeks as is the case here, we hypothesize that the result is a long-term reduction in sympathetic activity and in turn, blood pressure (Figure 8).

Lastly, IMT may exert its effects on blood pressure via the renin-angiotensin-aldosterone-system. Although there are no documented effects of IMT on RAAS function, 4 weeks of aerobic exercise reduces plasma AngII levels in a rabbit model of heart failure (Liu et al., 2000), and large lung inflation and alveolar pressures reduce the activation of ACE within the lungs of rabbits (Toivonen & Catravas, 1986). Because it is difficult to separate the effects of exercise on AngII synthesis from the effects of exercise on sympathetic nervous system activity, the relative contribution of each in the overall regulation of blood pressure is unclear. Importantly, because increases in respiratory muscle strength and exercise capacity in heart failure patients are correlated with reduced angiotensin converting
enzyme synthesis (Meyer et al., 1991; Coirault, 2001), this indicates that ACE production is indeed modifiable and that that IMT also may lower ACE and RAAS function.

Future directions for this work include devising studies to examine the effects of training on RAAS function, baroreflex sensitivity, vascular compliance, and cardiac output (Figure 8). While we were able to demonstrate IMT-related reductions in plasma norepinephrine, recording muscle sympathetic nerve activity would assist in identifying the component(s) of the sympathetic nervous system (i.e. skin vs. muscle vs. splanchnic vs. renal sympathetic activity) most affected by IMT and thus, the components most likely to underpin a reduction in blood pressure. Additionally, if IMT is implemented in clinical populations, it would be critical to determine how much training is enough (i.e., every day, every other day), how large a change in intrathoracic pressure is required in order to lower blood pressure, and how long the effects on blood pressure endure after training stops.
Findings from Studies 1 & 2 demonstrate that IMT reduces blood pressure, and study 2 identified the specific respiratory stimulus that required to lower blood pressure (large positive or negative intrathoracic pressures). Large inspiratory efforts against a resistance generate large intrathoracic pressure that favor venous return, and that lead to acute increases in stroke volume, cardiac output, and blood pressure during training. Such an acute increase in central blood volume and blood pressure will stimulate arterial baroreceptors and cardiopulmonary receptors. Afferent feedback arising in these receptor locations results in NTS mediated alterations in parasympathetic and sympathetic nervous system activity. When delivered daily over 6 weeks, as is the case with our training protocol, we suggest that this behaves as a chronic stimulus that reduces sympathetic nervous activity, as reflected in lowered plasma norepinephrine content. Based on the results from Studies 1 and 2 that show no change in heart rate, parasympathetic nervous system modulation of blood pressure is not supported. Study 1 results exclude diminution of immune system reactivity as a key contributor to the improvement in blood pressure. Future studies will need to incorporate additional measures of baroreflex sensitivity, cardiac output and/or plasma levels of RAAS hormones, if we are to identify the mechanism(s) underpinning the IMT-related reduction in blood pressure and plasma NE.

1Findings from Study 1; 2Findings from Study 2; *Potential future directions
REFERENCES


Male D, Brostoff J, Roth DB & Roitt IM (2013). Immunology, 8th edn. Elsevier.


APPENDIX A

Supplementary data for Study 1
Table 4. Age and anthropomorphric data

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67
Table 5. Mean (±SEM) for all parameters measure via overnight polysomnography, pre- vs. post-training for each group. NREM=non-rapid eye movement; REM=rapid eye movement; index=events/hour of sleep; AHI=apnea/hypopnea index; Nadir=at lowest; SaO2=arterial oxygen saturation (*P < 0.01, pre vs. post; significance adjusted for multiple comparisons)

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<td>Wake after sleep onset (min)</td>
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<td>NREM SaO2 at Nadir (%)</td>
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Table 6. Pimax and spirometric data, pre- vs. post-training.

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Table 7. Weekly blood pressure.

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Figure 9. Mean arterial pressure (MAP). Weekly MAP by subject, given for each training group and comparing males vs. females. Females had significantly lower MAP at week 1 than males, but we report sex-dependent differences in training effects. Note the gradual decline in MAP for the training group and no effect for the placebo group.
Table 8. Plasma catecholamine levels and percentage of T cells subsets, pre- vs. post-training.

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APPENDIX B

Daily respiratory training with large intrathoracic pressures, but not large lung volumes, lowers blood pressure in normotensive adults
ABSTRACT

Inspiratory muscle training holds promise as a non-pharmacologic treatment that can improve respiratory muscle strength, reduce blood pressure, and improve autonomic balance in hypertensive patients. There is a gap in knowledge regarding the specific respiratory stimulus that gives rise to these favorable outcomes. We implemented five respiratory training protocols that differed in the magnitude and direction of the lung volumes and/or intrathoracic pressures generated by subjects in training. Normotensive adults were randomly assigned to each group and trained daily for 6 weeks. Pre-post and weekly measures of blood pressure showed significant declines in systolic [-8.96 mmHg (95% CI, 7.39 to 10.53)] and diastolic [-5.25 mmHg (95% CI, 3.67 to 6.83)] blood pressures for subjects who trained with large positive or negative intrathoracic pressures. Subjects who trained with modest intrathoracic pressures or large lung volumes saw no improvement in blood pressure ($P > 0.3$). Large intra-thoracic pressures are the specific respiratory stimulus underpinning breathing training related improvements in blood pressure.

Key words: respiratory training; human; lung volume; intrathoracic pressure
1. INTRODUCTION
As the prevalence of hypertension grows worldwide, novel approaches are needed to combat what may become a public health epidemic (Brook et al., 2013; Mancia et al., 2013). Of the non-pharmacologic treatments available, a long-time respiratory strength training protocol (Abelson & Brewer, 1987; Aldrich et al., 1989) has yielded surprising results including improved blood pressure and autonomic balance in hypertensive patients (Ferreira et al., 2013) and improved baroreflex sensitivity in a rodent model of heart failure (Jaenisch et al., 2011). Despite the important secondary benefits of inspiratory muscle training (IMT) on cardiovascular health, the respiratory mechanisms responsible for the reported improvements in blood pressure and autonomic balance are not known. Accordingly, our purpose in this study was to determine if 6 weeks of IMT can lower blood pressure in normotensive adults and second, to identify the specific respiratory stimulus underpinning that result.

From the standpoint of respiratory mechanics, IMT requires that a subject generate large negative (i.e., inspiratory) intrathoracic pressures with attendant large increases in lung volume and chest wall excursion. Thus, it is unclear whether the large negative intrathoracic pressures and/or large lung volumes contribute to the lowering of blood pressure. In order to identify the stimulus, we devised five training groups that differed from one another in regard to the magnitude and direction of the lung volumes and/or intrathoracic pressures generated by the subject in the course of training. Individuals in Group 1 served as the positive control and performed standard IMT (Griffiths and Mcconnell, 2007; Held and Pendergast, 2014; Kellerman et al., 2000; Sapienza et al., 2011; Tzelepis et al., 1994; Volianitis et al., 2001; Weiner et al., 2003) to verify the effects of
training on respiratory strength and to assess its secondary effect on blood pressure in healthy adults. Individuals in Group 2 trained with large positive intrathoracic pressures and large lung volumes. Individuals in Group 3 trained with large negative intrathoracic pressures and minimal lung volume excursion. Individuals in Group 4 trained with large lung volumes but modest intrathoracic pressures and those in Group 5 trained with modest intrathoracic pressures and lung volume excursion comparable to tidal breathing, and served as the placebo control. By separating respiratory-related pressures from respiratory-related volume events, we sought to assess the effects of each separately and determine their significance in the long-term modulation of blood pressure. Based on previous work (Jaenisch et al., 2011) we hypothesized that large lung volumes (and chest wall excursions) would be the principle stimulus contributing to the lowering of blood pressure.

2. METHODS
We recruited 50 healthy young adults (25 women and 25 men, age 21.0±0.3 years; height 172.5±1.4 cm; weight 68.9±2.0 kg; BMI 22.9±0.4) to participate in a 6 week training protocol (Table 1). Subjects were nonsmokers, without history of hypertension, respiratory, neuromuscular, or cardiovascular disease and forced expiratory volume in 1.0 sec (FEV$_{1.0}$), expressed relative to forced vital capacity (FEV$_{1.0}$/FVC) was greater than 80% predicted in all cases. All experimental procedures were approved by the Human Subjects Protection Program at The University of Arizona and subjects gave their written informed consent prior to participation.

2.1 Assessment measures
Height and weight were recorded at intake and at the completion of 6 weeks training. A pre-assessment questionnaire recorded typical levels of physical activity which subjects were required to maintain throughout the 6 week training period. Tests of pulmonary function, respiratory muscle strength, and blood pressure were performed at study intake, at the beginning of each week prior to that day’s training, and at completion of the study, 24 hours after the final training session of week six.

Standard spirometric measures including FEV$_{1.0}$, FVC, forced inspiratory volume in 1.0 second (FIV$_{1.0}$), FEV$_{1.0}$/FVC, FIV$_{1.0}$/FVC, peak expiratory flow (PEF), and peak inspiratory flow (PIF) were performed in accordance with the guidelines of The American Thoracic Society (Miller et al., 2005). To assess respiratory muscle strength, subjects were required to generate maximal inspiratory (PI$_{\text{max}}$) and expiratory pressures (PE$_{\text{max}}$) by inspiring or expiring against a constant resistance (Black and Hyatt, 1969; Kellerman et al., 2000). PI$_{\text{max}}$ and PE$_{\text{max}}$ were measured via a pressure transducer (Omegadyne Inc., Sunbury, OH) and determined from the average of the three largest pressure values generated by the subject. Blood pressure was determined via sphygmomanometer and stethoscope at the brachial artery and measurement was performed in accordance with current guidelines (Mancia et al., 2013; Pickering et al., 2005). As such, subjects rested for 5 minutes prior to measurements, with back and arms supported. Measures were taken in triplicate, on alternating arms, and averaged to obtain the individual’s systolic and diastolic blood pressures. All measures were obtained at the same time and day each week by a certified emergency medical technician blind to subject training group.
2.2 Respiratory training protocols

Subjects were randomly assigned to one of the five training groups using selection of de-identified subject codes by a third party. Subjects were blind to the existence of multiple training groups and to the purpose of the study. As depicted in Figure 1, each protocol was distinct from any another in regard to the magnitude or direction of the intrathoracic pressure and/or lung volumes generated by the subject during the course of the training.

For all groups, training comprised 30 breaths per day, performed 5 days a week, for 6 weeks. Each subject trained at the same time each day and each training session was supervised by the same research technician (J.R.V.) to ensure training exercises were completed correctly and consistently. All training groups trained on the same respiratory training device comprising a two-way non-rebreathing valve (2600 series, Hans Rudolph, Shawnee, KS) and an attached mouthpiece. A tube attached to the device was coupled to a pressure transducer (Omegadyne Inc., Sunbury, OH) and detected intraoral pressure. The pressure signal was sampled at 500 Hz, digitized, stored using a Cambridge Electronic Design 1401 interface and Spike2 software (Cambridge Electronic Design, Cambridge UK), and displayed on a computer monitor. The monitor displayed each individual’s target training pressure (calculated percentage of the subject’s PImax or PEmax), and subjects were instructed to adjust their effort to achieve that target. A flow limitation end cap placed either on the inlet or outlet of the non-rebreathing valve offered a constant inspiratory or expiratory resistance and which yielded PImax and PEmax values comparable to previous reports (Griffiths and Mcconnell, 2007; Kellerman et al., 2000; Weiner et al., 2003). All subjects were coached to maintain a standard training pace of ~12 breaths per minute. Subjects in all groups performed 5 sets of 6 training breaths per day, and rested 1-2
minutes between sets. As training required subjects to attain large lung volume excursions that encompass the entire lung volume range extending from total lung capacity to residual volume (Hostettler et al., 2011), subjects in Groups 1, 2, and 4 were coached to attain the appropriate range. Subjects who were unwilling or unable to comply with instructions and/or any portion of the study were excluded. The details for each protocol are provided below.

**Group 1 (Positive control)**

Subjects were instructed to first exhale to residual lung volume and then to inhale against a resistance to the previously determined target pressure set at 75% $P_{I_{\text{max}}}$ and to hold 75% $P_{I_{\text{max}}}$ for ~1-2 seconds. No resistance was offered to expiration. In this task subjects generated *large negative* (inspiratory) intrathoracic pressures (-45.0 to -60.0 mmHg) and attained *large* end-inspiratory lung volumes (residual volume to total lung capacity). This training protocol is standard IMT and as such individuals in this group served as the *positive controls*.

**Group 2**

Subjects were instructed to first inhale to total lung capacity and then to exhale against the resistance to the previously determined target pressure set at 75% $P_{E_{\text{max}}}$ and to hold 75% $P_{E_{\text{max}}}$ for ~1-2 seconds. No resistance was offered to inspiration. In this configuration, subjects generated *large positive* (expiratory) intrathoracic pressures (+60.0 to +80.0 mmHg) and attained *large* end-inspiratory lung volumes (residual volume to total lung capacity).
Group 3

Subjects were instructed to first exhale to residual volume and then to inhale against the obstruction to the previously determined target pressure set at 75% PI$_{\text{max}}$ and to hold 75% PI$_{\text{max}}$ for ~1-2 seconds and then inhale as needed. In this configuration, the resistance to inspiration is near maximal and airflow is restricted to a pinhole leak sufficient to permit detection of pressure at the mouth (Hanly et al., 1989). No resistance was offered to expiration. Accordingly, subjects in this group generated large negative intrathoracic pressures (-45.0 to -60.0 mmHg) but attained negligible lung volume expansion similar to a Mueller maneuver (Morgan et al., 1993).

Group 4

Subjects were instructed to first exhale to residual lung volume and then to inhale to total lung capacity for ~1-2 seconds. No resistance was offered to either inspiration or expiration. In this configuration, subjects generated modest intrathoracic pressures (-5.0 to +5.0 mmHg) but attained large end-inspiratory lung volumes (residual volume to total lung capacity).

Group 5 (Placebo control)

Subjects were instructed to first exhale to residual lung volume and then inhale against the resistance to generate a previously determined target pressure of 15% PI$_{\text{max}}$ and to hold 15% PI$_{\text{max}}$ for ~1-2 seconds. In this configuration, subjects generated modest intrathoracic pressures (-10.0 to +5.0 mmHg) and modest (i.e., tidal) end-inspiratory lung volumes. Individuals in this group served as the placebo controls.
Note that because respiratory training improves muscle strength, training target pressures were reset weekly to new $P_{\text{I}_{\text{max}}}$ or $P_{\text{E}_{\text{max}}}$ values where appropriate.

2.3 Data analysis

All statistical analyses were performed by a statistician blinded to the study purpose, using commercially available software (SPSS, version 22; SPSS Inc.). Assessment was via general linear model ANOVA ($2 \times 2 \times 5$), testing for significant between-group differences in systolic (SBP), diastolic (DBP), mean arterial (MAP) pressures and $P_{\text{I}_{\text{max}}}$ and $P_{\text{E}_{\text{max}}}$ for the independent variables: sex, pre- vs. post-training, and training group. Significance was set at $P < 0.05$. Within group comparisons were performed using paired T tests, with significance adjusted according to the Bonferroni correction.

3. RESULTS

The retention rate for the study was 98%. One subject was disqualified due to noncompliance with training. An additional subject was recruited to achieve a total of 50 subjects allocated among the five training groups (i.e., 10 subjects per group). There was no difference in the number of males and females between training groups ($P = 0.401$), and we report no interaction between sex and any of the independent variables ($P > 0.1$).

Means and standard errors for respiratory muscle strength and blood pressure are provided in Tables 2 and 3. There were no between-group differences in baseline respiratory muscle strength or blood pressure at intake ($P > 0.8$). At study close, no differences were noted for spirometric measures as a result of any training protocol ($P > 0.1$), however differences in muscle strength were found for inspiratory training (31.3%
increase in $\text{PI}_{\text{max}} (P = 0.035)$) and expiratory training (36.2% increase in $\text{PE}_{\text{max}} (P = 0.026)$) groups.

Pulse pressure declined in Groups 1-3 (38.9±1.8 vs. 35.2±1.6; $P = 0.002$), but was unchanged in Groups 4 and 5 (38.6±2.0 vs. 39.7±2.4; $P = 0.467$). There was no change in resting heart rate pre- vs. post-training for any of the training groups as follows: Group 1 (60.0±5.7 vs. 65.5±3.6; $P = 0.135$) Group 2 (75.0±3.9 vs. 71.7±5.6; $P = 0.445$), Group 3 (68.3±5.3 vs. 70.2±4.6; $P = 0.551$), Group 4 (72.5±2.9 vs. 68.2±4.2; $P = 0.213$), Group 5 (79.7±6.1 vs. 78.3±4.8; $P = 0.650$).

Although mean arterial pressure was higher in men than women at intake ($P < 0.01$), the effect of training on blood pressure was comparable for men and women in each group ($P = 0.564$). A change in blood pressure was evident at week 2 for Group 1, at week 3 for Group 2, and at week 4 for Group 3 ($P < 0.05$) (Figure 2). The training performed by individuals in Groups 4 and 5 had no effect on blood pressure ($P > 0.05$).

The changes in systolic, diastolic, and mean arterial pressures (i.e., week 6 value – week 1 value) for each training group are presented in Figure 3. There was no difference in the magnitude of the reduction in blood pressure exhibited by individuals in Groups 1-3 ($P > 0.2$). The average change in BP for these groups was: -9.0±0.8 SBP, -5.3±0.8 DBP, and -6.5±0.6 MAP. Thus, individuals in these three groups exhibited lowered blood pressure pre versus post training ($P < 0.01$), and relative to Groups 4 & 5 ($P < 0.05$). By comparison, individuals training in Group 4 or Group 5 registered slight but non-significant increases in blood pressure as follows: +1.9±1.0 SBP, +0.8±1.2 DBP, +1.2±0.6 MAP ($P > 0.05$).
4. DISCUSSION

This study is the first randomized controlled single blind study to assess IMT with the objective of identifying the respiratory component(s) of training that impact on blood pressure. Importantly, lowered systolic, diastolic, and mean arterial blood pressures in the IMT group (Group 1) reproduce recently published results in hypertensive men and women (Ferreira et al., 2013) and demonstrate the capacity for IMT to lower blood pressure in normotensive adults. Second, contrary to our hypothesis, large lung volume excursions associated with training cannot explain the decline in blood pressure, but large positive or large negative intrathoracic pressures are sufficient to account for the result. Last, paced breathing in combination with modest or large lung volume excursions does not impact arterial blood pressures.

4.1 Respiratory muscle training protocols

Standard inspiratory muscle training protocols require subjects to perform 30 breaths per day but differ in regard to the magnitude of the inspiratory effort (e.g., 50-80% PI\text{max}) and duration (e.g., 5-7 days per week, for 4-12 weeks) (Chiappa et al., 2008; Griffiths and Mcconnell, 2007; Held and Pendergast, 2014; Hostettler et al., 2011; Kellerman et al., 2000; Markov et al., 2001; McCool and Tzelepis, 1995; Ramirez-Sarmiento et al., 2002; Ray et al., 2010; Sapienza et al., 2011; Tzelepis et al., 1994; Volianitis et al., 2001; Wang et al., 2002; Weiner et al., 2003). Inspiratory training undertaken by participants in Group 1 fell at the midpoint of the published range and required subjects to complete 30 supervised training breaths per day (75% PI\text{max}), 5 days a week for a period of 6 weeks.
Subjects in Group 2 performed at the equivalent effort level as for inspiratory training but with resistance imposed on expiration (i.e., 75% PE\textsubscript{max}). Expiratory resistance training is also well documented in the literature (Laciuga et al., 2012; Sapienza et al., 2011; Troche et al., 2010), although its potential to lower blood pressure has never been assessed. Its inclusion in this case permitted us to determine whether inspiratory and expiratory training would exert comparable effects on blood pressure. Subjects in Group 3 performed a maneuver akin to a Mueller maneuver commonly used in assessment of autonomic nervous system output (Hanly et al., 1989; Koshino et al., 2010; Morgan et al., 1993; Somers et al., 1993). Again, to our knowledge, this has not been implemented previously as a respiratory training technique. Subjects in this group attained the same inspiratory pressures generated by subjects performing IMT but in the absence of lung inflation. Note that subjects in Groups 1, 2, and 4 attained comparable lung volume expansion (i.e., total lung capacity – residual lung volume) (Hostettler et al., 2011). Individuals in the placebo trained Group 5, also breathed against an inspiratory resistance however the target pressure, and therefore the negative (inspiratory) pressure generated by these subjects during training, approximated only 15% PI\textsubscript{max}, also in accordance with the literature (Guy et al., 2014; Laoutaris et al., 2007; Volianitis et al., 2001).

4.2 Effect of training on pulmonary function and respiratory muscle strength

We report no difference in spirometric measures as a result of any of the training protocols ($P > 0.1$). This is consistent with previous findings for IMT (Ramirez-Sarmiento et al., 2002; Ray et al., 2010; Sapienza et al., 2011; Weiner et al., 2003). Only individuals in Group 1 who performed standard IMT exhibited increased inspiratory strength ($\text{PI}_{\text{max}}$) pre-post training ($P = 0.035$). Similarly, only subjects in Group 2 who trained against an expiratory
resistance exhibited improved expiratory strength or PE_max (P = 0.026). These findings reflect training specificity and are consistent with published findings (Weiner et al., 2003). Interestingly, although individuals in Group 3 also trained at 75% of their PI_max, they did not exhibit any change in inspiratory strength. This is in line with a previous report by Tzellepis et al. (1994), and suggests that improvements in respiratory muscle strength may depend on the lung volumes attained in training or the nature of the training task. For individuals in Groups 1 and 2, training entailed dynamic (inspiratory and expiratory) muscle contractions whereas individuals who performed a Mueller maneuver (Group 3) trained against a near infinite resistance akin to an isometric contraction (Hanly et al., 1989; Morgan et al., 1993). Although isometric contractions improve limb muscle strength (Davies et al., 1988; Jones and Rutherford, 1987), it is unclear if this is the case for respiratory muscles. Moreover, because processes of muscle adaptation differ for dynamic vs. isometric contractions, specifically the rate of tension development and maximal shortening velocity are greater for dynamic relative to isometric contractions (Duchateau and Hainaut, 1984), the dynamic movements performed by Groups 1 and 2 may have been more effective in strengthening respiratory muscles and may explain the observed differences in strength.

4.3 Paced-breathing
In addition to the volume and pressure manipulations imposed in each of the five training groups, individuals in each group trained at ~12 breaths/min, which falls at the midpoint of the range reported previously for device-guided breathing training (Altena et al., 2009; Grossman et al., 2001; Landman et al., 2013; Logtenberg et al., 2007; Schein et al., 2001). Device-guided breathing is distinct from IMT in using musical tones to regulate breathing
frequency, but like IMT, is performed each day for 10-15 minutes (Landman et al., 2014). Importantly for the current study, individuals in the placebo control group (Group 5) trained with lung volumes and intrathoracic pressures comparable to rest breathing but at a set pace. Because these individuals showed no change in blood pressure, paced breathing is excluded as the key factor in blood pressure control in agreement with recently published reviews (Landman et al., 2014, 2013; van Hateren et al., 2014).

4.4 Acute versus sustained blood pressure response

Subjects in inspiratory training, expiratory training, and Mueller maneuver training (Groups 1-3, respectively) showed a gradual decline in blood pressure over the first weeks of training that reached its nadir around day 30. This time frame resembles time frames reported previously for pharmacologic interventions and other lifestyle intervention programs for the treatment of hypertension (Bacon et al., 2004; Sacks et al., 2001; von Scholten et al., 2014). In the discussion that follows, we refer to the acute and long term effects of training. In this case, the term acute changes in blood pressure refers to changes that occurred either during or immediately following a training session (i.e., within minutes to hours) whereas long-term changes in blood pressure refers to changes that persisted beyond that day’s training and which influenced blood pressure over days and weeks.

There is an extensive literature that documents the acute effects of inspiratory and expiratory activation against resistance and Mueller maneuvers on blood pressure. Previous studies show that even very modest inspiratory resistances (~ 7cmH₂O) increase stroke volume, cardiac output, and systolic blood pressure secondary to increased venous return (Convertino et al., 2011, 2007, 2004). Larger inspirations also evoke acute increases
in arterial blood pressure of \(~5\text{-}10\) mmHg (McConnell and Griffiths, 2010) an effect that is attributed to the inspiratory muscle metaboreflex via an increase in sympathetic output (Sheel et al., 2001; St Croix et al., 2000). Similarly, expiratory muscle activation during resistive breathing (\(60\% \text{ PE}_{\text{max}}\)) evokes a \(~20\) mmHg increase in blood pressure (Derchak et al., 2002) and there is a \(~10\) mmHg increase in blood pressure reported upon release of the Mueller maneuver (Morgan et al., 1993; Somers et al., 1993). Note that in the latter studies, subjects sustained the Mueller maneuver for 20-40 seconds (relative to 1-2 sec in this study) and neither study had subjects repeat maneuvers, as was the case here.

Acute surges in blood pressure followed by long-term lowering of blood pressure are features of traditional dynamic or aerobic exercise (Arakawa, 1993; Halliwill, 2001) that typically are accompanied by declines in heart rate (Duncan et al., 1985). Traditional aerobic exercise training protocols performed by normotensive adults reportedly lower heart rate but do not affect blood pressure (Arida et al., 1996; Duncan et al., 1985). In this case, we report the converse finding -- a significant decline in blood pressure for subjects in Groups 1-3 in the absence of any change in heart rate. Thus, there is an important, but as yet unidentified distinction, between traditional forms of aerobic training and the respiratory training protocols implemented in this study.

The steady declines in blood pressure reported here over three weeks may have occurred secondary to the stimulation of carotid and/or aortic baroreceptors (Angell James, 1971). Thus, the magnitude of the intrathoracic pressures generated during training may be sufficient to stimulate baroreceptors directly or indirectly via repeated shifts in central blood volume (Bevegard et al., 1977; Convertino et al., 2011). Much as chronic electrical
stimulation of the carotid bodies affects long-term reductions in blood pressure (Heusser et al., 2010; Wustmann et al., 2009) repetitive exposure to a (negative pressure or blood volume) stimulus as experienced by subjects performing respiratory training may alter baroreceptor sensitivity (Brum et al., 2000; Krieger et al., 1998), and sympathetic/parasympathetic balance (Ferreira et al., 2013; Jaenisch et al., 2011), resulting in lowered systolic and diastolic blood pressures.

5. CONCLUSIONS
Identifying the specific component of respiratory training that improves blood pressure is of interest and importance if such training is to gain acceptance and if we are to determine its suitability for use in particular patient populations. The current findings provide proof of concept that the repeated daily generation of large (positive or negative) intrathoracic pressures, but not large lung volumes, can lower blood pressure in normotensive adults. Importantly, because respiratory training is simple, cost effective, and time efficient it holds considerable appeal as a training therapy for cardiovascular co-morbidities associated with many disorders. Future studies reasonably might address how much training is enough (i.e., every day, every other day), the magnitude of intrathoracic pressure necessary to lower blood pressure and how long the effects on blood pressure endure after training stops.
Conflicting interests

None.

Author contributions

Conception and design of the experiments: JRV and EFB. Collection and analysis of data: JRV. Interpretation and reporting of data: JRV and EFB. Draft and revision of the article: JRV and EFB. JRV and EFB had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES


Table 1. Sex and anthropomorphic data of subjects in each of the training groups. Values are displayed as averages (± SEM).

<table>
<thead>
<tr>
<th>Training group</th>
<th>Sex</th>
<th>Age (yrs)</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Male/Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 (n=10)</td>
<td>4/6</td>
<td>21.1 ± 0.4</td>
<td>170.8 ± 2.3</td>
<td>68.1 ± 3.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>68.1 ± 3.9</td>
</tr>
<tr>
<td>Group 2 (n=10)</td>
<td>6/4</td>
<td>22.0 ± 0.8</td>
<td>174.7 ± 1.9</td>
<td>67.0 ± 5.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>67.1 ± 5.0</td>
</tr>
<tr>
<td>Group 3 (n=10)</td>
<td>4/6</td>
<td>21.1 ± 0.6</td>
<td>173.7 ± 2.4</td>
<td>74.4 ± 5.8</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>74.1 ± 5.6</td>
</tr>
<tr>
<td>Group 4 (n=10)</td>
<td>5/5</td>
<td>20.8 ± 0.5</td>
<td>172.9 ± 2.1</td>
<td>68.4 ± 3.4</td>
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<td></td>
<td></td>
<td>68.3 ± 3.4</td>
</tr>
<tr>
<td>Group 5 (n=10)</td>
<td>6/4</td>
<td>19.8 ± 0.2</td>
<td>170.3 ± 2.6</td>
<td>66.7 ± 4.4</td>
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<tr>
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<td>67.7 ± 4.3</td>
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</table>
Table 2. Maximal inspiratory ($P_{I_{\text{max}}}$) and expiratory ($P_{E_{\text{max}}}$) pressures, pre- vs. post-training, given by training group. Values are displayed as averages ($\pm$ SEM).

$^*p < 0.05$, pre- vs. post-training

<table>
<thead>
<tr>
<th>Training group</th>
<th>$P_{I_{\text{max}}}$ (mmHg)</th>
<th>$P_{E_{\text{max}}}$ (mmHg)</th>
<th>$p$ value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>$p$ value</td>
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<tr>
<td>Group 1 (n=10)</td>
<td>-61.8 ± 6.7</td>
<td>-81.1 ± 6.4</td>
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<td>Group 2 (n=10)</td>
<td>-69.1 ± 5.5</td>
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<td>Group 3 (n=10)</td>
<td>-65.9 ± 7.8</td>
<td>-71.0 ± 7.4</td>
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<td>-62.2 ± 6.5</td>
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<td>Group 5 (n=10)</td>
<td>-69.7 ± 5.1</td>
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<td>0.72</td>
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Table 3. Systolic, diastolic, and mean arterial blood pressure, pre- vs. post-training, given by training group. Values are displayed as averages (± SEM).
*p < 0.01, pre- vs. post-training

<table>
<thead>
<tr>
<th>Training group</th>
<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
<th>MAP (mmHg)</th>
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<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td>Pre</td>
<td>Post</td>
<td>P value</td>
<td>Pre</td>
</tr>
<tr>
<td>Group 1 (n=10)</td>
<td>115.1 ± 2.3</td>
<td>105.4 ± 2.8</td>
<td>&lt;0.001*</td>
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<tr>
<td>Group 2 (n=10)</td>
<td>117.5 ± 3.5</td>
<td>106.7 ± 3.5</td>
<td>&lt;0.001*</td>
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<tr>
<td>Group 3 (n=10)</td>
<td>114.2 ± 3.6</td>
<td>107.8 ± 4.0</td>
<td>0.002*</td>
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<tr>
<td>Group 4 (n=10)</td>
<td>113.0 ± 4.5</td>
<td>115.8 ± 3.9</td>
<td>0.084</td>
</tr>
<tr>
<td>Group 5 (n=10)</td>
<td>113.7 ± 3.0</td>
<td>114.8 ± 3.4</td>
<td>0.461</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS.

Figure 1. Respiratory traces. Representative recordings of mouth pressure (mmHg) and chest wall expansion generated during each breath in each of the training protocols. Dashed lines denote $PI_{\text{max}}$, $PE_{\text{max}}$, and training level (% of $PI$ or $PE_{\text{max}}$) where appropriate. As shown, each group differed in regard to the end-inspiratory volume and/or intrathoracic pressure generated during training. Individuals in Group 1 generated large end-inspiratory lung volumes and large negative (inspiratory) intrathoracic pressures. Individuals in Group 2 generated large end-inspiratory lung volumes and large positive (expiratory) intrathoracic pressures. Individuals in Group 3 generated large negative intrathoracic pressures in the absence of lung volume expansion. Individuals in Group 4 generated large end-inspiratory volumes alone, and those in the placebo training Group 5 generated modest volumes and pressures typical of rest breathing.

Figure 2. Average (±SEM) mean arterial pressure (MAP) for each group over the course of 6 weeks training. MAP declined for subjects in Groups 1 - 3 but was unchanged for subjects in Groups 4 & 5. *$P < 0.05$, significantly different from week 1 for Group 1; #*$P < 0.05$, significantly different from week 1 for Group 2; †*$P < 0.05$, significantly different from week 1 for Group 3.

Figure 3. Net change in blood pressure as a result of six weeks of respiratory training. Group data (±SEM) depicting change in systolic (SBP), diastolic (DBP), and mean arterial (MAP) blood pressure for each treatment group. *$P < 0.05$, change significantly different pre vs. post-training; #*$P < 0.05$, change significantly different than Group 4; †*$P < 0.05$, change significantly different than Group 5.
Figure 1.
Figure 2.
Figure 3.
APPENDIX C

A comprehensive assessment of genioglossus electromyographic activity in healthy adults
ABSTRACT

The genioglossus (GG) is an extrinsic muscle of the human tongue that plays a critical role in preserving airway patency. In the last quarter century, more than fifty studies have reported on respiratory-related GG electromyographic (EMG) activity in human subjects. Remarkably, of the studies performed none have duplicated subject body position, electrode recording locations, and/or breathing task(s) making interpretation and integration of the results across studies extremely challenging. In addition, more recent research assessing lingual anatomy and muscle contractile properties has identified regional differences in muscle fiber type and myosin heavy chain expression giving rise to the possibility that the anterior and posterior regions of the muscle fulfill distinct functions. Here, we assessed EMG activity in anterior and posterior regions of the genioglossus, across upright and supine, in rest breathing and in volitionally modulated breathing tasks. We tested the hypotheses that GG EMG is greater in the posterior region and in supine, except when breathing is subject to volitional modulation. Our results show differences in the magnitude of EMG (% regional maximum) between anterior and posterior muscle regions (7.95±0.57 vs. 11.10±0.99, respectively; $P < 0.001$), and between upright and supine (8.63±0.73 vs. 10.42±0.90, respectively; $P = 0.008$). Although the nature of a task affects the magnitude of EMG ($P < 0.001$), the effect is similar for anterior and posterior muscle regions and across upright and supine ($P > 0.2$).
INTRODUCTION

The genioglossus (GG) muscle of the human tongue is involved in functions critical to survival including swallowing, speech, and breathing. In view of the muscle’s role as an airway dilator, the preponderance of research has focused on electromyographic (EMG) activity during sleep and wakefulness when subjects are in the supine or side-lying position (Eastwood et al., 2003; Malhotra et al., 2004; Fogel et al., 2005; Bailey et al., 2007; Eckert et al., 2009; Wilkinson et al., 2010; Jordan et al., 2010; Richardson and Bailey, 2010; Saboisky et al., 2010; Laine and Bailey, 2011; Trinder et al., 2013). Numerous other studies have incorporated manipulations that impact upon respiratory-related genioglossus activity, including head/body position (i.e., head-up vs. head-back; upright vs. supine) (Douglas et al., 1993; Wasicko et al., 1993; Ono et al., 1996; Otsuka et al., 2000; Tsuiki et al., 2000; Williams et al., 2000; Pae et al., 2002, 2004; Takahashi et al., 2002; Walsh et al., 2008), assessment of EMG activity in multiple muscle regions (Eastwood et al., 2003; Wilkinson et al., 2008, 2010; Nicholas et al., 2010; Richardson and Bailey, 2010; McSharry et al., 2013; Trinder et al., 2013), and in different tasks (i.e., rest breathing, voluntary hyperventilation, maximal inspiratory effort, or exercise) (Mezzanotte et al., 1992; Williams et al., 2000; Eastwood et al., 2003; Walls et al., 2013). In addition, studies of human tongue muscle tissue highlight regional differences in GG muscle fiber type, myosin heavy chain composition, cross sectional area, innervation, and motor end-plate banding (Sanguineti and Laboissi, 1997; Saigusa et al., 2001; Zur et al., 2004; Buchaillard et al., 2009; Mu and Sanders, 2010; Daugherty et al., 2012; Sanders et al., 2013) which suggest different regions of the muscle may fulfill different functions (Saigusa et al., 2001; Daugherty et al., 2012).
In this case, we sought to integrate across the breadth of approaches reported in the literature and recorded multiunit EMG activity in rest breathing, deep breathing, voluntary hyperventilation, and Mueller maneuvers. Given the suggestion that different regions of the muscle are driven preferentially by certain inputs (Eastwood et al., 2003), or that different regions of the muscle may have different mechanical effectiveness in dilation of the airway (Bilston and Gandevia, 2014), we recorded activity in the most anterior and most posterior regions of the muscle. In view of gravitational effects on the upper airway (Pae et al., 1994) and recently reported evidence that posterior region of the genioglossus muscle exhibits greater respiratory-related activation (Cheng et al., 2008), we predicted that EMG activity would be greatest in the posterior region and in supine. Second, given indications that (motor) cortical input may be a more potent driver of the anterior tongue (Laine and Bailey, 2011) and anatomical evidence that the anterior GG may be more important for volitional activities (Saigusa et al., 2001) we predicted that anterior EMG would exceed posterior EMG activity in the context of volitionally modulated breathing.

METHODS
We recruited fourteen healthy young adults (11 women and 3 men, age 20.6±2.1 years; height 167.7±9.0 cm; weight 62.0±11.2 kg; BMI 21.9±2.8). Subjects were nonsmokers, without history of sleep disorders, respiratory, neuromuscular, or cardiovascular disease and a forced expiratory volume (1.0 sec) to forced vital capacity ratio (FEV$_{1.0}$/FVC) greater than 80% predicted value based on height, weight, age, and sex (Miller et al., 2005). Experimental procedures were approved by The University of Arizona Human Subjects Protection Program and subjects gave their written informed consent prior to participation.
**General Procedures.** Subjects were fitted with an oronasal facemask that allowed for oral and nasal breathing (Series 8900, Hans Rudolph). The mask was held in place with a head cap (Series 7450, Hans Rudolph), and self-sealed to the face. The seal was verified by a vacuum leak test. Inspiratory and expiratory airflows were measured using a pneumotachometer attached in series to the mask (Figure 1) and connected to an amplifier (Model 1110, Hans Rudolph) that transmitted mask pressure and airflow signals to a data acquisition system (Cambridge Electronic Design, 1401 and Spike 2 software).

**Electromyography.** We recorded multiunit EMG activity in the genioglossus using two electrode types (see below). In the anterior region, EMG activities were recorded via bipolar intramuscular hook-wire electrodes (50 μm, California Finewire, Grover Beach, CA) inserted via the mouth, as described previously (Sauerland, E.K. and Harper, 1976; Williams et al., 2000; Pittman and Bailey, 2009; Richardson and Bailey, 2010). Each hook-wire was bared of ~ 2-3 mm insulation at the tip, threaded through a 30 gauge needle (0.3mm x 13mm, Becton, Dickinson & Co., Franklin Lakes, NJ) and inserted bilaterally, immediately posterior to the lingual sulcus at points equidistant from the lingual frenulum to a depth ~12 mm from the mucosal surface (Sauerland & Harper, 1976; Eastwood et al., 2003). The needle subsequently was removed leaving the hook-wire in the muscle belly. Hook wires were taped to the chin and anchored by the breathing mask. This recording area corresponds well with the region identified previously as GG-A comprising anterior and superior muscle fibers prior to entry into the tongue (Daugherty et al., 2012).

Multiunit EMG activity in the posterior GG was recorded via bipolar intramuscular tungsten microelectrodes (1-5 μm tip diameter, 250 μm shaft diameter, Frederick Haer & Co,
Bowdoin, ME). Electrodes were inserted bilaterally into the skin underlying the jaw, ~2.0 cm posterior to the mandible, ~0.5-1.0 cm from the midline and 1.0-2.0 cm from the other electrode. Based on our calculations and depending on subject size, electrodes were inserted ~15-18 mm posterior to the mandible at an angle ~120-130 degrees to the horizontal when seated in upright. This recording area is in the most posterior region of the muscle and corresponds well with the area GG-P identified previously as comprising inferior oblique and horizontal muscle fibers (Daugherty et al., 2012). For these recordings, the distance to the GG in each subject was determined via ultrasonography (Aloka Pro Sound 3500, Tokyo, Japan) (Eastwood et al., 2003), and this information was used to place a mark on the electrode to indicate the distance to the middle of the muscle. This mark served as an indicator of the target depth and was used to ensure that the electrode placement was preserved throughout. Subjects were grounded with a gold cup electrode ear clip (Grass Technologies, Warwick, RI).

Tungsten microelectrodes were manufactured without insulation on the last 5.0 mm (Frederick Haer & Co, Bowdoin, ME). Similarly, we prepared hook-wire electrodes baring ~2-3 mm insulation at the terminal tip to match the impedance of the tungsten electrodes. Both electrode types subsequently were assessed and found to have equivalent negligible impedances at 1000 Hz (Electrode Impedance Tester, Bak Electronics, Inc., Sanford, FL) and thus, equivalent recording surface areas. EMG signals were sampled at 5kHz and pre-amplified (3X), amplified (1000X), and band-pass filtered from 30-3,000 Hz using CED 1902 amplifiers and head stages (Cambridge Electronic Design, Cambridge, United Kingdom). The signals were digitized and stored using a Cambridge Electronic Design 1401 interface and Spike2 software (Cambridge Electronic Design, Cambridge UK).
Experimental Protocol. Subjects were assigned randomly to begin the experiment in supine or seated upright. Head placement was kept in the Frankfort plane throughout the experiment (Johnson, 1950).

GG EMG activity, inspiratory and expiratory airflow and mask pressure were subsequently recorded for two minutes in rest breathing via the mouth and rest breathing via the nose. Subjects next performed deep breathing, maximal voluntary hyperventilation, and Mueller maneuvers. For deep breathing, subjects were instructed to inspire to approximately twice their normal breath (Fox et al., 1986). For maximal voluntary hyperventilation, subjects were instructed to breathe as fast and as deeply as they could. In view of the high airflows associated with these tasks and to minimize airway resistance, subjects were directed to breathe through the mouth (Fregosi and Lansing, 1995; Williams et al., 2000). For Mueller maneuvers, subjects made an inspiratory effort at end-expiration against the occluded intake port on the pneumotachometer. For purposes of EMG normalization, subjects were asked to perform sharp sniffs, unimpeded tongue protrusions out of the mouth, and swallows at end-expiration to determine maximal GG activation. The maneuver in which the maximum EMG amplitude was observed served as the maneuver against which EMG in all other maneuvers or tasks were normalized. Note that subjects rested for 1-2 minutes between maneuvers/tasks to ensure that EMG and breathing frequency returned to baseline before proceeding. The entire protocol, including maximum maneuvers, was repeated for the seated upright or supine body position, whichever body position had yet to be completed.

Data analysis. All data were analyzed offline using Spike2 software and customized scripts. EMG signals were rectified and integrated at a time constant of 100 milliseconds.
Breathing frequency (breaths/min) was determined for each task. Measures of inspiratory flow were integrated to obtain inspiratory tidal volume ($V_T$) and ventilation ($V_E$). Mean inspiratory flow ($V_T/T_i$ (ml sec$^{-1}$) was calculated, as an accepted index of ventilatory drive (Milic-Emili and Grunstein, 1976; Boggs and Tenney, 1984) in each breathing task. Average multiunit EMG activity was determined for the phasic (i.e., inspiratory) and tonic (i.e., expiratory) portions of each breath cycle. As breathing frequencies varied between tasks, EMG measures at each electrode location were based on average EMG amplitude for 10 consecutive breaths (Mateika et al., 1999; Eastwood et al., 2003; Saboisky et al., 2006, 2007; Bailey et al., 2007; Richardson and Bailey, 2010). Because subtle differences in electrode impedance may alter the signal in terms of raw voltage, EMG values were normalized with regard to the regional maximal EMG activity (% of max) and averaged across breaths. The maximum EMG for both the anterior (range: 0.5-1.6 mV) and posterior (range: 0.2-1.3 mV) muscle regions occurred during unimpeded tongue protrusion in all subjects (Pittman and Bailey, 2009).

EMG averages were not normally distributed and were converted into logarithms for statistical purposes (Douglas et al., 1993). Statistical evaluation was by general linear model ANOVA ($2 \times 2 \times 2 \times 5$), testing for significant differences in EMG, airflow ($V_T/T_i$), and breathing frequency ($f_R$) as a function of muscle region (anterior vs. posterior), body position (upright vs. supine), and task (rest breathing via nose, rest breathing via mouth, deep breathing, voluntary hyperventilation, Mueller maneuver). Significance was set at $P < 0.05$. Post hoc comparisons were performed using paired T tests, with significance adjusted according to the Bonferroni correction.
RESULTS
Means and standard errors for ventilation parameters in upright and supine and for each task are reported in Table 1. There were no differences in inspiratory time \((P = 0.952)\), expiratory time \((P = 0.798)\), breathing frequency \((P = 0.847)\), tidal volume \((P = 0.514)\), inspiratory flow rate \((P = 0.806)\), or minute ventilation \((P = 0.741)\) between upright and supine within a task. \(V_{T}/T_{I}\) and \(f_{R}\) were comparable for rest breathing via the nose \((185.12 \pm 16.20 \text{ ml/sec}; 13.95 \pm 0.84 \text{ breaths/min})\) and rest breathing via the mouth \((222.51 \pm 18.92 \text{ ml/sec}; 14.12 \pm 0.86 \text{ breaths/min})\). Given the absence of any difference between the two conditions \((P > 0.3)\), only data for breathing via the mouth are presented in Figs. 2-5. As expected, inspiratory flow rates and breathing frequency at rest differed from deep breathing \((328.80 \pm 29.43 \text{ ml/sec}; 10.09 \pm 0.55 \text{ breaths/min})\) \((P < 0.005)\) and voluntary hyperventilation \((789.66 \pm 89.54 \text{ ml/sec}; 40.24 \pm 2.55 \text{ breaths/min})\) \((P < 0.001)\). Because a Mueller maneuver entails an inspiratory effort against an occlusion, no airflow or volume change was anticipated nor detected in this condition.

The three panels in Figure 2 contain representative recordings obtained from one subject during rest breathing (Fig. 2A), volitionally modulated deep breathing (Fig. 2B) and voluntary hyperventilation (Fig. 2C) in upright (left) and supine (right). The example recordings provided here are qualitatively similar to the patterns of activity in the subject pool more broadly, and show multiunit EMG activities in the anterior and posterior locations during rest breathing. As shown for rest breathing, the muscle is tonically active in the anterior and posterior regions across upright and supine, although additional unit(s) are recruited in supine and thus the magnitude of the activity appears somewhat greater in that position. A majority (10/14) of our young adult subjects exhibited tonic GG activation
during rest breathing. In contrast, deep breathing and voluntary hyperventilation were characterized by phasic activity, with the greatest phasic activation evident in the posterior region (Figure 2B and C).

Task averages for both the inspiratory and expiratory components of the genioglossus electromyogram are shown in Figure 3. Consistent with the representative recording shown in Figure 2, average data showed inspiratory and expiratory components of the EMG of comparable magnitude in rest breathing ($P = 0.7$) however, in deep breathing, Mueller maneuver and hyperventilation, evident differences in the magnitude of the inspiratory and expiratory components of the EMG emerged ($P < 0.05$). Thus, there is a shift from predominantly tonic to predominantly phasic activation in both the anterior and posterior muscle regions as subjects progressed from rest breathing to hyperventilation. As shown, the inspiratory component of the multi-unit EMG increases in the context of deep breathing, Mueller Maneuver and voluntary hyperventilation ($P < 0.05$). Increases in the expiratory component attained significance only in voluntary hyperventilation. Accordingly, average data for the inspiratory component of the breath cycle are presented Figures 4 and 5.

We report main effects for muscle region, posterior greater than anterior ($P < 0.001$) and body position, supine greater than upright ($P = 0.008$). There was a significant interaction between muscle region and body position ($P < 0.05$) (Figure 4), such that EMG activity in the anterior region alone was greater in supine than in upright. Note that activity in the posterior GG consistently exceeded the anterior EMG independent of body position. There were no systematic differences in regional muscle activities as a function of task. Thus, although voluntary hyperventilation and Mueller maneuver were associated with greater
EMG activation relative to rest breathing and deep breathing ($P < 0.001$) (Figure 5), these effects were not specific to muscle region or body position ($P > 0.2$). Interestingly, there is a progressive increase in the magnitude of the EMG, such that in rest breathing activity approaches $\sim 5\%$ max, deep breathing $\sim 7.5\%$ max, and voluntary hyperventilation and Mueller maneuvers approach $\sim 15\%$ max.

**DISCUSSION**

In summary, body position and breathing task are key determinants of the magnitude of genioglossus electromyographic activity in both the anterior and posterior regions of the genioglossus. These healthy young adults exhibited tonic activation in the anterior and posterior regions of the muscle across upright and supine that shifted to phasic activity in volitionally modulated deep breathing and hyperventilation. The increase in the magnitude of tonic and phasic components of the EMG from rest breathing to volitionally modulated breathing is consistent with the recruitment of increasing numbers of GG motor units (Richardson and Bailey, 2010; Bailey, 2011; Walls et al., 2013). We found no evidence of differential activation of the anterior versus posterior GG, rather the magnitude of electromyographic activities in each region was similarly impacted by the requirements of the particular task.

*Critique of Method.* We studied 14 healthy young adults reportedly free of sleep-disordered breathing. Although we did not perform overnight sleep studies, subjects were interviewed and health history was provided by self-report, which in low risk subjects is known to be effective in ruling out individuals with sleep apnea (Mezzanotte et al., 1992; Young et al., 1993). Second, the population comprised a majority of women whereas previous studies recruited exclusively male subjects (Tangel et al., 1992; Wasicko et al.,
1993; Mezzanotte et al., 1996; Tsuiki et al., 2000; Saboisky et al., 2007) or a majority of men (Sauerland, E.K. and Harper, 1976; Leiter and Andrew, 1990; Douglas et al., 1993; Williams et al., 2000; Jordan et al., 2010; Saboisky et al., 2010; Wilkinson et al., 2010). We do not consider that the proportion of women in the study lessens the credibility of the findings.

In light of studies showing heterogeneity in genioglossus contractile properties (Sanguineti and Laboissi, 1997; Saigusa et al., 2001; Zur et al., 2004; Buchaillard et al., 2009; Mu and Sanders, 2010; Sanders et al., 2013), we recorded from the most anterior and posterior regions of the muscle taking care to avoid the mid region of the muscle that is considered an area of transition (Saigusa et al., 2001; Sanders et al., 2013). To access the anterior muscle we used traditional hook-wire electrodes inserted per-orally into the floor of mouth, ~5.0 mm from the internal aspect of the mandible close to the muscle’s origin on the mandible (Sauerland, E.K. and Mitchell, 1975; Sauerland, E.K. and Harper, 1976). This approach is well documented, well tolerated by subjects, and yielded excellent recordings in these fourteen subjects.

To access the posterior region of the GG we used tungsten microelectrodes inserted via the skin under the chin, using ultrasound to establish distance to the GG muscle in each subject. In our experience, tungsten electrodes cause less discomfort upon percutaneous insertion than hook-wire electrodes (which require a hypodermic needle) and permit the monitoring of electrode position and depth throughout the experiment. Tungsten electrodes also yield remarkably stable recordings across diverse behaviors and protocols as has been demonstrated previously (Pittman and Bailey, 2009; Richardson and Bailey, 2010; Laine and Bailey, 2011; Walls et al., 2013). Finally, the signal detection properties of the two types of electrodes are virtually identical, and which depends primarily on the surface area
of exposed i.e., un-insulated electrode. In both cases, the electrode head was without insulation and confirmed by the equivalent negligible impedances measured at ~1000 Hz for both electrode types. Although electrodes were of equivalent impedance and EMG activity was normalized to the regional maximum, we acknowledge that the surface areas of the two electrodes differed and as a result electrode pick-up also differed slightly. In this case, the hook-wire electrode had the smaller pick-up which may have favored detection of single motor unit activity in the anterior muscle region.

**Ventilatory-related findings.** The averages reported here for inspiratory flow rates during breathing at rest are comparable with previous reports (Williams et al., 2000; Parreira et al., 2010) and the average $V_T/T_i$ attained in voluntary hyperventilation tasks approximates the values reported previously by Eastwood *et al.* (2003) for hyperventilation in supine and during moderate cycling exercise in upright and supine (Williams et al., 2000; Walls *et al.*, 2013). Previous studies show voluntary hyperventilation lasting ~20-30 seconds exerts a negligible effect on EMG amplitude (Shea *et al.*, 2000) nevertheless any resultant hypocapnia may have reduced the magnitude of overall EMG activity.

** Electromyographic findings.** Genioglossus EMG amplitude during rest breathing in supine was greater than in upright but was significant only in the anterior muscle region. Although average EMG in the posterior region showed a trend toward increased activation in supine (see Figure 4), the increase failed to attain significance. The overall greater magnitude of EMG activity in the posterior tongue may have obscured the more subtle effects of posture on EMG activity. Our findings in supine are consistent with previously published work by Douglas *et al.* (1993) and Pae *et al.* (2002) but differ from other studies that documented transient increases in EMG activity followed by a decrease in activity.
(Wasicko et al., 1993), and no effect of body position (Williams et al., 2000). Divergent outcomes are to be expected if the conditions and/or tasks performed by the subjects also differ. For example, Wasicko et al. (1993) manipulated vestibular input and/or blood pressure secondary to passive tilt whereas Williams et al. (2000) assessed GG EMG in upright and supine within the context of cycling exercise. Thus, the stimulus may exert a unique and/or combinatorial effect on the GG electromyogram that renders meaningful comparison difficult.

The results obtained here are consistent with prior results also demonstrating predominantly tonic activation during rest breathing (Mezzanotte et al., 1992; Mateika et al., 1999; Laine et al., 2012; Walls et al., 2013) and underscore the genioglossus’ role as a pharyngeal airway dilator active in both the inspiratory and expiratory phases of the respiratory cycle in the healthy adult (Bailey, 2011). Phasic activation has been observed previously in deep breathing associated with moderate exercise (Williams et al., 2000; Walls et al., 2013). The apparent absence of phasic activation in hyperventilation (Figure 3) is an intriguing finding and resembles increased tonic GG activation seen under conditions of inspiratory loading (Tangel et al., 1992). Tonic activity may be a more energetically efficient means of ensuring airway patency especially at very high breathing rates.

Importantly, this study is distinct from the earlier study by Eastwood and colleagues (2003). Eastwood et al., recorded genioglossus EMG in rest breathing, in a 10-breath transition from rest breathing to hyperventilation, in response to negative pressure application, and during inspiration against an occluded airway. Moreover, all tasks were performed with subjects in supine position. This study documents EMG activity in rest
breathing via nose, rest breathing via the mouth, deep breathing, voluntary hyperventilation and Mueller maneuvers, performed both in upright and in supine. The recording locations accessed in this study are somewhat more anterior and further posterior than those reported by Eastwood et al. (2003).

*Volitionally modulated breathing versus rest breathing.* Relative to rest breathing, the various volitionally modulated breathing tasks were associated with significantly greater magnitude EMG overall as well as differences in the phasic and tonic EMG components. These effects held true across recording location and body positions. As noted above, the progression from rest breathing to deep breathing, hyperventilation, and Mueller maneuver was characterized by successive increases in the magnitude of the GG electromyogram (% max) in each subject (see Figure 5). This increase in the magnitude of the EMG activity may be related to the source(s) of neural drives converging onto hypoglossal motoneurons (MN). For example, rest breathing presumably is driven primarily by the respiratory central pattern generator and by mechanoreceptor and chemoreceptor inputs. For volitionally modulated breathing however, additional inputs arise in motor cortex but may also include inputs arising in pontine, parabrachial nuclei that control respiratory phase-switching and permit adjustment of the respiratory cycle to fit the particular behavioral requirements (Sawczuk and Mosier, 2001; Martelli et al., 2013; Dutschmann and Dick, 2014). Although reflex activation secondary to negative pressure pulse application was not attempted in this protocol, voluntary hyperventilation and Mueller maneuvers, large negative (inspiratory) pressures also may have contributed to genioglossus activation via stimulation of upper airway receptors resulting in reflex
augmentation of the EMG activity in a manner distinct from cortical drive (Horner et al., 1991; Eastwood et al., 2003).

Finally, studies in other respiratory motoneuron pools including intercostal and abdominal muscles have documented greater EMG activation in the context of volitionally modulated breathing (McKenzie et al., 1988; Gandevia et al., 1990), however none assessed the magnitude of EMG activity across the number of tasks attempted here. The present data obtained across a range of behaviors may be of use in assaying regional muscle activation and in gauging the proportion of the MN pool that can be recruited into activity in healthy adults.

Summary

We selected for use the traditional per-oral hook-wire electrodes and lesser known tungsten microelectrodes inserted percutaneously. Importantly, both electrodes were constructed to equivalent impedance specifications and yielded excellent recordings from their respective muscle locations. We conducted a comprehensive assessment of upper airway EMG in healthy adults across a range of breathing behaviors, in two body positions and two recording locations. Respiratory behaviors were selected for inclusion as tasks that reasonably might be encountered in the course of daily life and in the case of the Mueller maneuvers, to approximate the response of the muscle to airway obstruction (Hanly et al., 1989; Andreas et al., 1992; Morgan et al., 1993; Somers et al., 1993; Koshino et al., 2010; Camen et al., 2013). Given evidence that individuals with sleep apnea exhibit augmented GG activity in wakefulness (Mezzanotte et al., 1992; Saboisky et al., 2007, 2012) findings obtained from healthy individuals will serve as a valuable data set against which to compare anterior and posterior genioglossus activity in this population.
REFERENCES


ADDITIONAL INFORMATION

Competing interests

None.

Author contributions

Conception and design of the experiments: JRV and EFB. Collection and analysis of data: JRV. Interpretation and reporting of data: JRV and EFB. Draft and revision of the article: JRV and EFB.

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Table 1. Means (±SEM) for inspiratory time ($T_I$), expiratory time ($T_E$), breathing frequency ($f_R$), tidal volume ($V_T$), inspiratory flow ($V_T/T_I$), and minute ventilation ($V_E$) in each task and as a function of body position (i.e., upright and supine).

<table>
<thead>
<tr>
<th>Task</th>
<th>Nasal breathing</th>
<th>Oral breathing</th>
<th>Deep breathing</th>
<th>Hyper-ventilation</th>
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<td></td>
<td>Upright</td>
<td>Supine</td>
<td>Upright</td>
<td>Supine</td>
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<tr>
<td>$T_I$ (sec)</td>
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<td>$T_E$ (sec)</td>
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<td>2.75 ±0.25</td>
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<tr>
<td>$f_R$ (br/min)</td>
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<td>14.08 ±1.30</td>
<td>14.04 ±1.19</td>
<td>14.19 ±1.30</td>
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<tr>
<td>$V_T$ (mL)</td>
<td>306.01 ±39.21</td>
<td>312.62 ±38.36</td>
<td>345.29 ±47.65</td>
<td>391.83 ±47.83</td>
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<tr>
<td>$V_T/T_I$ (mL/sec)</td>
<td>189.19 ±24.41</td>
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<td>215.27 ±27.63</td>
<td>229.76 ±26.73</td>
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<tr>
<td>$V_E$ (L*br/min)</td>
<td>4.23 ±0.55</td>
<td>4.40 ±0.60</td>
<td>4.85 ±0.56</td>
<td>5.56 ±0.59</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS.

Figure 1. A) Schematic of experimental set-up. B) Lateral view of the tongue and mandible showing approximate locations of hook-wire and tungsten electrodes in the anterior and posterior GG muscle regions.

Figure 2. Representative recordings obtained from one subject during (A) rest breathing via the mouth, (B) deep breathing, and (C) voluntary hyperventilation. Airflow (l/min⁻¹) where inspiration is negative (top most trace), anterior multiunit muscle GG EMG (middle trace), and posterior multiunit muscle GG EMG (lower trace). Dashed lines demonstrate inspiratory (I) and expiratory (E) breath phase information to illustrate which portions of the electromyogram were used during analysis. (Note for this individual, average EMG (mV) recorded in the maximum maneuver was 1.3 mV in the anterior region and 1.1 mV in the posterior region.)

Figure 3. Average (±SEM) multiunit muscle EMG (%max), comparing phasic vs. tonic activation in each breathing task. Phasic activation represents EMG activity during the inspiratory phase of the breath cycle, while tonic activation represents EMG activity during the expiratory phase of the breath cycle. In the case of Mueller maneuver, tonic activity was determined from the exhale immediately following the inspiratory maneuver. (*P < 0.05, phasic vs. tonic; #P < 0.05, relative to rest breathing).

Figure 4. Average (±SEM) multiunit inspiratory GG EMG. GG EMG (% max) in anterior and posterior muscle regions, in upright and in supine. (*P < 0.05, upright vs. supine body position; #P < 0.05, anterior vs. posterior muscle region).

Figure 5. Effect of breathing task on average (±SEM) multiunit muscle EMG (% max), collapsed across muscle region and body position. (*P < 0.005 between factors; brackets indicate significantly different pairwise comparisons between breathing tasks)
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.