Reactive oxygen species mediated diaphragm fatigue in a rat model of chronic intermittent hypoxia.

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**Citation**

Reactive Oxygen Species Mediated Diaphragm Fatigue in a Rat Model of Chronic Intermittent Hypoxia

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New Findings:

What is the central question of this study?
The effects of chronic intermittent hypoxia (CIH) on respiratory muscles are relatively underexplored. It is speculated that muscle dysfunction, and other key morbidities associated with sleep apnoea, are the result of CIH-induced oxidative stress. We sought to investigate the putative role of CIH-induced reactive oxygen species in the development of respiratory muscle dysfunction.

What is the main finding and its importance?
CIH-induced diaphragm muscle fatigue is time- and intensity-dependent and is associated with a modest oxidative stress. N-acetyl cysteine supplementation prevents CIH-induced diaphragm muscle dysfunction suggesting that antioxidant supplementation may have therapeutic value in respiratory muscle disorders characterised by CIH, such as obstructive sleep apnoea.
Abstract
Respiratory muscle dysfunction documented in sleep apnoea patients is perhaps due to oxidative stress secondary to chronic intermittent hypoxia (CIH). We sought to explore the effects of different CIH paradigms on respiratory muscle form and function in a rodent model. Adult male Wistar rats were exposed to CIH (n=32) consisting of 90s normoxia/90s hypoxia [either 10% or 5% oxygen at the nadir; SaO₂ ~ 90% / 80% respectively], for 8h/day or to sham treatment (air/air, n=32) for 1 or 2 weeks. Three additional groups of CIH-treated rats (5% O₂ for 2 weeks) had free access to water containing N-acetyl cysteine (1% NAC, n=8), tempol (1mM, n=8) or apocynin (2mM, n=8). Functional properties of the diaphragm muscle were examined ex vivo at 35°C. MHC and SERCA isoform distribution, SDH and GPDH enzyme activity, Na⁺/K⁺ ATPase pump content, TBARS concentration, DNA oxidation and antioxidant capacity were determined. CIH (5% oxygen at the nadir; 2 weeks) decreased diaphragm muscle force and endurance. All three drugs reversed the deleterious effects of CIH on diaphragm endurance but only NAC prevented CIH-induced diaphragm weakness. CIH increased diaphragm muscle MHC 2B areal density and GSSG/GSH ratio. We conclude that CIH-induced diaphragm dysfunction is ROS-dependent. NAC was most effective in reversing CIH-induced effects on diaphragm. Our results suggest that respiratory muscle dysfunction in sleep apnoea may be the result of oxidative stress, and as such antioxidant treatment could prove a useful adjunct therapy for the disorder.
Introduction

Chronic intermittent hypoxia (CIH) is a central feature of many respiratory disorders, most notably obstructive sleep apnoea (OSA). This debilitating disorder affects at least 2-4% of the population (Young et al., 1997) and is associated with a wide spectrum of disorders including cardiovascular, metabolic and neurocognitive dysfunctions (Verstraeten, 2007; Levy et al., 2009; Butt et al., 2010).

Growing evidence suggests that upper airway muscle dysfunction is implicated in the pathophysiology of OSA syndrome. Both OSA patients (Carrera et al., 1999) and the English bulldog (Petrof et al., 1994), a naturally occurring animal model of OSA, show signs of upper airway dilator muscle dysfunction. Of note, CIH has been shown to alter upper airway muscle function (McGuire et al., 2002a, b; Pae et al., 2005; Dunleavy et al., 2008; Ding & Liu, 2011) and induce structural changes in upper airway muscles (McGuire et al., 2002a; Pae et al., 2005). The evidence suggesting that diaphragm muscle dysfunction is implicated in the pathophysiology of OSA is somewhat controversial. Diaphragm dysfunction is reported in OSA patients (Griggs et al., 1989; Chien et al., 2010), and in animals exposed to transient tracheal occlusion (Smith et al., 2012) and CIH (McGuire et al., 2003). However, other studies report that diaphragm remodelling is not present in OSA patients (Montserrat et al., 1997) or CIH-treated animals (Clanton et al., 2001; Pae et al., 2005). We speculated that the apparent discrepancy in CIH studies relates to the different CIH paradigms employed in studies and that CIH-induced respiratory muscle remodelling is dependent on the pattern, intensity and duration of exposure to hypoxia. Therefore in this study we sought to examine the effect of different CIH intensities (5 or 10% O₂ at the nadir) and durations (1 and 2 weeks) on diaphragm muscle structure and function. We hypothesized that the effects of CIH on diaphragm form and function are both time- and intensity-dependent.

It is well established that CIH is associated with an increased production of reactive oxygen species (ROS) (Prabhakar, 2001; Peng & Prabhakar, 2003; Yuan et al., 2004; Shan et al., 2007; Dunleavy et al., 2008; Dutta et al., 2008; Raghuraman et al., 2009; Sharma et al., 2009; Khan et al., 2011). Evidence suggests that ROS may contribute to the development of skeletal muscle dysfunction (Jackson, 2008). Pro-oxidants can exacerbate (Dunleavy et al., 2008; Shortt & O’Halloran, 2013), while antioxidant strategies can ameliorate (Skelly et al., 2006).
2010), respiratory muscle dysfunction. CIH-induced production of excess ROS is implicated in respiratory muscle dysfunction (Dunleavy et al., 2008; Ding & Liu, 2011; Skelly et al., 2012). These observations suggest that ROS are important modulators of respiratory muscle performance. Therefore we sought to further investigate the putative role of CIH-induced ROS in the development of respiratory muscle dysfunction, examining the efficacy of antioxidant strategies (N-acetyl cysteine and Tempol) in one of our CIH paradigms (5% O₂ at the nadir for 2 weeks).

Although it is well established that CIH increases ROS production, the source of these free radicals in respiratory muscle is less clear, though this has been very well characterised in other tissues. CIH-induced oxidative damage in the liver (Jun et al., 2008), cardiovascular system (Nisbet et al., 2009) and brain (Hui-guo et al., 2010) is ameliorated by the inhibition of NADPH oxidase (NOX) activity suggesting that this membrane-bound superoxide generating enzyme is a major source of CIH-induced ROS. In addition, ROS generation by IH-induced NOX activation inhibits complex I activity and thus increases mitochondrial ROS showing a relationship between NOX and the electron transport chain (Khan et al., 2011). In skeletal muscle fibres, NOXs are localized to the SR (Xia et al., 2003), transverse tubules (Javesghani et al., 2002) and plasma membranes (Javesghani et al., 2002). Inhibition of NOXs in skeletal muscle reduces the levels of superoxide (Javesghani et al., 2002; Pattwell et al., 2004). These studies implicate a role for NOX-derived ROS in skeletal muscle (dys)function. Therefore, to compliment the antioxidant studies described above, we examined whether in vivo apocynin supplementation would ameliorate the deleterious effects of CIH (5% at the nadir; 2 weeks) on respiratory muscle function.
Methods

Full details of the methods are provided in the online supplementary material.

Ethical approval

All protocols were performed under licence from the Irish Government, Department of Health and Children and were carried out in accordance with National and European guidelines and legislation, following Royal College of Surgeons in Ireland and University College Dublin Research Ethics Committee approval.

Chronic intermittent hypoxia

All appropriate measures were undertaken to minimize any pain or discomfort caused to the animals during these experiments in accordance with institutional guidelines and National and EU legislation. Adult male Wistar rats were exposed to CIH consisting of 90s normoxia/90s hypoxia [5% oxygen at the nadir; SaO₂ ~ 80%], for 8h/day for 1 (n=8) or 2 (n=8) weeks or to sham treatment (air/air, n=16) for the same durations. Separate studies exploring 90s normoxia/90s hypoxia (10% O₂ at the nadir; SaO₂ ~ 90%) for 8h/day for 1 (n=8) or 2 (n=8) weeks with corresponding sham controls (n=16) were also conducted. Following gas exposures, the animals were anaesthetized with 5% isoflurane by inhalation and killed humanely by cervical spinal cord transaction in accordance with guidelines provided by the Royal College of Surgeons in Ireland and University College Dublin animal welfare committees. The right ventricle of the heart was dissected free from the left ventricle (plus septum) and both were weighed individually. Haematocrit concentration was determined from blood samples which were collected in triplicate in microcapillary tubes from the inferior vena cava. The left hemi-diaphragm with central tendon and rib intact was excised for functional studies. Additional muscle samples were snap frozen in isopentane cooled in liquid nitrogen and stored at -80°C for structural and biochemical analysis.

Respiratory muscle function

Contractile and endurance properties of the diaphragm muscle were examined ex vivo at 35°C under control (95% O₂/5% CO₂) conditions. Respiratory and limb muscles were snap frozen and stored at -80°C for subsequent structural and biochemical analysis.
SDH and GPDH enzyme histochemistry

Muscle sections were stained for succinate dehydrogenase (SDH) and glycerol phosphate dehydrogenase (GPDH) activity as previously described (McMorrow et al., 2011; Skelly et al., 2012; Shortt et al., 2013). Sham and CIH-treated muscles were processed in parallel.

Myosin heavy chain immunohistochemistry

Indirect immunofluorescence was used to tag for myosin heavy chain (MHC) type 1, 2A, 2X and 2B fibres as previously described (McMorrow et al., 2011; Skelly et al., 2012; Shortt et al., 2013).

SERCA immunohistochemistry

Indirect immunofluorescence was performed to identify the distribution of SERCA1 and SERCA2 isoforms (Shortt et al., 2013).

Na\(^+\)/K\(^+\)-ATPase pump content

Na\(^+\)/K\(^+\) pump α\(_2\) isoform was determined in diaphragm muscle using the vanadate-facilitated \(^3\)Houabain binding method as previously described (McMorrow et al., 2011).

Assessment of Oxidative Stress

Lipid peroxidation in diaphragm muscle samples was determined using a Thiobarbituric Acid Reactive Substances (TBARS) assay kit (CAT no. 10009055, Cayman’s Chemical Company). A commercial glutathione assay kit (CAT no. 703002, Cayman’s Chemical Company) was used to quantify total glutathione (tGSH) concentration and GSSG/GSH ratio. Muscle sections were probed immunocytochemically for 4-HNE (a marker of oxidative stress) and 8-OHdG (a marker of DNA oxidation).

Chronic antioxidant/apocynin supplementation in CIH-treated animals

Three additional groups of CIH-treated rats (5% O\(_2\) at the nadir; 2 weeks) had free access to water containing N-acetyl cysteine (1% NAC, n=8), tempol (1mM, n=8) or apocynin (2mM, n=8) beginning one day prior to treatment and continuing throughout the 2 week study. Doses were informed by previous published studies (Dunleavy et al., 2008; Johns et al., 2010; Skelly et al., 2012).
Data analysis

Specific force was calculated in N/cm$^2$ of muscle cross-sectional area (CSA). Muscle fatigue was assessed by expressing tetanic contractions at 1 and 2 minutes of a repeated muscle stimulation trial as a percentage of the initial force. To determine SDH and GPDH enzymatic activities, optical density of the muscle sections were measured using Scion Image™ software. MHC fibre distribution, CSA and SERCA isoform distribution was calculated using Cell A™ software using stereological principles. All data per animal were first averaged before computing group means. Data are presented as mean ± SEM. All statistical analyses were performed using Graph Pad Prism software. A two-way ANOVA (stimulation frequency x drug) was employed to compare diaphragm muscle force-frequency relationships across the groups. All other data was statistically analyzed using Student’s unpaired t tests or a one-way ANOVA with Tukey post-hoc test where appropriate. In all tests, $p < 0.05$ was taken as significant.
Results

Body Mass, Haematocrit and Right Ventricular Mass
CIH (2 weeks; 5% O\textsubscript{2} at the nadir) decreased body mass (298 ± 5 vs. 250 ± 5 g, sham (n=8) vs. CIH (n=8), mean ± SEM, p<0.001, Student’s t-test) and increased haematocrit concentration (42±1 vs. 47±1 %, sham (n=8) vs. CIH (n=8), mean ± SEM, p<0.001, Student’s t-test). Right ventricle mass was unaffected by CIH (0.22 ± 0.01 vs. 0.26 ± 0.01 right ventricular/left ventricular ratio, sham (n=8) vs. CIH (n=8), mean ± SEM, p=0.42, Student’s t-test).

Diaphragm Function (10% O\textsubscript{2} at the nadir)
Diaphragm force was unaffected by 1 week of CIH treatment but was decreased (p=0.02, 2-way ANOVA) following 2 weeks of exposure (Fig S2, supplementary material). CIH (1 or 2 weeks) did not affect diaphragm muscle fatigue (Fig S2, supplementary material).

Diaphragm Function (5% O\textsubscript{2} at the nadir)
One week of CIH exposure significantly improved diaphragm force (Fig 1a; p<0.0001, 2-way ANOVA) but had no effect on fatigue (Fig 1b). CIH treatment for 2 weeks had a significant negative inotropic effect on diaphragm (Fig 2e; p=0.0034, 2-way ANOVA). In addition, diaphragm endurance was significantly reduced following 1 minute (p=0.03, Student’s t-test) and 2 minutes (p=0.02, Student’s t-test) of the fatigue trial (Fig 2f).

SDH & GPDH Enzyme Activities
CIH treatment had no effect on SDH (141 ± 5 vs. 135 ± 10 arbitrary units (AU), sham (n=5) vs. CIH (n=5), mean ± SEM, p=0.61, Student’s t-test) or GPDH (37 ± 2 vs. 34 ± 1 AU, sham (n=5) vs. CIH (n=5), mean ± SEM, p=0.32, Student’s t-test) enzymatic activity in the diaphragm.

MHC Immunofluorescence
Type 1, type 2A and type 2X muscle fibre areal density was not affected by CIH treatment. Type 2B areal density was significantly increased (Fig 3e; p=0.04, Student’s t-test) in the diaphragm with CIH treatment. The fibre CSA of type 1, type 2A, type 2X and type 2B was unchanged following CIH treatment (Fig 3f). CIH did not affect areal density or CSA of the EDL or soleus muscles (Fig S3, supplementary material).
**SERCA Isoform Distribution**

CIH did not alter SERCA1 (77 ± 4 vs. 76 ± 1, % areal density, sham (n=5) vs. CIH (n=5), mean ± SEM, p=0.72, Student’s t-test) or SERCA2 (30 ± 2 vs. 30 ± 2, % areal density, sham (n=5) vs. CIH (n=5), mean ± SEM, p=0.94, Student’s t-test) isoform distribution in rat diaphragm muscle.

**Sodium Potassium ATPase Pump Content**

CIH did not alter Na⁺/K⁺-ATPase pump content in the diaphragm (433 ± 28 vs. 437 ± 47 ouabain binding sites occupied pmol/g wet weight, sham (n=6) vs. CIH (n=6), mean ± SEM, p=0.95, Student’s t-test).

**Oxidative Stress Levels Following CIH±Antioxidants/Apocynin**

8-OHdG was positively labelled in myonuclei of 5/6 animals treated with CIH (Fig S4, supplementary material). There was no evidence of increased 4-HNE immunolabelling in diaphragm muscle following CIH (data not shown). CIH did not affect diaphragm muscle malondialdehyde (MDA) concentration (1.8 ± 0.6 vs. 3.7 ± 1.4 nmol MDA/ mg protein, sham (n=6) vs. CIH (n=6), mean ± SEM, p=0.24, Student’s t-test) or total glutathione concentration (Fig 4a; p>0.05, ANOVA). GSSG/GSH ratio was significantly increased in CIH diaphragm (Fig 4b; p<0.05, ANOVA) and this was significantly ameliorated by NAC administration (Fig 4b; p<0.05, ANOVA) but not by chronic Tempol or apocynin supplementation (Fig 4b; p>0.05, ANOVA).

**Effect of NAC, Tempol and Apocynin on Diaphragm Muscle Function**

NAC fully prevented the deleterious effects of CIH on diaphragm force (Fig 5a; p<0.05, 2-way ANOVA). Tempol however did not reverse CIH-induced diaphragm muscle weakness (Fig 5a; p=0.58, 2-way ANOVA). The CIH-induced reduction in diaphragm muscle endurance was fully prevented by NAC (Fig 5b; p<0.01, 2-way ANOVA) and tempol (Fig 5b; p<0.01, 2-way ANOVA) supplementation in vivo. Apocynin did not reverse CIH-induced loss of diaphragm force (Fig 5c; p=0.7, 2-way ANOVA) but the drug prevented CIH-induced diaphragm fatigue (Fig 5d; p<0.05, 2-way ANOVA).
Discussion

The main findings of the present study are: 1) CIH-induced diaphragm muscle dysfunction is time- and intensity-dependent; 2) CIH-induced diaphragm muscle fatigue may relate in part to a structural alteration (‘slow-to-fast’ fibre-type transformation) within the muscle; 3) CIH treatment caused DNA oxidation and decreased an endogenous antioxidant defence system in diaphragm muscle without evidence of lipid peroxidation; 4) NAC, but not tempol or apocynin, prevents CIH-induced diaphragm muscle weakness; 5) Antioxidants and apocynin prevent CIH-induced diaphragm muscle fatigue.

CIH-induced respiratory muscle dysfunction

CIH, a central feature of OSA, has been shown to alter skeletal muscle function (McGuire et al., 2002a, 2003; Pae et al., 2005). Despite its clinical relevance, the effect of CIH on diaphragm muscle function is relatively underexplored. We found that CIH treatment caused a marked reduction in diaphragm muscle force and endurance in a time- and intensity-dependent manner. Diaphragm weakness is consistent with observations in OSA patients (Chien et al., 2010) and unfavourable effects of CIH on diaphragm endurance was also reported following a 5 week CIH exposure in rats (McGuire et al., 2003). Short exposures (10 days) were previously shown to be insufficient to elicit diaphragm fatigue (Clanton et al., 2001) consistent with the hypothesis that the effects of CIH are pattern and duration sensitive. In this study we showed that duration (1 week vs. 2 weeks) and intensity (10% vs. 5% oxygen at the nadir) are important factors in the determination of the effects of hypoxia on muscle function. Increased intensity and duration of IH exposure causes modest oxidative stress and diaphragm weakness and fatigue.

Effect of CIH on skeletal muscle structure

We examined the effects of CIH on respiratory muscle structure and hypothesized that CIH-induced changes in diaphragm function are related to a structural alteration within the muscle as seen in other animal models of OSA (McGuire et al., 2002a; Pae et al., 2005; O'Connell et al., 2013). We found no change in SDH or GPDH activity suggesting that an alteration in metabolic enzymes is not likely responsible for increased diaphragm fatigue observed in our model. Other studies have also shown that metabolic properties are not always directly related to the fatigability of the muscle (Sieck et al., 1989). However, the question of whether
other oxidative enzymes like cytochrome c oxidase or pyruvate dehydrogenase are implicated in CIH-induced respiratory muscle fatigue was not investigated in this study.

We observed evidence of subtle structural remodelling in CIH diaphragm within the fast fibres resulting in an increase in type 2B density which may partly contribute to increased muscle fatigue. Type 2B density and diaphragm endurance were negatively correlated (see Fig S5, supplementary material). However, we speculate that this structural remodelling is not the major factor driving aberrant muscle function following CIH exposure. Antioxidant supplementation attenuated CIH-induced increase in type 2B areal density (data not shown), but did not fully reverse areal density to control values whereas diaphragm endurance was fully recovered suggesting that fibre transition in CIH diaphragm, though consistent with the functional change, is not a major contributor to CIH-induced diaphragm fatigue.

Studies have shown that SERCA function is closely linked with skeletal muscle fatigue (Aubier & Viïres, 1998; Tupling, 2004). Our laboratory has previously shown that SERCA1 isoform distribution is increased, while fibres expressing SERCA2 isoform are decreased following CIH (Shortt et al., 2013). We hypothesized that a SERCA2 to SERCA1 transition was responsible for the increased diaphragm fatigue observed in our CIH model. However, we found that CIH had no effect on SERCA isoform distribution. This is consistent with other studies showing an alteration in muscle fatigue independent of a shift in fibres expressing SERCA (Kim et al., 2003; McMorrow et al., 2011). However, it must be noted that we did not measure SERCA protein expression, SERCA activity or phospholamban phosphorylation. Alteration in SERCA expression or activity could contribute to CIH-induced diaphragm muscle fatigue (Aubier & Viïres, 1998). However, of note, we found that half-relaxation time of diaphragm twitch kinetics was not altered following CIH treatment suggesting that SERCA activity was unchanged.

The Na⁺/K⁺-pump is important in maintaining Na⁺ and K⁺ concentrations in skeletal muscle preserving membrane excitability and thus force production. A reduction or inhibition (with ouabain) of Na⁺/K⁺-pump activity leads to a decline in muscle contractile function (Leppik et al., 2004; McKenna et al., 2006). Increased Na⁺/K⁺-pump content was observed in diaphragm from chronic hypoxic animals, associated with improved diaphragm endurance (McMorrow et al., 2011) suggesting that pump content is affected by hypoxia and is implicated in muscle fatigue. However, we found that CIH treatment had no effect on Na⁺/K⁺-pump content in
diaphragm showing that decreased Na\(^+/\)K\(^+/\)-pump content is not responsible for CIH-induced diaphragm fatigue, a finding supported by others (Madsen et al., 1994). It must be noted however that pump content and not pump activity was measured in our study and therefore it is possible that pump activity may have been reduced independent of expression. This scenario was previously observed in humans (Leppik et al., 2004) and animals (McKenna et al., 2003).

**CIH and Oxidative Stress**

It is well recognized that CIH increases the production of ROS in humans and animals (Shan et al., 2007; Dunleavy et al., 2008; Dutta et al., 2008). CIH-induced oxidative damage has been observed in skeletal muscle (Dutta et al., 2008) and other tissues (Veasey et al., 2004; Raghuraman et al., 2009; Khan et al., 2011). In addition, ROS scavengers such as superoxide dismutase (SOD) and N-acetylcysteine (NAC) prevent CIH-induced muscle (Dunleavy et al., 2008) and neuronal (Veasey et al., 2004) damage. These studies suggest a putative role for CIH-induced oxidative stress via the production of ROS in diaphragm dysfunction. Lipid peroxidation (MDA concentration and 4-HNE staining) was not evident in CIH-treated diaphragm muscle. This is perhaps not surprising as Raghuraman and colleagues have demonstrated that short bouts of IH (15 seconds hypoxia; 5 minutes normoxia) produces higher levels of ROS compared to 90 second hypoxic/normoxic cycles (Raghuraman et al., 2009). Other studies have also shown that the detrimental effects of CIH manifest in a “dose”-dependent manner and the effects of oxidative stress may be organ-specific (Shan et al., 2007; Jun et al., 2008). In consideration of this, it seems likely that the deleterious effects of CIH in muscle take time to manifest suggestive of a cumulative stress or change in redox status of key regulatory targets. We observed DNA oxidation in myonuclei of 5/6 CIH diaphragms and an increase in GSSG, both indicative of a relatively modest increase in oxidative stress associated with our most intense paradigm of CIH.

**Effect of Antioxidants on Muscle Function**

Antioxidants reversed CIH-induced diaphragm fatigue in our animal model. These results further suggest a role for CIH-induced ROS in diaphragm dysfunction and this finding is supported by many studies indicating a correlation between oxidation and skeletal muscle dysfunction. Animal studies have shown that NAC protects against diaphragm fatigue *ex vivo* (Diaz et al., 1994) and *in vivo* (Shindoh et al., 1990). In addition, NAC can improve diaphragm endurance (Supinski et al., 1995) and decrease diaphragm oxidative stress...
(Supinski et al., 1995) following inspiratory resistive breathing. These studies together with our findings suggest that NAC may be a very useful compound for the prevention of respiratory muscle fatigue in the context of hypoxia-related dysfunction.

Tempol is considered a powerful inotropic agent; it increases upper airway muscle force *ex vivo* (Skelly et al., 2010) and improves CIH-induced upper airway muscle dysfunction (Skelly et al., 2012). The effects of *in vivo* tempol supplementation on diaphragm muscle function are not well investigated but studies have shown that SOD and other SOD mimetics improve diaphragm contractility (Fujimura et al., 2000; Supinski & Callahan, 2005). In addition, tempol has been shown to reverse the inhibitory effects of elevated temperature on tetanic force of isolated intact mouse and rat EDL (Edwards et al., 2007). Our study extends this body of work showing that tempol supplementation prevents CIH-induced diaphragm fatigue though it is interesting to note that the drug, unlike NAC, was not effective in significantly reversing diaphragm glutathione oxidation and CIH-induced muscle weakness.

*Potential Sources for ROS Production*

The evidence above suggests that an increased production of ROS due to recurrent hypoxia/reoxygenation is detrimental for respiratory muscle function. Although the origin of ROS remains debatable, there are a number of potential sources for CIH-induced ROS generation in skeletal muscle. These include the mitochondrial electron transport chain and enzymatic pathways such as xanthine oxidase (XO), phospholipase A₂ (PLA₂) and NOX. Some studies suggest oxidative stress is mediated by XO (Barclay & Hansel, 1991; Stofan et al., 2000; Judge & Dodd, 2004) while others disagree with this potential pathway (Supinski et al., 1999a; Heunks et al., 2001; Javesghani et al., 2002). PLA₂ releases arachidonic acid from phospholipids which acts as a substrate for ROS-generating enzymes such as lipoxygenase and cyclooxygenase. Some studies show that this pathway is implicated in the generation of ROS (Nethery et al., 1999; Gong et al., 2006). Activation of PLA₂ can stimulate NOXs (Zhao et al., 2002) and therefore may also generate ROS through this pathway. Mitochondrial electron transport chains are a major source of ROS in skeletal muscle with approximately 1-2% of molecular oxygen consumed during normal physiological respiration converted to superoxide (Orrenius et al., 2007). In addition, it has been shown that during CIH (Yuan et al., 2004; Shan et al., 2007; Khan et al., 2011) and intense exercise (Kanter, 1994), the rate of mitochondrial ROS production is increased. NOXs are membrane-associated enzymes, which catalyze the one electron reduction of
molecular oxygen using either NADH or NADPH as electron donors. Studies have identified NOX enzymes associated with the SR (Xia et al., 2003), transverse tubules and plasma membrane (Javesghani et al., 2002) of skeletal muscle, all of which have been associated with superoxide generation. Interestingly, inhibitors of cyclo-oxygenases, XOs and mitochondrial enzymes were shown to have no dampening effect on muscle superoxide production (Javesghani et al., 2002). These findings were supported by Michaelson et al. (2010) who found that inhibition of NOX abolished contraction-induced ROS production in single living myofibres (Michaelson et al., 2010). These studies suggest a role for NOX-derived ROS in skeletal muscle dysfunction. We hypothesized that NOX-dependent superoxide is implicated in CIH-induced diaphragm muscle dysfunction and that supplementation with apocynin would ameliorate the deleterious effects of CIH on diaphragm muscle performance suggesting that NOX may be a major source of CIH-derived ROS in the context of our model. Apocynin is a naturally occurring methoxy-substituted catechol, experimentally used as an inhibitor of NOX. We demonstrated that in vivo administration of apocynin prevents CIH-induced diaphragm fatigue suggesting that NOX may be implicated as a major source of ROS production in respiratory muscle during CIH but this requires further study. Our finding is consistent with other studies showing that apocynin ameliorates diaphragmatic oxidative stress and contractile dysfunction (Supinski et al., 1999b; McClung et al., 2009). However, similar to tempol, it should be noted that apocynin did not significantly reverse increased GSSG/GSH levels in CIH diaphragm muscle and the drug was ineffective in preventing CIH-induced diaphragm muscle weakness.

It is also important to highlight that apocynin may act as an antioxidant rather than a specific NOX inhibitor (Heumuller et al., 2008). The molecular structure of apocynin contains a phenol group with potential scavenging capacity, suggesting a potential role as an antioxidant scavenger. Recent studies demonstrate that apocynin can specifically inhibit NOX but this only occurs in cells that generate large amounts of ROS and express myeloperoxidase (Heumuller et al., 2008). In the absence of these molecules apocynin is suggested to function as an antioxidant (Heumuller et al., 2008). Of interest, an increase in the levels of myeloperoxidases has been identified in the liver following CIH treatment (Feng et al., 2010) suggesting that under these conditions apocynin can act as a NOX inhibitor. The drug has been widely used in vivo and is recognized as having greater specificity than other pharmacological inhibitors of NOX such as diphenylene iodonium (Williams & Griendling, 2007). Therefore, at this time we are unable to exclude the possibility that apocynin acted as a
general non-specific antioxidant though the source of the ROS may still have been predominantly from the NOX enzymes. As such, our results with apocynin should be interpreted with caution as additional studies are required to determine if NOX is the primary source of ROS underpinning CIH-induced diaphragm dysfunction. In future work, it will be important to demonstrate that CIH increases NOX expression and/or activity in CIH-treated respiratory muscle. Moreover, studies utilising NOX null mice (Peng et al., 2009; Khan et al., 2011) would prove useful in establishing if there is an absolute requirement for NOX-derived ROS in CIH-induced diaphragm muscle dysfunction.

**Conclusion**

We have shown that CIH-induced respiratory muscle dysfunction is mediated by ROS. Antioxidant supplementation especially with NAC was effective in reversing CIH-induced diaphragm muscle impairment. We speculate on the basis of our observations with apocynin that the deleterious effects of ROS on diaphragm muscle performance are at least in part due to an increase in NOX-derived ROS but this hypothesis requires further study. Our findings suggest that enhancing ROS scavenging capabilities of skeletal muscle via the administration of exogenous antioxidants or perhaps through the inhibition of NOX is a potentially attractive therapeutic approach for the treatment of respiratory muscle dysfunction seen in OSA patients.

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**Competing Interests:** The authors declare that there are no competing interests.

**Conflict of Interest:** The authors declare that there are no conflicts of interest.
References:


Figure 1: Diaphragm muscle function (5% O₂ at the nadir; 1 week)
(a) Group data (mean ± S.E.M) showing that CIH treatment improves diaphragm force (p<0.001, 2-way ANOVA). Group data (mean ± S.E.M) showing diaphragm endurance (b) is not altered by CIH (p>0.05, Student’s unpaired t-test).

![Graph showing diaphragm muscle function](image)

- a) Specific force vs. frequency
- b) % of initial force vs. time

Frequency: p<0.003, Treatment: p<0.0001, Interaction: p<0.05.
Figure 2: Diaphragm muscle function (5% O$_2$ at the nadir; 2 weeks)
Original traces of diaphragm muscle force-frequency relationship from a sham (a) and CIH-treated (c) animal. (e) Group data (mean ± S.E.M) showing that CIH treatment reduces diaphragm force (p=0.003, 2-way ANOVA). Original traces of diaphragm muscle fatigue with initial and final contractions superimposed from a sham (b) and CIH-treated (d) animal. Note how diaphragm muscle force in the CIH-treated animal decreases to a greater extent with continuous contractions over time compared to the sham animal. (f) Group data (mean ± S.E.M) showing diaphragm endurance is reduced following 1 minute (p=0.03) and 2 minutes (p=0.02) of the fatigue trial in CIH compared to sham (*p<0.05, Student’s unpaired t-test).
Figure 3: Respiratory muscle myosin heavy-chain (MHC) areal density and fibre cross-sectional area (CSA)

Representative images of diaphragm muscle triple-labelled with monoclonal antibodies from a sham (a) and CIH (b) treated muscle showing MHC 1 (blue), MHC 2a (red), and MHC 2b (green); MHC 2x fibres are untagged. Representative images of diaphragm muscle double-labelled with monoclonal antibodies tagging the basement membrane protein laminin (green) and all MHC isoforms (red) except for MHC 2x (untagged) from a sham (c) and CIH (d) treated muscle. (e) Group data (mean ± S.E.M) showing that type 2B areal density was significantly increased in the CIH-treated diaphragm (*p=0.04, Student’s unpaired t-test). (f) Group data (mean ± S.E.M) showing that fibre CSAs were not affected by CIH treatment.
Figure 4: Oxidative stress in the diaphragm

(a) Group data (mean ± SEM) showing GSH concentration in the diaphragm. GSH concentration was not altered by CIH ± drug intervention. (b) Group data (mean ± SEM) showing that CIH treatment results in a significant increase in GSSG (oxidised glutathione) in the diaphragm (*p<0.05 Sham vs. CIH, ANOVA). NAC supplementation significantly negated CIH-induced increase in GSSG levels (†p<0.05 CIH vs. CIH+NAC; ANOVA). Neither tempol nor apocynin supplementation significantly reduced GSSG levels.
**Figure 5: Effect of antioxidants/apocynin on diaphragm muscle function**

(a) Group data (mean ± S.E.M) showing that tempol does not reverse the negative inotropic effect of CIH on diaphragm muscle (p=0.58, CIH vs. CIH+tempol; 2-way ANOVA). NAC reversed the effect of CIH on diaphragm force-frequency relationship (p<0.05, CIH vs. CIH+NAC; 2-way ANOVA). (b) Group data (mean ± S.E.M) showing that both NAC and tempol supplementation during CIH exposure reversed the effects of CIH on diaphragm fatigue (*p<0.05 Sham vs. CIH, ANOVA; †p<0.05 CIH vs. CIH+drug). (c) Group data (mean ± S.E.M) showing that apocynin treatment in vivo does not reverse the negative inotropic effects of CIH on diaphragm muscle (p=0.71, CIH vs. CIH+apocynin; 2-way ANOVA). (d) Group data (mean ± S.E.M) showing that apocynin reversed the effects of CIH on diaphragm fatigue (*p<0.05 Sham vs. CIH, ANOVA; †p<0.05 CIH vs. CIH+apocynin).