THE EFFECTS OF N-ACETYLCYSTEINE ON RESPIRATORY MUSCLE FATIGUE DURING HEAVY EXERCISE

by

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Abstract

Diaphragmatic fatigue is known to limit endurance performance during heavy exercise in humans. Previous reports have shown that diaphragmatic fatigue is reduced in rats with N-acetylcysteine (NAC; a nonspecific antioxidant) infusion, suggesting that oxidative stress contributes to this fatigue. However, it is not known if oral supplementation of NAC will reduce respiratory muscle fatigue during heavy exercise in humans. Therefore, the purpose of this study was to determine the effect of an acute oral dose of NAC on respiratory muscle fatigue during whole body heavy exercise. Eight healthy, non-smoking men (22±2 yrs), with no history of cardiovascular or lung disease, completed baseline pulmonary function tests followed by an incremental cycle VO_{2peak} test. A randomized, double blind crossover design was then used where subjects were given either placebo (PLA) or NAC (1800 mg) 45 min prior to a 30 minute constant load (85% VO_{2peak}) discontinuous (six-five minute stages) or continuous (cycle until volitional exhaustion) exercise test. Tests were separated by approximately one week. Maximum pressures (inspiratory, P_{Imax}; expiratory, P_{Emax}) and venous blood samples (plasma lactate and total plasma glutathione) were made prior to- and following each 5-min of exercise in discontinuous tests and pre- and post-exercise in continuous tests. Subject's VO_{2peak} was 43±5 ml/kg/min. There was no difference ($p>0.05$) in P_{Imax} between NAC (127.9±34.1 cmH_2O) or PLA (134.1±28.1 cmH_2O) at rest. During exercise, P_{Imax} was significantly lower (~14%) in 6 of 8 subjects with PLA compared to NAC at minutes 25 and 30 of the discontinuous test indicating respiratory muscle fatigue. With NAC, P_{Imax} did not change

($p>0.05$) from rest throughout exercise indicating no respiratory muscle fatigue. There was no difference ($p>0.05$) in P_{Emax}, plasma glutathione, lactate, oxygen uptake (VO_2), ventilation (VE), heart rate (HR), or rating of perceived exertion between PLA and NAC at rest or during exercise. Time to exhaustion was not different ($p>0.05$) during the continuous tests (PLA: 1263 $\pm$ 334 sec; NAC: 1047 $\pm$ 136 sec). These results suggest that an acute dose of NAC reduces respiratory muscle fatigue during high intensity exercise but does not alter other ventilatory or metabolic indices. The significance of this reduced respiratory muscle fatigue with NAC on whole body exercise performance remains to be determined.

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CHAPTER 1 - Introduction

The pulmonary system has been shown to limit exercise performance in some healthy subjects. Specifically, at high intensity exercise ($\geq$80%VO_{2peak}), respiratory muscles have been shown to fatigue (Johnson, Saupe, & Dempsey, 1992). This respiratory muscle fatigue has been hypothesized to be a primary determinant in pulmonary performance, as well as exercise performance (Dempsey, Romer, Rodman, Miller, & Smith, 2006). Respiratory muscle fatigue has been shown to be associated with hydrogen ion production, glycogen depletion, and, recently, the production of reactive oxygen species (ROS) within the respiratory and locomotor muscles (Barreiro et al., 2006; Davies, Quintanilha, Brooks, & Packer, 1982; Juel, 2006; Kerksick & Willoughby, 2005; McKenna et al., 2006; Medved et al., 2004b; Medved, Brown, Bjorksten, & McKenna, 2004a; Reid, Stokic, Koch, Khawli, & Leis, 1994; Reid, 2001a; Supinski, 1998). At the cellular level, ROS can alter chemical balances, blood flow, damage cell membranes, and cause DNA morphing (Davies et al., 1982; Kerksick & Willoughby, 2005; Medved et al., 2004a). ROS are produced in fatiguing muscle and free radical scavengers/antioxidants are needed to combat ROS and prevent cellular damage and maintain homeostasis.

N-acetylcysteine (NAC) is a free radical scavenger that has been associated with decreased levels of fatigue in limb muscles (Barreiro et al., 2006; Kerksick & Willoughby, 2005; McKenna et al., 2006; Medved et al., 2004b; Sen & Packer, 2000; Shindoh, DiMarco, Thomas, Manubay, & Supinski, 1990) and, more recently in the respiratory muscles (Sen & Packer, 2000; Shindoh et al., 1990; Supinski, Stofan, Ciufo,

& DiMarco, 1997b; Travaline et al., 1997). Glutathione (GSH), an endogenous antioxidant, is oxidized in a naturally occurring cellular process (Sen & Packer, 2000; Sjodin, Hellsten Westing, & Apple, 1990; Smith & Reid, 2006), but when it is produced it increases the number of ROS (Sen & Packer, 2000; Sjodin et al., 1990; Smith & Reid, 2006). When NAC is present in the cell, it dissociates into cysteine and aids in the process of returning oxidized glutathione (GSSG) back into GSH in a process called GSH biosynthesis (Sen, Rankinen, Vaisanen, & Rauramaa, 1994b; Sen & Packer, 2000; Sjodin et al., 1990; Smith & Reid, 2006). This same process allows GSH to combine with hydrogen peroxide, a ROS, to form water (Sen & Packer, 2000; Sjodin et al., 1990; Smith & Reid, 2006) neutralizing the cellular environment.

NAC supplementation has been shown in several studies, primarily animals, to reduce respiratory muscle fatigue (Barreiro et al., 2006; Kerksick & Willoughby, 2005; McKenna et al., 2006; Medved et al., 2004b; Shindoh et al., 1990). However, the mechanism of how NAC delays this fatigue has not been examined (Juel, 2006; McKenna et al., 2006; Medved et al., 2003; Medved et al, 2004a; Mishima, Yamada, Matsunaga, & Wada, 2005; Sen & Packer, 2000; Shindoh et al., 1990). It is presently unknown whether an acute oral doses of NAC will reduce respiratory muscle fatigue and/or improve exercise performance in humans.

CHAPTER 2 - Review of Literature

High intensity exercise (>85%VO_{2max}) has been shown to cause respiratory muscle fatigue (Babcock, Pegelow, Harms, & Dempsey, 2002; Dempsey et al., 2006; Johnson, Babcock, Suman, & Dempsey, 1993; Ozkaplan, Rhodes, Sheel, & Taunton, 2005) which has been reported to limit exercise tolerance (Dempsey et al., 2006). Therefore, it is of interest to identify factors which either contribute to this fatigue, or help alleviate it. Reactive oxygen species (ROS) have been identified in promoting respiratory muscle fatigue. Antioxidants may therefore, be useful to combat ROS and potentially reduce respiratory muscle fatigue. Infusion of one specific antioxidant, N-Acetylcysteine (NAC), has been shown to decrease respiratory muscle fatigue and increase whole body exercise performance (Koechlin et al., 2004; Reid et al., 1994). It is not known if an acute oral dose will show similar effects. This review will first examine respiratory muscles during exercise, followed by the role of ROS on skeletal muscle fatigue, and finally, the role of antioxidants, specifically NAC, on respiratory muscle performance.

Respiratory Muscles during Exercise

The primary respiratory muscle is the diaphragm, while the internal and external intercostals, sternocleidomastoids, and abdominals serve accessory roles. The diaphragm tends to resist fatigue due to its high oxidative capacity and increased amount of blood flow during exercise (Dempsey et al., 2006). As exercise intensity exceeds 80% VO_{2peak},

the diaphragm reportedly fatigues, as shown by bilateral phrenic nerve stimulation (Johnson et al., 1993), and accessory muscles are recruited to help maintain ventilation (Babcock et al., 2002; Dempsey et al., 2006; Johnson et al., 1993; Ozkaplan et al., 2005). This respiratory muscle fatigue seen with high intensity exercise may limit whole body exercise performance by decreasing vascular conductance and blood flow to the locomotor limb musculature (Babcock et al., 2002; Harms et al., 1997; Harms et al., 1998) and increasing work of breathing (Romer, Lovering, Haverkamp, Pegelow, & Dempsey, 2006; Sheel et al., 2001).

Significance of Respiratory Muscles during Exercise

Respiratory Muscles and Work of Breathing

At rest, the amount of oxygen needed (the cost of breathing) is negligible compared to what is needed during exercise. In fact, Aaron, Seow, Johnson, and Dempsey. (1992) reported that approximately 2% of total body VO_2 (6-10 ml/min) is required to sustain ventilation. As ventilation increases during voluntary hyperpnea, the cost or work of breathing, and thus the cost of oxygen has been shown to increase as well (Coast & Krause, 1993; Shephard, 1966). Therefore, as the body transitions from rest to moderate exercise, the work of breathing is increased from 2% to 3-5% and 10-15% (300-600 ml/min) of total body VO_2 in sedentary and highly fit individuals, respectively (Aaron et al., 1992). This increase in work of breathing is accompanied by changes in metabolic effects, such as an increase in cardiac output; however, these changes are usually seen during heavy exercise (Aaron et al., 1992; Harms et al., 1998).

Respiratory Muscles, Exercise, and Blood Flow

Respiratory muscle fatigue during exercise leads to cardiovascular adjustments. In particular, during whole body exercise, there is a "competition" between respiratory muscles and locomotor muscles for the available cardiac output such that respiratory muscles apparently "steal" blood flow and oxygen from locomotor muscles (Harms et al., 1997). Although this "competition" has not been measured directly in the human model, radioactive microspheres and flow probes have been utilized in the animal model to determine changes in blood flow during exercise (Musch et al., 1987; Poole et al., 2000). During incremental treadmill running, the mongrel dog showed an increase in blood flow (~16-25%) to the respiratory muscles (Musch et al., 1987). Similarly, Poole et al. (2000) showed an increase in blood flow (~260%) to the rat diaphragm during maximal exercise when compared to resting values. These authors also reported that accessory respiratory muscles, such as the intercostals, scalenus, and abdominals, also show an increase in blood flow of 6- to 10-fold (68 ± 6, 152 ± 36, and 148 ± 21 ml/min/100g, respectively). Vascular conductance also increased in these accessory muscles suggesting the necessity to increase blood flow to the respiratory muscles due to increased hyperpnea and the high oxidative capacity of the diaphragm (Poole et al., 2000).

Although a shift in blood flow between vascular beds during exercise has not been directly measured in humans, cardiac output to the respiratory muscles has been previously determined by use of a Swann-Ganz catheter and the direct Fick method for measuring cardiac output. Harms et al. (1998) found that approximately 14-16% of total cardiac output is directed to the respiratory muscles during maximal exercise. The cardiac output in this case is limited to the working locomotor musculature, and the exercising limbs and respiratory muscles must therefore compete for the available blood

flow. Thus, the exercising limbs may be affected by the blood flow competition between the respiratory muscles and the locomotor muscles. Local reflexes, leading to sympathetic vasoconstriction, are the likely mechanism for this effect (Harms et al., 1997; St Croix, Morgan, Wetter, & Dempsey, 2000).

Respiratory muscle fatigue is thought to contribute to increasing ventilatory requirements and influence exercise performance by reducing locomotor muscle blood flow and oxygen transport, leading to whole body fatigue (Dempsey et al., 2006; Harms, Wetter, St Croix, Pegelow, & Dempsey, 2000). The effect of respiratory muscle fatigue on exercise performance was further demonstrated by McConnell and Lomax (2006). These authors reported that during inspiratory muscle fatigue and plantar flexion exercise, left ventricular contraction decreased, indicating that inspiratory muscle work increases with skeletal muscle fatigue (McConnell & Lomax, 2006). This data supports the blood flow steal phenomenon. Others have shown a decrease in limb blood flow and an increase in limb vascular resistance during inspiratory muscle fatigue (Sheel et al., 2001). These authors found that cardiovascular adjustments are limiting exercise performance due to increasing resting limb blood flow during respiratory muscle fatigue (Sheel et al., 2001). However, the mechanism for blood flow distribution is likely, though not proven, to be an activated metaboreflex (type III and IV afferents) which is linked to respiratory muscle fatigue (Dempsey et al., 2006; Sheel et al., 2001; St Croix et al., 2000).

Respiratory Muscles and Exercise Performance

Decreasing the work of breathing during heavy exercise has been shown to improve whole body exercise performance (Harms et al., 2000; Romer et al., 2006).

During high-intensity cycling, exercise time to fatigue is inversely related to the level of prevailing inspiratory muscle work. Thus, as respiratory muscle fatigue is reduced, exercise time increases, suggesting that inspiratory muscle fatigue limits limb performance (McConnell & Lomax, 2006). Moreover, Romer et al. (2006) have shown increases in peripheral muscle fatigue during incremental cycling exercise with proportional assist ventilator (loaded breathing to increase the work of breathing). These authors have suggested when fatigue of the respiratory muscles is enhanced by increasing the work of breathing, the peripheral muscles, the quadriceps in this case, fatigue much quicker (Romer et al., 2006).

Another strategy to reduce respiratory muscle work and enhance exercise performance is to increase respiratory muscle strength by inspiratory muscle training. Downey et al. (2007) showed that inspiratory muscle training increased diaphragm thickness (8-12%), increased respiratory muscle strength (25%), and decreased respiratory muscle fatigue by ~7.5% during exercise at 80% VO_{2max}. In agreement, Verges et al. (2007) found that respiratory muscle fatigue was reduced >10% with respiratory muscle training. However, neither of these investigators saw a change in cycling endurance exercise, implying a change in respiratory muscle fatigue, via respiratory muscle training, does not necessarily influence whole body exercise performance (Downey et al., 2007; Verges, Lenherr, Haner, Schulz, & Spengler, 2007). Therefore, the effect of reducing respiratory muscle fatigue on whole body exercise performance is not clear.

Reactive Oxygen Species Increase Fatigue

Reactive oxygen species are oxidized molecules (molecules with an unpaired electron) which react with reduced molecules (molecules with all paired electrons). This transfer of electrons or hydrogen ions from one molecule to the other is called oxidation-reduction reactions. The over consumption of oxygen, leading to the production of reactive oxygen species, results from: 1) an error in a mitochondrial oxidative process, 2) a hypoxic environment inside the capillary endothelium (i.e. exercise and disease states), and/or 3) muscle and tissue damage from an oxidative burst from inflammatory cells (Sjodin et al., 1990) (i.e. prolonged intense exercise or eccentric-based exercise states (Kerksick & Willoughby, 2005)). Reactive oxygen species (ROS) have been suggested to increase fatigue (Juel, 2006) and impair contracting skeletal muscle (Davies et al., 1982; Reid et al., 1992). Although moderate exposure of ROS to the musculature increases their ability to generate force, excessive ROS production can significantly reduce muscle force generation (Shindoh et al., 1990). With regards to exercise, levels of ROS also increase; therefore, ROS levels may have a significant contribution to exercise performance (Juel, 2006) and, more specifically, muscle fatigue (Davies et al., 1982; Reid et al., 1992).

Antioxidants

Antioxidants work in the body as reducing agents and are oxidized when ROS "steal" a paired electron. The purpose of antioxidants is to produce water and a stable molecule. The body's endogenous antioxidant system (antioxidants that originate in the body) is unable to remove excess ROS if there are not enough antioxidants (molecules

capable of slowing or preventing oxidation-reduction reactions). These conditions occur if there is: 1) inadequate antioxidant content in the normal diet, 2) a high intake of pro-oxidants, 3) exposure to chemicals or ultraviolet light, 4) injury, and/or 5) intense exercise (Kerksick & Willoughby, 2005). The production of ROS augments muscle fatigue in the rat (Reid et al., 1992) and canine diaphragm (Nashawati, Dimarco, & Supinski, 1993; G. Supinski, Nethery, Stofan, & DiMarco, 1997) and the skeletal muscle of the mouse (Barclay & Hansel, 1991). Therefore, enhancing exogenous antioxidant intake (the antioxidants that are taken in as food, supplements, or vitamins) may have a favorable effect on muscle performance.

Antioxidants Decrease Fatigue

Increasing certain antioxidants (i.e. glutathione, n-acetylcysteine, ά-lipoid acid, and vitamins A, E, and C) in the body's circulation will prevent the accumulation of ROS thereby reducing oxidative stress (Kerksick & Willoughby, 2005; Matuszczak et al., 2005; Medved, Brown, Bjorksten, & McKenna, 2004). [Oxidative stress indices are exceeded due to the ROS increased levels in the cytosol (Reid et al., 1992), extracellular space (Reid, Shoji, Moody, & Entman, 1992), and vascular compartment (Kolbeck, She, Callahan, & Nosek, 1997; O'Neill, Stebbins, Bonigut, Halliwell, & Longhurst, 1996). Oxidative stress also elevates glutathione oxidation (Mishima et al., 2005) and malondialdehyde (Reid, 2001b).] The use of antioxidant compounds has aided in the reduction of fatigue in numerous preparations such as canine diaphragm (G. Supinski et al., 1997) and gastrocnemius muscle (Barclay & Hansel, 1991), rat diaphragm bundles (Reid et al., 1992), and human diaphragm (Travaline et al., 1997). They have also shown

to reduce fatigue in voluntary exercise in mice (Novelli, Falsini, & Bracciotti, 1991), humans (Lands, Grey, & Smountas, 1999), and human limb electrical stimulation (Reid et al., 1994). Subsequently, maintaining pre-fatigue levels of oxidation-reduction sensitive functions and oxidative stress is the potential outcome when using supplemental antioxidants (Reid et al., 1994).

N-Acetylcysteine

One specific antioxidant which has been suggested to decrease respiratory muscle fatigue is N-Acetylcysteine (NAC). This antioxidant is a ROS scavenger (Aruoma, Halliwell, Hoey, & Butler, 1989), that maintains glutathione status in muscle cells (Kerksick & Willoughby, 2005), and has been shown to decrease exercise-induced oxidative stress (Koechlin et al., 2004). NAC, a reduced cysteine donor, acts in the cell supporting glutathione (GSH) synthesis and resynthesis and opposing oxidative stress (Ruffmann & Wendel, 1991). NAC works in the cell by scavenging superoxide anion radicals, hydroxyl radicals, and H_2O_2. It also supplies cysteine for *de novo* synthesis of GSH (Shindoh et al., 1990). Meister (1998) has shown that GSH is used in the body by GSH peroxidases. GSH peroxidases are known to reduce H_2O_2 and organic peroxides and form GSSG (oxidized glutathione). They also protect the cell proteins and cell membranes against oxidation (Meister, 1988). NAC is an acetylated version of cysteine, which is the rate limiting substrate for glutathione synthesis in the body (Sen, 2001). Supplementation of NAC has also been shown to modify vascular tone and redistribute cardiac output, which may have an effect on respiratory and skeletal muscle fatigue (Harrison, Wendon, Gimson, Alexander, & Williams, 1991).

N-Acetylcysteine and Skeletal Muscle Fatigue

Muscle fatigue in humans is associated with oxidative stress and an increase in ROS production. To reduce the impact of ROS, Reid et al. (1994) discovered that pretreatment with NAC is useful for reducing the amount of human skeletal muscle fatigue. This finding was established using intravenous loading (150mg/kg) and repetitive, low-frequency electrical stimulation (10Hz) in the tibialis anterior. NAC increased force production compared to placebo conditions, starting at minute three and continued until trials concluded. However, using the same protocol, with high-frequency stimulation (40 Hz), NAC was not significantly different than placebo conditions. Therefore, this landmark study influenced others to use pretreatment of NAC during whole body exercise (McKenna et al., 2006; Medved et al., 2003) and voluntary muscle contraction (Koechlin et al., 2004). Medved et al. (2003) used intravenous NAC (125mg/kg/h for 15 minutes and 25mg/kg/h during testing session) during high-intensity (130% VO_{2peak}) intermittent cycling in healthy humans. These authors found that NAC altered blood redox status and impaired plasma potassium (K^+) regulation, but did not enhance performance during the high-intensity intermittent cycling exercise. Although these authors did not see an improvement in exercise performance, McKenna et al. (2006) did see an improvement of ~24% during submaximal cycling exercise in healthy men. In a different human population, Koechlin et al. (2004) used chronic oral dosing (600mg 3xday with an additional 600mg on testing day) in chronic obstructive pulmonary disease patients. These authors found that NAC prevented exercise-induced oxidative stress and enhanced quadriceps endurance by 25%. To date, results are controversial and the effect of NAC on the respiratory muscles has not been sufficiently established.

N-Acetylcysteine and Respiratory Muscle Fatigue

During strenuous exercise, contractions in the diaphragm and other respiratory muscles release ROS (Shindoh et al., 1990; Supinski et al., 1997a; Supinski et al., 1997b) and it is possible that ROS may contribute to fatigue (Reid et al., 1992b). Due to NAC's ability to increase GSH levels, it is possible for NAC to establish a more cellular favorable environment (a balance between antioxidants and ROS), therefore improving exercise performance (Kerksick & Willoughby, 2005). Low-frequency diaphragmatic fatigue has been attributed to an increased amount of ROS (Reid et al., 1992b; Supinski et al., 1997b), but NAC has also been shown to attenuate this fatigue during low-frequency diaphragmatic electrical stimulation (Barclay & Hansel, 1991; Hida et al., 1996; Nashawati et al., 1993; Reid et al., 1994; Shindoh et al., 1990; Travaline et al., 1997). To date, the majority of studies have utilized infusion of NAC to determine its effect on respiratory muscle fatigue.

Intravenous loading has been found to be effective in decreasing respiratory muscle fatigue (Shindoh et al., 1990; Supinski et al., 1997a; Travaline et al., 1997), decreasing skeletal muscle fatigue (Matuszczak et al., 2005; Reid et al., 1994), changing muscle GSH levels (Matuszczak et al., 2005; Medved et al., 2003; Supinski et al., 1997a), and affect exercise performance (Medved et al., 2004b; Reid et al., 1994; Travaline et al., 1997). A study by Supinski et al. (1997b) determined that rats infused with NAC (150mg/kg) were able to tolerate and maintain higher inspiratory pressure, and sustain higher inspired volumes. These authors also demonstrated that rats infused with NAC were able to tolerate greater inspiratory pressures and volumes prior to respiratory arrest development and that diaphragmatic GSH levels were significantly reduced compared to unloaded controls (Supinski et al., 1997). In the rabbit diaphragm strip, Shindoh et al.

(1990) found similar results to that of Supinski et al. (1997b). Intravenous NAC decreased fatigue compared to control groups during rhythmic, repetitive isometric contraction (Shindoh et al., 1990). The force-frequency relationship also decreased with placebo. In addition to these animal models, Travaline et al. (1997) demonstrated, in healthy human subjects, that increased inspiratory resistive loading and task endurance were greater after NAC administration (150mg/kg). This study utilized transdiaphragmatic twitch occlusion to initiate diaphragmatic fatigue. These authors also found that high-frequency diaphragmatic fatigue had less of an effect with NAC than the low-frequency diaphragmatic fatigue effect with NAC. These results are likely due to improvements in both diaphragm strength and endurance as well as ROS mediated effects by NAC treatment.

To date, there have been few studies examining the effects of oral supplementation of NAC on skeletal muscle fatigue. Some reports suggest that oral supplementation seems to have similar effects as intravenous loading. In dogs, oxidative stress was reduced by oral supplementation of NAC (3mmol/kg every 24 hours) during high intensity respiratory loading (Barreiro et al., 2006). Oxidative stress was also reduced in chronic obstructive pulmonary disease (COPD) patients who supplemented with oral NAC (3x200mg-three times per day for four days with an additional 600mg on test day) (Koechlin et al., 2004). This increased the dynamic one-repetition maximum leg extension by 40% and increased endurance by 25%. Another study using a human model showed no change in blood GSH with NAC dosing (4x200mg per day for two days with an additional 800mg on test day) (Sen, Atalay, & Hanninen, 1994). However, this study tested the difference on blood and plasma levels of GSH and GSSG with

differing exercise intensities. Although these studies have shown a decrease in oxidative stress with chronic oral supplementation in animal and human models and an increase in endurance and strength performance, it is still not known if an acute oral dose will have similar results.

Summary

Respiratory muscles fatigue during high-intensity exercise. With the onset of respiratory muscle fatigue, cardiac output may redistribute from the limbs to the respiratory muscles compromising locomotor blood flow and adversely affecting performance. Reactive oxygen species have been implicated to contribute to respiratory muscle fatigue. N-Acetylcysteine may reduce reactive oxygen species in respiratory muscles and potentially negate respiratory muscle fatigue. To date, decreased respiratory muscle fatigue in humans using an acute oral dose of NAC has never been determined.

Statement of the Problem

The purpose of the study is to determine the effects of an acute oral dose of N-Acetylcysteine on respiratory muscle fatigue in healthy, active college-aged males during heavy exercise.

Hypotheses

We hypothesized that, compared to placebo based control trials, acute NAC supplementation prior to 30 minutes of heavy, constant-load exercise, will increase plasma glutathione levels and decrease respiratory muscle fatigue.

CHAPTER 3 - Methods

Nine healthy men were recruited to participate in this study. All were free from pulmonary and heart disease as determined from medical history questionnaire. The subjects were active, but not competitively trained, and maintained their normal activity status throughout testing. Each participant was told not to consume alcohol or participate in vigorous activity during 24 hours prior to testing, not to consume antioxidants, vitamins, or supplementations of any kind throughout testing, and not to consume food within two hours prior to testing. Informed consent was obtained from each subject and all procedures were approved by the Kansas State University Human Research Board, Manhattan, KS.

Experimental Design

Pulmonary function tests (PFT) include total lung capacity (TLC), inspiratory and expiratory pressures (P_{Imax} and P_{Emax}, respectively), and maximum flow-volume loops (MFVL) were determined during the first testing session. A peak oxygen uptake (VO_{2peak}) test on an electromagnetically braked cycle ergometer (800 Ergometer, SensorMedics, USA) was then performed by each subject. Seat height was measured and used for all future tests and toe clips were in place for every exercise test on the cycle ergometer. After VO_{2peak} was established, 2-4 practice sessions took place within one week to determine a workload that elicited ~85% VO_{2peak} and to familiarize the subjects

with all equipment. After all submax tests were complete, one participant dropped out for personal reasons leaving eight subjects for data analysis.

Following the practice sessions, a randomized, double blind cross-over study was used. Subjects were initially randomly assigned to either two discontinuous or two continuous protocols. After the first protocol was complete, the subject completed the alternative protocol for a total of four testing sessions. Each protocol consisted of either a placebo (PLA: cornstarch) or experimental (NAC: N-Acetylcysteine) trial in random order. A dosage of 1800mg (3x600mg) of NAC was used during the loading sessions based on the study by Koechlin et al. (2004), who showed significant decreases in oxidative fatigue in the human quadriceps using this level of oral dosing. PLA were in identical pill casings as NAC (3 pills). A senior investigator remained un-blinded throughout the study, and administered the supplements.

Pulmonary Function Tests

Total lung capacity (TLC) and residual volume (RV) were measured using a nitrogen washout technique. Maximal inspiratory pressure (P_{Imax}) was measured from RV and maximal expiratory pressure (P_{Emax}) was measured from TLC. P_{Imax} and P_{Emax} are measurements of respiratory muscle strength measured at the mouth and have been shown to be a good estimate of respiratory muscle strength (Huang, Martin, & Davenport, 2003; Inbar, Weiner, Azgad, Rotstein, & Weinstein, 2000; Romer, McConnell, & Jones, 2002a; Romer, McConnell, & Jones, 2002b; Sonetti, Wetter, Pegelow, & Dempsey, 2001). All PFT measurements were performed in triplicate except

during the discontinuous bouts when P_{Imax} and P_{Emax} were measured in duplicate due to time constraints. The average values of acceptable trials were used in analysis.

Peak Oxygen Uptake (VO_{2peak})

An incremental exercise cycle ergometer (800 Ergometer, SensorMedics, USA) test to exhaustion was performed to determine VO_{2peak} on a breath by breath basis using a metabolic cart (Vmax Series 229, SensorMedics, USA). PFTs (MFVL, P_{Imax}, and P_{Emax}) were measured to establish resting values. Four minutes of baseline with participant sitting on the cycle ergometer were taken followed by four minutes of warm-up at 20 watts (W). At the end of the warm-up, incremental exercise began and the resistance of the cycle ergometer was increased 25W every minute until volitional fatigue. Pedaling frequency was maintained at 60-70 revolutions per minute (RPM). The end of the test was determined when subjects could not maintain a pedal frequency of $\geq$50 RPM for five consecutive pedal revolutions. Following a four minute cool-down, post-PFTs were made. VO_{2peak} was determined by the average VO_2 in the last minute of exercise. Arterial oxygen saturation (SaO_2) was determined by a pulse oximeter (Datex-Ohmeda 3900P, Madison, WI) which was attached and secured to the earlobe to ensure accuracy and minimize movement artifacts. The workload associated with 85% of VO_{2peak} was calculated which was used in sub-maximal exercise. Workload was adjusted if during the VO_2 practice session was <80% VO_{2peak} or > 90% VO_{2peak}.

Discontinuous Exercise Protocol

Pulmonary function measurements and a venous blood sample were made prior to submaximal exercise. Subjects then ingested NAC or PLA and rested 45 minutes in the lab for peak blood concentration to occur (Borgstrom, Kagedal, & Paulsen, 1986; De Bernardi di Valserra,M. et al., 1989; Matuszczak et al., 2005; Sen et al., 1994). At 45 minutes, a second blood sample was taken followed by four minutes of baseline metabolic data, followed by four minutes of warm-up (20W). Subjects then cycled for five minutes at their predetermined 85% VO_{2peak} workload, at which time they stopped pedaling for two minutes while repeat P_{Imax} and P_{Emax} measurements and a venous blood sample were made. Following this two minute rest, subjects immediately began cycling at the specified workload and followed this protocol for a total of six bouts of exercise (30 minutes total). Post-PFTs and bloods were taken at the conclusion of a three minute cool-down.

Continuous Protocol

Subjects also completed two continuous exercise bouts at ~85% VO_{2peak}. PFT measurements and venous blood samples were made prior to exercise. Participants then loaded with NAC or PLA and waited for an absorption period of 45 minutes. At the conclusion of the 45 minutes, another blood sample was taken followed by baseline data collection for four minutes, and finally four minutes of warm-up at 20W. After warm-up, exercise was performed at 85% VO_{2peak} work-rate until volitional fatigue. Pedal frequency was maintained at 60-70 RPM. If a participant voluntarily stopped despite

verbal encouragement or pedal frequency $\leq$ 50 RPM, the test was terminated. A three minute cool-down was performed followed by post-PFTs and a venous blood sample.

Venous Blood Sampling

A 22gauge, in-dwelling catheter was placed in the right antecubital vein of each subject at the beginning of each testing session. Samples were taken in 3cc syringes treated with heperanized saline, transferred to vacutainers treated with EDTA, and placed on ice. After each draw, the catheter was flushed with heperanized saline to keep the line patent. All samples were kept on ice before and immediately after being centrifuged. Resulting plasma was frozen for later determination of both plasma total glutathione concentration (Biovision ApoGSH Glutathione Colorimetric Detection Kit, Mountain View, CA) and plasma lactate concentration (YSI 2300 STAT Plus Glucose and Lactate Analyzer, Yellow Springs Instruments, Yellow Springs, OH).

Statistics

SigmaSTAT statistical software (Jandel Scientific Software) was used for data analysis. Data is presented as mean $\pm$ standard deviation. Differences were determined by a 2x2 (group x time) ANOVA. A Tukey post hoc analysis was performed to determine where significant differences existed. Statistical significance was set at $P \leq 0.05$ for all analysis.

CHAPTER 4 - Results

Subject Characteristics

Subject characteristics are shown in Table 1. Subjects were active, but not competitively trained. All subjects pulmonary function values were within normal predicted values. Also, subjects stated that they maintained activity status throughout the study. No known side effects of NAC were reported by the subjects.

Table 1: Subject Characteristics

Age (yr)	22.0 $\pm$ 2.3
Weight (kg)	77.3 $\pm$ 15.7
Height (cm)	181.0 $\pm$ 11.1
BMI (kg/m^2)	23.6 $\pm$ 4.2
PEF (l/sec)	7.65 $\pm$ 1.7
FEF_{25-75} (l/sec)	4.48$\pm$ 1.2
FEV_1 (l/sec)	4.22 $\pm$ 0.47
FVC (l)	5.06 $\pm$ 0.34
FEV_1/FVC (%)	83.0 $\pm$ 6.5
TLC (l)	6.43 $\pm$ 1.56
VC (l)	4.65 $\pm$ 0.68
RV(l)	1.78 $\pm$ 1.25

Table 1: All values are mean $\pm$ SD. BMI: body mass index; PEF: peak expiratory flow; $FEF_{25-75\%}$: forced expiratory flow during 25-75% of vital capacity; FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; TLC: total lung capacity; VC: vital capacity; RV: residual volume.

VO_{2peak} Data

Table 2 shows data recorded at VO_{2peak}. At end exercise, all subjects had an RER>1.10, HR within 10% of maximal HR, and with minimal desaturation (>94% SaO_2).

Table 2: VO_{2peak} Data

VO_2 (l/min)	3.25 ± 0.42
VO_2 (ml/kg/min)	42.7 ± 5.1
VE (l/min)	118.3 ± 19.4
VE/VO_2	36.9 ± 5.7
VE/VCO_2	32.6 ± 4.2
RER	1.13 ± 0.05
SaO_2 (%)	96.9 ± 1.5
HR (bpm)	177.1 ± 9.4

Table 2: All values are mean ± SD. VE: minute ventilation; RER: respiratory exchange ratio.

Plasma Glutathione

Glutathione (GSH) during submaximal exercise testing is presented in Table 3. There was no change ($p>0.05$) in GSH in each stage; i.e., pre-loading (PRE), post-loading (POST), minutes 5-30, or post-exercise (POST-EX); nor was there a change ($p>0.05$) in plasma GSH between groups (PLA vs. NAC).

Maximum Inspiratory and Expiratory Pressure

Mean maximal inspiratory and expiratory pressures ($P_{Imax,}$ P_{Emax} respectively) taken during submax exercise tests are shown in Table 5. Figure 1 shows mean P_{Imax} at rest and each time point during exercise. There were no differences ($p>0.05$) in P_{Imax} at

rest between groups. During exercise, P_{Imax} was significantly lower (~14%) during placebo at minutes 25 and 30 compared to rest, suggesting significantly less respiratory muscle fatigue with NAC. P_{Imax} did not change from rest to exercise with NAC supplementation. Individual values at minute 30 are shown in Figure 2. There was significantly lower P_{Imax} in PLA in 6 of 8 subjects. There was no change ($p>0.05$) between placebo and NAC at rest or during exercise (minute 5-30) in P_{Emax}. Also, for the constant load continuous exercise trials, P_{Imax} was not different ($p>0.05$) prior to exercise (NAC: 137.5$\pm$21.7cmH_2O; PLA: 129$\pm$24.3cmH_2O), but was significantly lower (~16%) following exercise with placebo compared to NAC (PLA: 117.4$\pm$28.1cmH_2O; NAC: 135.2$\pm$23.7cmH_2O).

Table 3: Plasma Glutathione (Discontinuous Exercise Bout)

	Rest		Post-loading		5-min		10-min		15-min		20-min		25-min		30-min		Post-Exercise	
	PLA	NAC	PLA	NAC	PLA	NAC	PLA	NAC	PLA	NAC	PLA	NAC	PLA	NAC	PLA	NAC	PLA	NAC
GSH (ng/L)	26.7 ± 13.3	22.6 ± 4.9	24.9 ± 5.4	23.4 ± 6.1	21.6 ± 6.5	26.9 ± 9.2	25.7 ± 10.0	24.4 ± 7.0	23.9 ± 7.9	24.7 ± 6.3	23.6 ± 8.8	23.8 ± 8.8	22.6 ± 5.1	22.0 ± 4.4	24.5 ± 9.8	24.9 ± 7.8	22.7 ± 7.6	21.5 ± 5.3

Table 3: Discontinuous plasma glutathione (GSH) levels shown in placebo (PLA) and N-Acetylcysteine (NAC) conditions during rest, post-loading of supplementation (PLA or NAC), five minute exercise bouts, and post-exercising conditions. GSH was not different between conditions or across time ($p>0.05$). All values are mean ± SD.

Table 4: Plasma Glutathione (Continuous Exercise Bout)

	Rest		Post-Loading		Post-Exercise	
	PLA	NAC	PLA	NAC	PLA	NAC
GSH (ng/L)	24.1 ± 5.5	32.8 ± 23.1	23.5 ± 6.1	24.0 ± 9.7	28.3 ± 15.6	27.4 ± 14.1

Table 4: Plasma glutathione (GSH) levels during continuous exercise bouts are shown with placebo (PLA) and N-Acetylcysteine (NAC) conditions during rest, post-loading of supplementation (PLA or NAC), and post-exercise. GSH was not different between conditions or across time ($p>0.05$). All values are mean ± SD.

Table 5: Mean Maximum Inspiratory and Expiratory Pressures

	Rest		5-minute		10-minute		15-minute		20-minute		25-minute		30-minute		Post-Exercise	
	PLA	NAC	PLA	NAC	PLA	NAC	PLA	NAC	PLA	NAC	PLA	NAC	PLA	NAC	PLA	NAC
PI max (cmH_2O)	138.5 ± 27.1	140.2 ± 31.7	138.1 ± 28.0	141.3 ± 29.7	137.1 ± 30.9	140.3 ± 29.4	135.7 ± 27.5	139.1 ± 26.1	128.5 ± 28.7	137.9 ± 25.7	123.7 ± 27.3	139.7 ± 25.7*	124.9 ± 22.7	135.9 ± 27.1*	125.7 ± 23.1	136.8 ± 24.7
PE max (cmH_2O)	154.5 ± 35.2	153.6 ± 25.8	153.8 ± 28.5	138.4 ± 37.0	149.6 ± 29.8	151.4 ± 31.5	140.1 ± 29.1	155.6 ± 31.4	134.3 ± 28.9	152.1 ± 33.5	142.4 ± 28.6	150.3 ± 34.0	139.0 ± 32.2	149.6 ± 30.3	141.7 ± 39.5	140.9 ± 29.1

Table 5: Inspiratory and Expiratory pressures, taken at rest, each five-minute exercise bout, and post-exercise. All values are mean ± SD, * = $p \leq 0.05$

Figure 1: Maximum Inspiratory Pressure versus Time

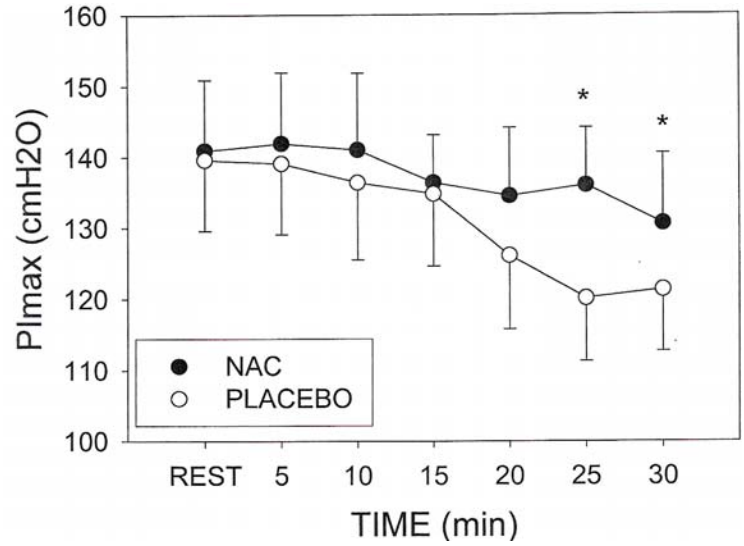

Figure 1: Maximal inspiratory pressures with placebo (PLA) showed a significant decrease at minute 25 and 30 of constant load exercise (~85% VO_{2peak}) compared to resting conditions. N-Acetylcysteine (NAC) trials show no difference ($p>0.05$) from rest throughout exercise. Therefore, P_{Imax} was significantly lower with PLA at minutes 25 and 30 versus NAC.

Figure 2: Individual and Mean Inspiratory Pressures at minute 30

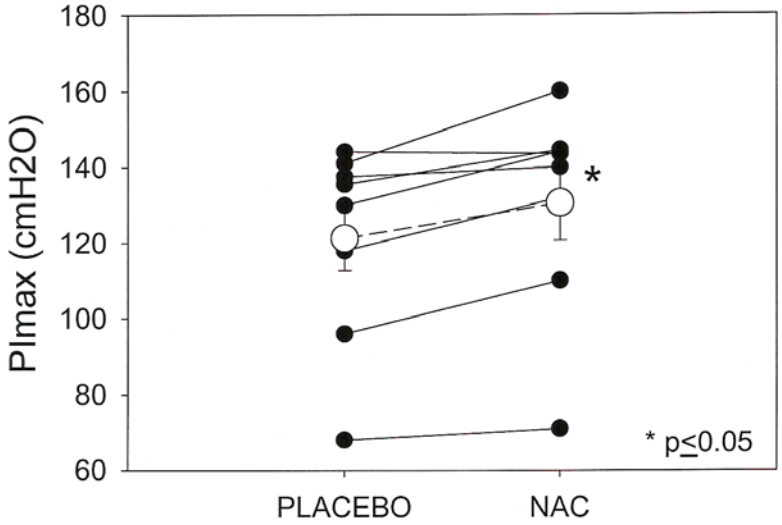

Figure 2: An increase in maximal inspiratory pressures was observed in 6 of 8 subjects with N-Acetylcysteine (NAC) with a mean increase of ~8%; *p≤0.05.

Exercise Response

Exercise VO_2 (percent of VO_{2peak}) averaged 79.5 ± 4.9% at minute 5, 84.8 ± 4.1% at minute 10, 88.4 ± 8.1% at minute 15, 87.4 ± 10.9% at minute 20, 87.9 ± 7.6% at minute 25, and 86.6 ± 9.9% at minute 30. Table 6 shows metabolic data during the discontinuous exercise test. Figure 3 shows VO_2 versus time, Figure 4 shows heart rate versus time, and Figure 5 shows ventilation versus time. There was no significant difference between PLA and NAC in any measured variable throughout exercise. Individual and mean time to exhaustion (T_{lim}), during the continuous exercise bout at 85% VO_{2peak}, is presented in Figure 6. Although there was no significant difference between PLA and NAC (1263 ± 334 sec PLA; 1047 ± 136 sec NAC), time to exhaustion was less in 6 of 8 subjects during NAC trials.

Figure 3: VO_2 versus Time

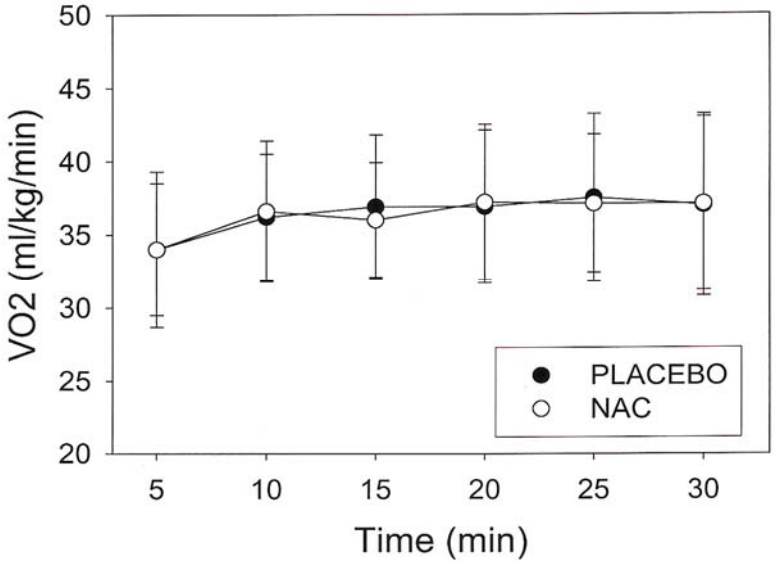

Figure 3: Whole body oxygen uptake (VO_2) versus time (in five-minute bouts) in both PLA (placebo) and NAC (N-Acetylcysteine). There were no differences between PLA or NAC ($p>0.05$).

Table 6: Submaximal Discontinuous Exercise

	Rest		5-minute		10-minute		15-minute		20-minute		25-minute		30-minute	
	PLA	NAC	PLA	NAC	PLA	NAC	PLA	NAC	PLA	NAC	PLA	NAC	PLA	NAC
VO_2 (l/min)	0.27 ± 0.05	0.30 ± 0.09	2.58 ± 0.37	2.60 ± 0.40	2.75 ± 0.30	2.78 ± 0.31	2.80 ± 0.31	2.74 ± 0.34	2.80 ± 0.37	2.83 ± 0.43	2.85 ± 0.44	2.84 ± 0.46	2.81 ± 0.47	2.86± 0.44
VO_2 (ml/kg/min)	3.6 ± 0.6	3.9 ± 0.9	34.0 ± 5.3	34.0 ± 4.5	36.2 ± 4.3	36.6 ± 4.8	36.9 ± 4.9	36.0 ± 3.9	36.9 ± 5.2	37.2 ± 5.3	37.5 ± 5.7	37.1 ± 4.7	37.0 ± 6.2	37.1 ± 5.9
VE (l/min)	11.7 ± 2.4	13.0 ± 3.7	63.1 ± 23.8	77.6 ± 15.6	80.7 ± 12.7	85.2 ± 18.0	88.5 ± 16.9	91.5 ± 19.6	93.2 ± 21.7	96.8 ± 24.0	94.9 ± 19.7	96.0 ± 22.2	98.4 ± 23.8	96.2 ± 25.2
VE/VO_2	58.9 ± 28.1	60.9 ± 22.9	28.4 ± 2.8	29.9 ± 4.0	29.3 ± 2.7	30.4 ± 4.2	31.6 ± 5.2	33.3 ± 5.6	33.1 ± 6.0	34.1 ± 5.5	33.3 ± 6.1	33.6 ± 4.9	35.0 ± 6.8	33.4 ± 4.9
VE/VCO_2	69.3 ± 26.0	67.5 ± 21.8	27.8 ± 2.2	29.6 ± 3.2	30.6 ± 2.4	31.6 ± 3.6	32.9 ± 4.0	34.6 ± 5.0	34.8 ± 5.3	35.4 ± 4.5	34.9 ± 5.3	35.5 ± 4.8	36.6 ± 5.6	35.7 ± 4.0
RER	0.82 ± 0.06	0.86 ± 0.7	1.02 ± 0.04	1.02 ± 0.03	0.96 ± 0.02	0.97 ± 0.03	0.96 ± 0.04	0.97 ± 0.3	0.96 ± 0.04	0.96 ± 0.02	0.96 ± 0.03	0.95 ± 0.02	0.95 ± 0.4	0.94 ± 0.03
SaO_2 (%)	98.0 ± 0.8	98.5 ± 0.9	97.0 ± 2.0	96.8 ± 1.8	96.5 ± 1.9	96.8 ± 1.8	96.6 ± 2.4	96.5 ± 2.5	95.6 ± 4.4	95.8 ± 2.9	96.4 ± 2.4	95.9 ± 1.9	95.9 ± 3.2	96.0 ± 2.6
HR (bpm)	64.9 ± 11.1	65.3 ± 6.8	145.4 ± 10.8	140.9 ± 22.1	151.3 ± 23.1	148.9 ± 21.7	158.4 ± 17.4	153.3 ± 30.1	155.3 ± 32.6	166.8 ± 11.8	160.4 ± 27.5	164.9 ± 14.9	165.3 ± 14.9	162.9 ± 15.3
RPE Borg (1-10)	-----	-----	3.3 ± 1.9	3.4 ± 0.9	4.1 ± 1.8	4.7 ± 1.4	5.3 ± 1.4	6.3 ± 1.6	6.3 ± 1.4	7.4 ± 1.6	7.9 ± 1.6	8.1 ± 1.0	8.6 ± 2.1	8.6 ± 1.1
RPD Borg (1-10)	-----	-----	3.3 ± 1.9	2.3 ± 0.5	3.7 ± 1.6	4.0 ± 0.9	4.6 ± 1.7	5.7 ± 2.3	5.7 ± 1.6	6.4 ± 2.2	6.7 ± 1.9	6.3 ± 1.5	8.0 ± 2.4	7.2 ± 1.9
Lactate (mM)	1.92 ± 0.74	2.14 ± 1.03	5.50 ± 1.59	5.36 ± 1.03	8.35 ± 1.30	8.40 ± 1.57	10.23 ± 1.61	9.31 ± 1.24	9.79 ± 1.76	9.79 ± 1.45	9.26 ± 3.21	9.63 ± 1.84	10.76 ± 2.57	9.75 ± 1.94

Table 6: Metabolic indices during discontinuous exercise bouts in both placebo (PLA) and N-Acetylcysteine (NAC). All values are mean ± SD. There were no differences ($p>0.05$) in any measured variable across time. RPE: rating of perceived exertion; RPD: rating of perceived dyspnea.

Figure 4: HR versus Time

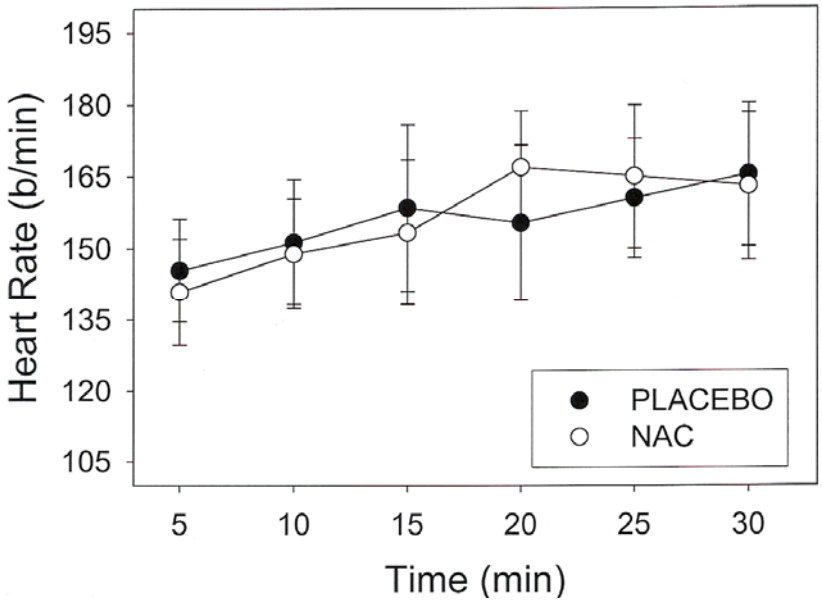

Figure 4: Heart rate versus time in placebo (PLA) and N-Acetylcysteine (NAC). No differences between PLA and NAC ($p>0.05$) were observed.

Figure 5: Ventilation (VE) versus Time

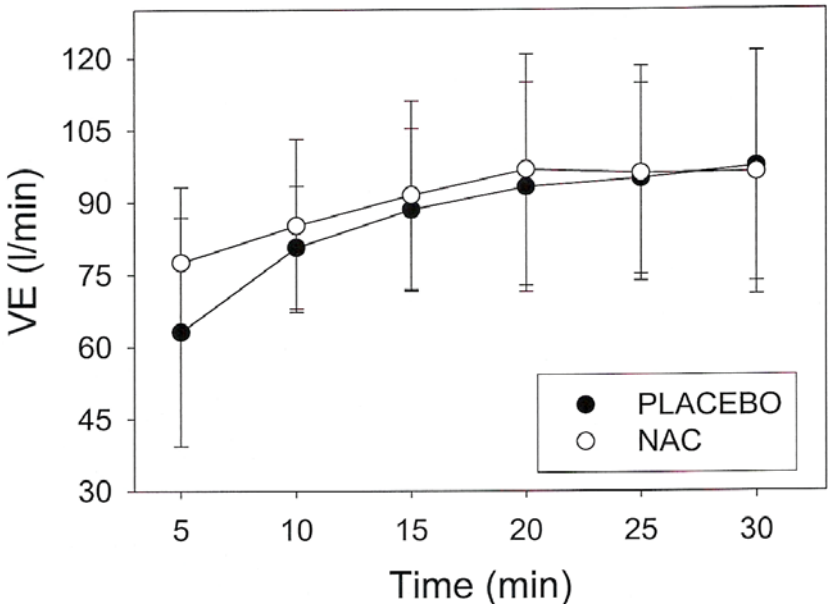

Figure 5: Minute ventilation (VE) in placebo (PLA) and N-Acetylcysteine (NAC). No differences between PLA and NAC were detected ($p>0.05$).

Figure 6: Time to Exhaustion (T_{lim})

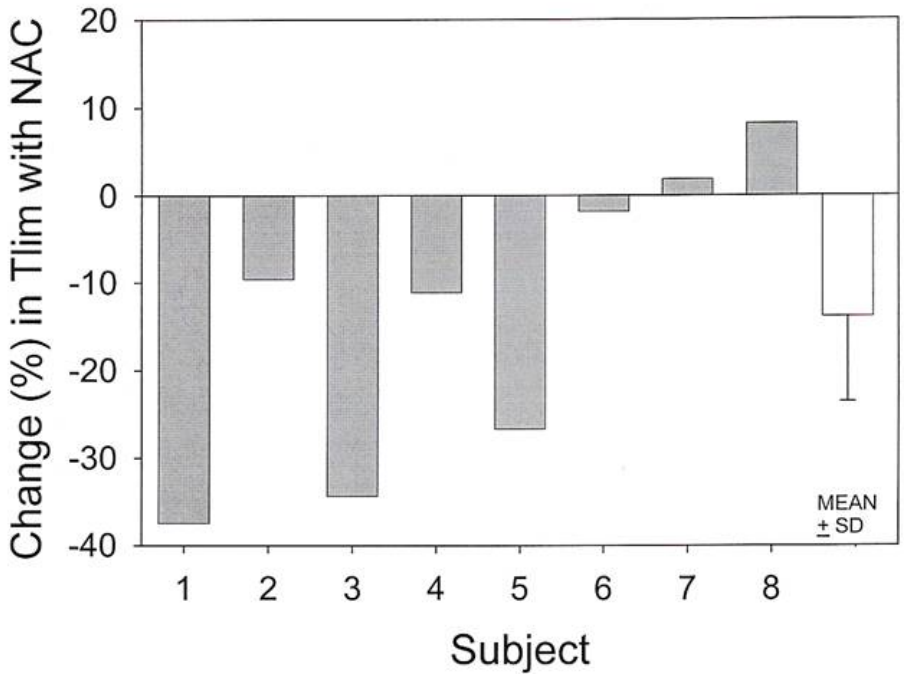

Figure 6: Individual and mean time to exhaustion (T_{lim}) during continuous exercise bout comparing placebo (PLA) and N-Acetylcysteine (NAC) conditions. No difference between PLA and NAC was found ($p>0.05$).

CHAPTER 5 - Discussion

Major Findings

The purpose of this study was to determine the effect of an acute, oral dose of N-Acetylcysteine on respiratory muscle fatigue during heavy exercise. Our major original findings support our hypothesis that an acute oral dose of N-Actylcysteine (NAC) reduces respiratory muscle fatigue during heavy intermittent exercise in healthy humans. Our results also demonstrated that NAC supplementation did not alter any of our measured ventilatory or metabolic measurements during exercise, nor was time to exhaustion changed compared to placebo. These results support the belief that oxidative stress contributes to respiratory muscle fatigue during heavy exercise.

Respiratory Muscle Fatigue

Our findings that NAC reduced respiratory muscle fatigue during heavy exercise are in agreement with several previous studies using both animal and human models. However, the vast majority of studies have infused NAC rather than administered NAC orally. Specifically, it has been shown that intravenous loading of NAC, 0.75ml/kg of a 200mg/ml NAC solution over a time period of five minutes, exhibited higher esophageal pressure and volume generation during loaded inspiratory pressures in rats (Supinski et al., 1997b). Also in the rat model, under tetanic stimulation, bundles of diaphragm strips incubated in 10mM of NAC dissolved in Krebs-Ringer solution showed a 1.8-fold higher force development (Mishima et al., 2005). Collectively, the reports demonstrate that intravenous loading of NAC can decrease fatigue and increase force development in the

respiratory muscles of the rat. Similar effects have been shown in humans. Travaline et al. (1997) demonstrated an increase in task endurance, determined by the sum of four diaphragmatic fatigue times during resistive breathing maintained at 80% targeted diaphragmatic twitch pressure, by NAC infusion (150 mg/kg in 250 ml of D_5W) during inspiratory resistive loading in healthy human subjects. Although this study used electrophrenic twitch stimulation and transdiaphragmatic twitch occlusion to establish diaphragmatic fatigue (see Travaline et al. 1997 for protocol detail), which was induced by loaded inspiratory breathing, and NAC infusion, the results are similar to our study in that NAC was able to improve diaphragm strength and reduce fatigue.

There is minimal research demonstrating the effects of oral supplementation of NAC on muscle fatigue in humans. The only known human oral supplementation studies are by Koechlin et al. (2004) who used 1800mg/day for 4 days with an additional 600mg on testing day in COPD patients, Sen et al. (1994b) who used 800mg/day for 2 days with an additional 800mg on testing day in healthy humans, and Matuszczak et al. (2005) who used 150mmol in 100mL of 0.9% saline in healthy humans. However, unlike our study, other skeletal muscles (i.e. the quadriceps) were examined. Koechlin et al. (2004) showed an improvement of 25% (208 $\pm$ 23 seconds) quadriceps endurance with NAC supplementation, compared to our study of 14% improvement of respiratory muscles, as well as decreased whole body oxidative stress (measured by superoxide anion release) (Koechlin et al., 2004). Sen et al. (1994b) determined the effect of NAC supplementation on different exercise intensities and changing levels of glutathione (GSH) and reduced glutathione (GSSG) in the blood. These authors did not observe GSH or GSSG values changing during either aerobic or anaerobic threshold exercises. Matusczcak et al. (2005)

determined that NAC supplementation can increase force production in the forearm (assessed by handgrip dynamometers). However, these studies are the only three studies to our knowledge to use oral dosing in humans. Koechlin et al. (2004) associated improvements in quadriceps endurance with reduced oxidative stress in the body. From these reports, it suggests that a decrease in oxidative stress, without a change in blood plasma values, while still improving muscle function, makes it possible for NAC to be effective in reducing respiratory muscle fatigue. Specific mechanisms responsible for the reduced respiratory muscle fatigue with NAC are not known.

Respiratory muscles fatigue can be due to both peripheral and central (stemming from the central nervous system) mechanisms. Since peripheral fatigue reflects a change in biochemical processes directly at the muscle, the effect of increased accumulation of ROS may influence peripheral muscle fatigue via Type III and IV muscle afferents (see below) to compromise blood flow from locomotor muscles and increase respiratory muscle blood flow (Amann & Calbet, 2008). Central fatigue may influence respiratory muscle fatigue by enhancing an inhibitory feedback on central command (Calbet et al., 2003). However, this may only be seen in hypoxic conditions. During central fatigue, exercise induced changes in the neurotransmitter systems, mostly demonstrated by inadequate oxygen delivery to the capillaries and mitochondria, also influences peripheral fatigue (Davis & Bailey, 1997; Meeusen, Watson, Hasegawa, Roelands, & Piacentini, 2006). The sensory feedback, linking the fatiguing locomotor and respiratory muscles to the regulation of central motor drive, links peripheral and central fatigue together. This inhibitory neural feedback has been suggested to be in proportion to the magnitude of

peripheral locomotor fatigue and that the rate at which peripheral fatigue develops has dose-dependent relationship to the trigger of central fatigue (Amann & Dempsey, 2008).

Significance of Respiratory Muscle Fatigue

Reduced respiratory muscle fatigue during exercise with NAC may improve whole body exercise performance. This theory is proposed by Dempsey, Romer, Rodman, Miller, and Smith (2006), who state that with respiratory muscle fatigue, exercise performance is limited via several processes. It has been previously determined that up to ~14-16% of total cardiac output is shifted to the respiratory muscles during sustained hyperventilation and high-intensity exercise (Harms et al., 1997). With this increased cardiac output directed to the respiratory muscles when respiratory muscles are likely fatiguing, there is an activated respiratory muscle metaboreflex which increases sympathetic outflow and locomotor muscle vasoconstriction (decreased cardiac output to the locomotor muscles) (Dempsey et al., 2006; Harms et al., 1997). It has been hypothesized that reducing respiratory muscle fatigue will improve exercise performance by attenuating a muscle metaboreflex in central command causing Type III and IV muscle afferents to de-activate the blood flow steal phenomena (Dempsey et al., 2006; Harms et al., 1997; Harms et al., 1998) directing respiratory muscle cardiac output to the skeletal muscles. Also, if respiratory muscle fatigue is cause for locomotor muscle fatigue (Dempsey et al., 2006), and if respiratory muscle fatigue can be decreased and/or prevented, as in the present study, time to exhaustion during whole body exercise may theoretically increase.

In the present study, respiratory muscle fatigue was reduced with NAC supplementation, but time to exhaustion was not changed. However, our protocol was not specifically designed to test this performance question. Multiple exercise sessions are necessary to reduce intra-test variability to accurately assess performance issues. Our study, however, is in agreement with a recent study by Medved et al. (2003) who found that intravenous loading of NAC did not increase exercise performance during high-intensity intermittent cycling. The effect on respiratory muscles is unknown; however, these authors suggest NAC may have an effect on low-frequency fatigue protocols, but not high-intensity exercise (Medved et al., 2003).

Although we did not see an increase in time to exhaustion, several studies have found NAC increases endurance performance. With human subjects, time to fatigue increased by ~23% during submaximal (90%VO_{2max}) cycling exercise (McKenna et al., 2006) with intravenous loading (125mg/kg/hr for 15 minutes and 25mg/kg/hr for duration of exercise) of NAC. During electrical stimulation of the tibialis anterior, NAC increased time to fatigue during low-frequency, but not high-frequency stimulation. This is in agreement with Medved et al. (2003), who suggest that high-intensity exercise may not be affected by NAC. In consensus, time to exhaustion is most likely influenced by the type of exercise and its intensity, high versus low, when supplementing with NAC. However, if NAC affects endurance performance in low-frequency fatigue, it is therefore not feasible to use a protocol in which respiratory muscle fatigue is shown since respiratory muscle fatigue only occurs during high-intensity exercise (Babcock et al., 2002; McKenna et al., 2006). Therefore, we would likely not see exercise at lower

intensities affected by NAC supplementation due to a decrease in respiratory muscle fatigue.

Plasma Glutathione

Plasma glutathione (GSH) did not change with oral supplementation of NAC in our study, which is in agreement with others (Gohil, Viguie, Stanley, Brooks, & Packer, 1988; Medved et al., 2003). The purpose of testing GSH was to identify an increase in GSH due to supplemented NAC (pre- to post-supplementation of NAC) and not GSH's effect during exercise. Increases are typically observed in whole blood GSH, not plasma. Although the following studies tested whole blood and plasma GSH during exercise, it is possible to understand why the current study did not detect any plasma GSH differences. Medved et al. (2003) determined that whole blood GSH decreased and whole blood GSSG increased with intense intermittent cycling, but, as stated previously, they were unable to detect plasma GSH status. In another study, NAC increased red blood cell GSH during hand grip exercise (50% maximal voluntary contraction, 3 seconds on, 3 seconds off) (Matuszczak et al., 2005). To ensure NAC was responsible for these increases in total blood GSH, Medved et al. (2004a) measured total NAC, whole blood NAC, and whole blood GSH pre- and post-submax cycling exercise. Significant changes were found in total NAC, whole blood NAC, and whole blood GSH suggesting NAC is responsible for the increase in whole blood GSH (Medved et al., 2004a). To accurately account for NAC supplementation in the body, it may perhaps be best to use whole blood assays versus plasma only assays.

Metabolic and Ventilatory Indices

During NAC supplementation, whole body VO_2, HR, VE, or lactate did not differ when compared to placebo trials. To date, reports on the effect of NAC on these measures during sustained heavy exercise has yet to be determined. Reid et al. (1994) and Travaline et al. (1997) did report no significant differences between heart rate and blood pressure with NAC infusion during low (10 Hz) and high (20 Hz) frequency stimulation in the tibialis anterior and resistive breathing at ~80% inspiratory pressure using diaphragmatic twitch occlusion, respectively. Therefore, it would be important to assess changes in these variables in future studies to possibly establish a connection between reduced respiratory muscle fatigue with NAC and improved exercise performance.

Limitations

There are several potential limitations which may have influenced our results. First, it is not possible to measure respiratory muscle fatigue directly in human subjects. Historically, bilateral phrenic nerve stimulation (BPNS) is the gold standard for assessing diaphragmatic fatigue. However, measuring respiratory muscle pressure at the mouth, as was performed in our study, has been shown to elicit similar effects as BPNS and been used in many previous studies (Huang et al., 2003; Inbar et al., 2000; Romer, McConnell, & Jones, 2002a; Romer, McConnell, & Jones, 2002b; Sonetti et al., 2001). Since P_{Imax} maneuvers are effort-dependent, subjects in our study were highly encouraged to perform maximal effort, tests were made in triplicate, and lung volume measures were made with each trial to ensure consistency. Although pressures taken at the mouth tend to have a

learning effect, it has been shown that keeping the number of trials to 3 or fewer with a warm-up session decreases the learning effect and allows for less subject variability (Volianitis, McConnell, & Jones, 2001) and does not contribute itself to respiratory muscle fatigue.

Determining exercise performance was not the primary goal of this study. However, our subjects did perform a continuous single volitional exercise test at 85% VO_{2peak} while under placebo and NAC conditions. However, we realize that this is not sufficient evidence to address performance questions. To determine the effects of NAC on time to exhaustion, multiple exercise sessions should be analyzed during both placebo and NAC trials to reduce subject and test variability. Also, our measurements did not take into account any mechanistic significance for respiratory muscle fatigue. For example, leg blood flow was not measured so we do not know if a cardiac output shift from the locomotor muscles to the respiratory muscles occurred during PLA trials, nor do we know if the blood flow shift during the NAC trials failed to occur because of the reduction in respiratory muscle fatigue.

Whole blood GSH measurements appear to be more effective than plasma GSH (Medved et al., 2003; Medved et al., 2004a). These authors explain that whole blood GSH measurements may be more responsive than plasma GSH measurements. The current study did not show an increase in plasma GSH, therefore, we may have seen an increase following NAC supplementation if we measured whole blood GSH.

Future Directions

Future research is worthwhile to expand our understanding of NAC's effect on respiratory muscle fatigue and exercise performance. First, it would be interesting to determine NAC's influence on exercise performance. Does reduced respiratory muscle fatigue with NAC lead to an increase in exercise performance? If so, under what exercise conditions or subject population? Also, if respiratory muscle fatigue is alleviated, is there a reverse direction of blood flow shift from the respiratory muscles to the exercising locomotor muscles, or perhaps less of a "steal"? Furthermore, experimenting with greater or lesser doses of NAC would be of interest to see if similar effects occur; i.e. do higher doses of oral NAC elicit greater amounts of fatigue alleviation, or will a chronic dose be more beneficial than an acute oral dose? Finally, subject selection is important in future research. Our subjects were healthy active males. Would we see similar effects with women, older adults, or diseased populations, such as COPD? Is work of breathing reduced because of NAC's effect on decreasing oxidative stress and decreased respiratory muscle fatigue? Will different populations receive greater benefits from NAC's effect on reducing oxidative stress due to aging and disease effects on the cardiopulmonary system? Our results obviously lead to many worthwhile future studies on the effect of NAC on both respiratory muscles and exercise performance.

Summary

In conclusion, this study has confirmed recent evidence that oxidative stress contributes to respiratory muscle fatigue by showing that an acute oral dose of NAC will reduce respiratory muscle fatigue during heavy exercise. We believe this is important

because respiratory muscle work and respiratory muscle fatigue has been attributed to limiting exercise performance in healthy subjects during heavy exercise. To date, NAC supplementation during exercise has had contradictory results regarding time to fatigue during high versus low intensity exercise and loading conditions (chronic versus acute, oral versus intravenous). Also, specific mechanisms responsible for reduced respiratory muscle fatigue with NAC are not yet known. Future research is obviously needed and warranted to gain a greater understanding of factors contributing to respiratory muscle fatigue during exercise.

References

Aaron, E. A., Seow, K. C., Johnson, B. D., & Dempsey, J. A. (1992). Oxygen cost of exercise hyperpnea: Implications for performance. *Journal of Applied Physiology (Bethesda, Md.: 1985), 72*(5), 1818-1825.

Amann, M., & Calbet, J. A. (2008). Convective oxygen transport and fatigue. *Journal of Applied Physiology (Bethesda, Md.: 1985), 104*(3), 861-870.

Amann, M., & Dempsey, J. A. (2008). Locomotor muscle fatigue modifies central motor drive in healthy humans and imposes a limitation to exercise performance. *The Journal of Physiology, 586*(1), 161-173.

Aruoma, O. I., Halliwell, B., Hoey, B. M., & Butler, J. (1989). The antioxidant action of N-acetylcysteine: Its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radical Biology & Medicine, 6*(6), 593-597.

Babcock, M. A., Pegelow, D. F., Harms, C. A., & Dempsey, J. A. (2002). Effects of respiratory muscle unloading on exercise-induced diaphragm fatigue. *Journal of Applied Physiology (Bethesda, Md.: 1985), 93*(1), 201-206.

Barclay, J. K., & Hansel, M. (1991). Free radicals may contribute to oxidative skeletal muscle fatigue. *Canadian Journal of Physiology and Pharmacology, 69*(2), 279-284.

Barreiro, E., Galdiz, J. B., Marinan, M., Alvarez, F. J., Hussain, S. N., & Gea, J. (2006). Respiratory loading intensity and diaphragm oxidative stress: N-acetyl-cysteine effects. *Journal of Applied Physiology (Bethesda, Md.: 1985), 100*(2), 555-563.

Borgstrom, L., Kagedal, B., & Paulsen, O. (1986). Pharmacokinetics of N-acetylcysteine in man. *European Journal of Clinical Pharmacology, 31*(2), 217-222.

Calbet, J. A., Boushel, R., Radegran, G., Sondergaard, H., Wagner, P. D., & Saltin, B. (2003). Determinants of maximal oxygen uptake in severe acute hypoxia. *American Journal of Physiology.Regulatory, Integrative and Comparative Physiology, 284*(2), R291-303.

Coast, J. R., & Krause, K. M. (1993). Relationship of oxygen consumption and cardiac output to work of breathing. *Medicine and Science in Sports and Exercise, 25*(3), 335-340.

Davies, K. J., Quintanilha, A. T., Brooks, G. A., & Packer, L. (1982). Free radicals and tissue damage produced by exercise. *Biochemical and Biophysical Research Communications, 107*(4), 1198-1205.

Davis, J. M., & Bailey, S. P. (1997). Possible mechanisms of central nervous system fatigue during exercise. *Medicine and Science in Sports and Exercise, 29*(1), 45-57.

De Bernardi di Valserra,M., Mautone, G., Barindelli, E., Lualdi, P., Feletti, F., & Galmozzi, M. R. (1989). Bioavailability of suckable tablets of oral N-acetylcysteine in man. *European Journal of Clinical Pharmacology, 37*(4), 419-421.

Dempsey, J. A., Romer, L., Rodman, J., Miller, J., & Smith, C. (2006). Consequences of exercise-induced respiratory muscle work. *Respiratory Physiology & Neurobiology, 151*(2-3), 242-250.

Downey, A. E., Chenoweth, L. M., Townsend, D. K., Ranum, J. D., Ferguson, C. S., & Harms, C. A. (2007). Effects of inspiratory muscle training on exercise responses in normoxia and hypoxia. *Respiratory Physiology & Neurobiology, 156*(2), 137-146.

Gohil, K., Viguie, C., Stanley, W. C., Brooks, G. A., & Packer, L. (1988). Blood glutathione oxidation during human exercise. *J.Appl.Physiol, 64*(1), 115-115-119.

Harms, C. A., Babcock, M. A., McClaran, S. R., Pegelow, D. F., Nickele, G. A., Nelson, W. B., et al. (1997). Respiratory muscle work compromises leg blood flow during maximal exercise. *Journal of Applied Physiology (Bethesda, Md.: 1985), 82*(5), 1573-1583.

Harms, C. A., Wetter, T. J., McClaran, S. R., Pegelow, D. F., Nickele, G. A., Nelson, W. B., et al. (1998). Effects of respiratory muscle work on cardiac output and its distribution during maximal exercise. *Journal of Applied Physiology (Bethesda, Md.: 1985), 85*(2), 609-618.

Harms, C. A., Wetter, T. J., St Croix, C. M., Pegelow, D. F., & Dempsey, J. A. (2000). Effects of respiratory muscle work on exercise performance. *Journal of Applied Physiology (Bethesda, Md.: 1985), 89*(1), 131-138.

Harrison, P. M., Wendon, J. A., Gimson, A. E., Alexander, G. J., & Williams, R. (1991). Improvement by acetylcysteine of hemodynamics and oxygen transport in fulminant hepatic failure. *The New England Journal of Medicine, 324*(26), 1852-1857.

Hida, W., Shindoh, C., Satoh, J., Sagara, M., Kikuchi, Y., Toyota, T., et al. (1996). N-acetylcysteine inhibits loss of diaphragm function in streptozotocin-treated rats. *American Journal of Respiratory and Critical Care Medicine, 153*(6 Pt 1), 1875-1879.

Huang, C. H., Martin, A. D., & Davenport, P. W. (2003). Effect of inspiratory muscle strength training on inspiratory motor drive and RREP early peak components. *Journal of Applied Physiology (Bethesda, Md.: 1985), 94*(2), 462-468.

Inbar, O., Weiner, P., Azgad, Y., Rotstein, A., & Weinstein, Y. (2000). Specific inspiratory muscle training in well-trained endurance athletes. *Medicine and Science in Sports and Exercise, 32*(7), 1233-1237.

Johnson, B. D., Babcock, M. A., Suman, O. E., & Dempsey, J. A. (1993). Exercise-induced diaphragmatic fatigue in healthy humans. *The Journal of Physiology, 460*, 385-405.

Johnson, B. D., Saupe, K. W., & Dempsey, J. A. (1992). Mechanical constraints on exercise hyperpnea in endurance athletes. *Journal of Applied Physiology (Bethesda, Md.: 1985), 73*(3), 874-886.

Juel, C. (2006). Muscle fatigue and reactive oxygen species. *The Journal of Physiology, 576*(Pt 1), 1.

Kerksick, C., & Willoughby, D. (2005). The antioxidant role of glutathione and N-acetyl-cysteine supplements and exercise-induced oxidative stress. *Journal of the International Society of Sports Nutrition, 2*(2), 38-38-44.

Koechlin, C., Couillard, A., Simar, D., Cristol, J. P., Bellet, H., Hayot, M., et al. (2004). Does oxidative stress alter quadriceps endurance in chronic obstructive pulmonary disease? *American Journal of Respiratory and Critical Care Medicine, 169*(9), 1022-1027.

Kolbeck, R. C., She, Z. W., Callahan, L. A., & Nosek, T. M. (1997). Increased superoxide production during fatigue in the perfused rat diaphragm. *American Journal of Respiratory and Critical Care Medicine, 156*(1), 140-145.

Lands, L. C., Grey, V. L., & Smountas, A. A. (1999). Effect of supplementation with a cysteine donor on muscular performance. *Journal of Applied Physiology (Bethesda, Md.: 1985), 87*(4), 1381-1385.

Matuszczak, Y., Farid, M., Jones, J., Lansdowne, S., Smith, M. A., Taylor, A. A., et al. (2005). Effects of N-acetylcysteine on glutathione oxidation and fatigue during handgrip exercise. *Muscle & Nerve, 32*(5), 633-638.

McConnell, A. K., & Lomax, M. (2006). The influence of inspiratory muscle work history and specific inspiratory muscle training upon human limb muscle fatigue. *The Journal of Physiology, 577*(Pt 1), 445-457.

McKenna, M. J., Medved, I., Goodman, C. A., Brown, M. J., Bjorksten, A. R., Murphy, K. T., et al. (2006). N-acetylcysteine attenuates the decline in muscle na+,K+-pump

activity and delays fatigue during prolonged exercise in humans. *The Journal of Physiology, 576*(Pt 1), 279-288.

Medved, I., Brown, M. J., Bjorksten, A. R., Leppik, J. A., Sostaric, S., & McKenna, M. J. (2003). N-acetylcysteine infusion alters blood redox status but not time to fatigue during intense exercise in humans. *Journal of Applied Physiology (Bethesda, Md.: 1985), 94*(4), 1572-1582.

Medved, I., Brown, M. J., Bjorksten, A. R., & McKenna, M. J. (2004a). Effects of intravenous N-acetylcysteine infusion on time to fatigue and potassium regulation during prolonged cycling exercise. *Journal of Applied Physiology (Bethesda, Md.: 1985), 96*(1), 211-217.

Medved, I., Brown, M. J., Bjorksten, A. R., Murphy, K. T., Petersen, A. C., Sostaric, S., et al. (2004b). N-acetylcysteine enhances muscle cysteine and glutathione availability and attenuates fatigue during prolonged exercise in endurance-trained individuals. *Journal of Applied Physiology (Bethesda, Md.: 1985), 97*(4), 1477-1485.

Meeusen, R., Watson, P., Hasegawa, H., Roelands, B., & Piacentini, M. F. (2006). Central fatigue: The serotonin hypothesis and beyond. *Sports Medicine (Auckland, N.Z.), 36*(10), 881-909.

Meister, A. (1988). Glutathione metabolism and its selective modification. *The Journal of Biological Chemistry, 263*(33), 17205-17208.

Mishima, T., Yamada, T., Matsunaga, S., & Wada, M. (2005). N-acetylcysteine fails to modulate the in vitro function of sarcoplasmic reticulum of diaphragm in the final phase of fatigue. *Acta Physiologica Scandinavica, 184*(3), 195-202.

Musch, T. I., Friedman, D. B., Pitetti, K. H., Haidet, G. C., Stray-Gundersen, J., Mitchell, J. H., et al. (1987). Regional distribution of blood flow of dogs during graded dynamic exercise. *Journal of Applied Physiology (Bethesda, Md.: 1985), 63*(6), 2269-2277.

Nashawati, E., Dimarco, A., & Supinski, G. (1993). Effects produced by infusion of a free radical-generating solution into the diaphragm. *The American Review of Respiratory Disease, 147*(1), 60-65.

Novelli, G. P., Falsini, S., & Bracciotti, G. (1991). Exogenous glutathione increases endurance to muscle effort in mice. *Pharmacological Research : The Official Journal of the Italian Pharmacological Society, 23*(2), 149-155.

O'Neill, C. A., Stebbins, C. L., Bonigut, S., Halliwell, B., & Longhurst, J. C. (1996). Production of hydroxyl radicals in contracting skeletal muscle of cats. *Journal of Applied Physiology (Bethesda, Md.: 1985), 81*(3), 1197-1206.

Ozkaplan, A., Rhodes, E. C., Sheel, A. W., & Taunton, J. E. (2005). A comparison of inspiratory muscle fatigue following maximal exercise in moderately trained males and females. *European Journal of Applied Physiology, 95*(1), 52-56.

Poole, D. C., Sexton, W. L., Behnke, B. J., Ferguson, C. S., Hageman, K. S., & Musch, T. I. (2000). Respiratory muscle blood flows during physiological and chemical

hyperpnea in the rat. *Journal of Applied Physiology (Bethesda, Md.: 1985), 88*(1), 186-194.

Reid, M. B. (2001a). Invited review: Redox modulation of skeletal muscle contraction: What we know and what we don't. *Journal of Applied Physiology (Bethesda, Md.: 1985), 90*(2), 724-731.

Reid, M. B. (2001b). Nitric oxide, reactive oxygen species, and skeletal muscle contraction. *Medicine and Science in Sports and Exercise, 33*(3), 371-376.

Reid, M. B., Haack, K. E., Franchek, K. M., Valberg, P. A., Kobzik, L., & West, M. S. (1992a). Reactive oxygen in skeletal muscle. I. intracellular oxidant kinetics and fatigue in vitro. *Journal of Applied Physiology (Bethesda, Md.: 1985), 73*(5), 1797-1804.

Reid, M. B., Stokic, D. S., Koch, S. M., Khawli, F. A., & Leis, A. A. (1994). N-acetylcysteine inhibits muscle fatigue in humans. *The Journal of Clinical Investigation, 94*(6), 2468-2474.

Reid, M. B., Shoji, T., Moody, M. R., & Entman, M. L. (1992b). Reactive oxygen in skeletal muscle. II. extracellular release of free radicals. *Journal of Applied Physiology (Bethesda, Md.: 1985), 73*(5), 1805-1809.

Romer, L. M., Lovering, A. T., Haverkamp, H. C., Pegelow, D. F., & Dempsey, J. A. (2006). Effect of inspiratory muscle work on peripheral fatigue of locomotor muscles in healthy humans. *The Journal of Physiology, 571*(Pt 2), 425-439.

Romer, L. M., McConnell, A. K., & Jones, D. A. (2002a). Effects of inspiratory muscle training on time-trial performance in trained cyclists. *Journal of Sports Sciences, 20*(7), 547-562.

Romer, L. M., McConnell, A. K., & Jones, D. A. (2002b). Inspiratory muscle fatigue in trained cyclists: Effects of inspiratory muscle training. *Medicine and Science in Sports and Exercise, 34*(5), 785-792.

Ruffmann, R., & Wendel, A. (1991). GSH rescue by N-acetylcysteine. *Klinische Wochenschrift, 69*(18), 857-862.

Sen, C. K. (2001). Antioxidant and redox regulation of cellular signaling: Introduction. *Medicine and Science in Sports and Exercise, 33*(3), 368-370.

Sen, C. K., & Packer, L. (2000). Thiol homeostasis and supplements in physical exercise. *The American Journal of Clinical Nutrition, 72*(2 Suppl), 653S-69S.

Sen, C. K., Atalay, M., & Hanninen, O. (1994a). Exercise-induced oxidative stress: Glutathione supplementation and deficiency. *Journal of Applied Physiology (Bethesda, Md.: 1985), 77*(5), 2177-2187.

Sen, C. K., Rankinen, T., Vaisanen, S., & Rauramaa, R. (1994b). Oxidative stress after human exercise: Effect of N-acetylcysteine supplementation. *Journal of Applied Physiology (Bethesda, Md.: 1985), 76*(6), 2570-2577.

Sheel, A. W., Derchak, P. A., Morgan, B. J., Pegelow, D. F., Jacques, A. J., & Dempsey, J. A. (2001). Fatiguing inspiratory muscle work causes reflex reduction in resting leg blood flow in humans. *The Journal of Physiology, 537*(Pt 1), 277-289.

Shephard, R. J. (1966). The oxygen cost of breathing during vigorous exercise. *Quarterly Journal of Experimental Physiology and Cognate Medical Sciences, 51*(4), 336-350.

Shindoh, C., DiMarco, A., Thomas, A., Manubay, P., & Supinski, G. (1990). Effect of N-acetylcysteine on diaphragm fatigue. *Journal of Applied Physiology (Bethesda, Md.: 1985), 68*(5), 2107-2113.

Sjodin, B., Hellsten Westing, Y., & Apple, F. S. (1990). Biochemical mechanisms for oxygen free radical formation during exercise. *Sports Medicine (Auckland, N.Z.), 10*(4), 236-254.

Smith, M. A., & Reid, M. B. (2006). Redox modulation of contractile function in respiratory and limb skeletal muscle. *Respiratory Physiology & Neurobiology, 151*(2-3), 229-241.

Sonetti, D. A., Wetter, T. J., Pegelow, D. F., & Dempsey, J. A. (2001). Effects of respiratory muscle training versus placebo on endurance exercise performance. *Respiration Physiology, 127*(2-3), 185-199.

St Croix, C. M., Morgan, B. J., Wetter, T. J., & Dempsey, J. A. (2000). Fatiguing inspiratory muscle work causes reflex sympathetic activation in humans. *The Journal of Physiology, 529 Pt 2*, 493-504.

Supinski, G.S. (1998). Free radical induced respiratory muscle dysfunction. *Mol Cell Biochem, 179*, 99-99-110.

Supinski, G.S., Nethery, D., Stofan, D., & DiMarco, A. (1997a). Effect of free radical scavengers on diaphragmatic fatigue. *American Journal of Respiratory and Critical Care Medicine, 155*(2), 622-629.

Supinski, G. S., Stofan, D., Ciufo, R., & DiMarco, A. (1997b). N-acetylcysteine administration alters the response to inspiratory loading in oxygen-supplemented rats. *Journal of Applied Physiology (Bethesda, Md.: 1985), 82*(4), 1119-1125.

Travaline, J. M., Sudarshan, S., Roy, B. G., Cordova, F., Leyenson, V., & Criner, G. J. (1997). Effect of N-acetylcysteine on human diaphragm strength and fatigability. *American Journal of Respiratory and Critical Care Medicine, 156*(5), 1567-1571.

Verges, S., Lenherr, O., Haner, A. C., Schulz, C., & Spengler, C. M. (2007). Increased fatigue resistance of respiratory muscles during exercise after respiratory muscle endurance training. *American Journal of Physiology.Regulatory, Integrative and Comparative Physiology, 292*(3), R1246-53.

Volianitis, S., McConnell, A. K., & Jones, D. A. (2001). Assessment of maximum inspiratory pressure. prior submaximal respiratory muscle activity ('warm-up') enhances maximum inspiratory activity and attenuates the learning effect of repeated measurement. *Respiration; International Review of Thoracic Diseases, 68*(1), 22-27.