Upper airway dilator muscle weakness following intermittent and sustained hypoxia in the rat: effects of a superoxide scavenger

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Abstract

Obstructive sleep apnoea syndrome (OSAS) is a common disorder associated with upper airway muscle dysfunction. Agents that improve respiratory muscle performance may have considerable therapeutic value. We examined the effects of sustained and intermittent hypoxia on rat pharyngeal dilator muscle function. Additionally, we sought to test the efficacy of antioxidant treatment in ameliorating or preventing hypoxia-related muscle dysfunction. Isometric contractile and endurance properties of isolated rat sternohyoid muscle bundles were examined at 35ºC *in vitro*. Muscle bundles were exposed to one of four gas treatments: hyperoxia (control), sustained hypoxia (SH), intermittent hypoxia (IH) or hypoxia/re-oxygenation (HR), in the absence or presence of the superoxide scavenger - Tempol (10mM). Stress-frequency relationship was determined in response to electrical stimulation (10-100Hz in increments of 10-20Hz, train duration: 300msec). Muscle performance was also assessed during repetitive muscle stimulation (40Hz, 300ms every 2s for 2.5 min). Compared to control, IH and HR treatments significantly decreased sternohyoid muscle force. The negative inotropic effect of the two gas protocols was similar, but both were of lesser magnitude than the effects of SH. SH, but not IH and HR, increased muscle fatigue. Tempol significantly increased sensitivity to stimulation in all muscle preparations and caused a leftward shift in the stress-frequency relationship of IH and SH treated muscles. Tempol did not ameliorate sternohyoid muscle fatigue during SH. We conclude that Tempol increases upper airway muscle sensitivity to stimulation but only modestly ameliorates respiratory muscle weakness during intermittent and sustained hypoxic conditions *in vitro*. Respiratory muscle fatigue during sustained hypoxia appears unrelated to oxidative stress.

Keywords: Antioxidants; Fatigue; Hypoxia/Re-oxygenation; Intermittent Hypoxia; Superoxide scavenger; Obstructive sleep apnoea syndrome; Upper airway muscles.

1. Introduction

Skeletal muscles of the upper airway function to stabilize and dilate the pharyngeal airspace during inspiration. Impaired upper airway muscle function is implicated in obstructive sleep apnoea syndrome (OSAS) – a common and devastating respiratory disorder (White et al., 2005). Of interest, there is evidence of upper airway muscle remodelling and dysfunction in OSAS (Stauffer *et al.* 1989, Sériès *et al.* 1996b, Ferini-Strambi *et al.* 1998, Carrera *et al.* 1999) effects that resolve with continuous positive airway pressure (CPAP) treatment (Carrera *et al.* 1999). In recent years, translational animal models have emerged providing strong evidence that intermittent hypoxia – a hallmark feature of OSAS due to recurrent apnoea – is a key factor in the development of significant morbidities in OSAS patients. Several groups have demonstrated that intermittent hypoxia alters respiratory control (Ling *et al.* 2001, O'Halloran *et al.* 2002, Veasey *et al.* 2004), and causes respiratory muscle dysfunction (McGuire *et al.* 2002a, Pae *et al.* 2005) which may have special relevance to OSAS and other respiratory muscle weakness disorders.

OSAS is now widely recognized as an oxidative stress disorder (Lavie *et al.* 2003, 2004, 2009) and antioxidant supplementation was recently shown to be effective in the treatment of OSAS patients (Sadasivam *et al*. 2011). There is substantial evidence to indicate that reactive oxygen species (ROS) are produced following bouts of hypoxia and re-oxygenation in skeletal muscle. Though basal levels of ROS play an important role in a variety of physiological functions including optimum contractile function in muscle (Reid *et al.* 1993), ROS-induced skeletal muscle dysfunction is well described (Anzueto *et al.* 1994, Supinski 1998, Dunleavy *et al.* 2008) and may contribute to the pathophysiology of OSAS (Lavie 2009). The first aim of this study was to compare the effects of sustained hypoxia, intermittent hypoxia and, hypoxia

followed by re-oxygenation on rat pharyngeal dilator muscle function. We hypothesized that sternohyoid muscle dysfunction would be greatest in intermittent hypoxia treated muscles due to increased oxidative stress. The sternohyoid muscle was selected for study as it is readily accessible and has fibres running in a uniform direction facilitating the interpretation of isometric force measurements *in vitro*. Supra- and infra-hyoid muscles contribute to the regulation of pharyngeal airway calibre and sternohyoid muscle dysfunction has been reported in animal models of sleep-disordered breathing (Petrof *et al.* 1994, McGuire *et al.* 2002b, c, O'Halloran *et al.* 2002, Bradford *et al.* 2005, Pae *et al.* 2005, Dunleavy *et al.* 2008, Skelly *et al.* 2010b).

Pharmacotherapy for OSAS is considered a viable clinical option (Hudgel & Thanakitcharu 1998, Veasey 2003, Hedner *et al.* 2008) especially for patients for whom CPAP – the gold standard treatment for OSAS – is not well tolerated. Drugs that improve pharyngeal dilator muscle performance are likely to benefit some patients and anti-oxidant agents are promising in this regard (Jordan *et al.* 2006, Lee *et al.* 2009, Skelly *et al.* 2010a, Sadasivam *et al.* 2011, Skelly *et al.* 2012). Therefore, the second aim of this study was to examine the efficacy of Tempol, a membrane-permeant superoxide dismutase (SOD)-mimetic, in mitigating respiratory muscle dysfunction secondary to gas treatments. We hypothesized that Tempol would be more effective in rescuing force following hypoxia/re-oxygenation protocols compared to sustained hypoxia because of its free radical scavenging properties.

2. Methods

2.1 Animals

Experiments were performed on 32 adult male Wistar rats (body mass 195-315g). Animals were assigned to one of four groups: control (n=7), sustained hypoxia (SH; n=8), intermittent hypoxia (IH; n=9) or hypoxia/re-oxygenation (HR; n=8). All protocols described in this study were approved and carried out in accordance with local institutional guidelines.

2.2 In vitro muscle preparation

The animals were anaesthetized with 5% isoflurane and euthanized by cervical spinal cord section. The paired sternohyoid muscles were separated along their medial border and excised. The muscles were placed in a tissue bath at room temperature containing continuously gassed (95% O_2/5% CO_2) Krebs solution. The solution contained the following components: NaCl 120mM, KCl 5mM, Ca^{2+} gluconate 2.5mM, $MgSO_4$ 1.2mM, NaH_2PO_4 1.2mM, $NaHCO_3$ 25mM and glucose 11.5mM. Longitudinal strips of muscle (~1mm diameter) were prepared and placed vertically in Plexiglas tissue holders in Krebs solution in water-jacketed organ baths at 35°C gassed with a 95% O_2/5% CO_2 gas mixture. The neuromuscular paralyzing agent, d-tubocurarine (25μM), was added to the bathing medium to eliminate activation of intramuscular nerve branches thus ensuring that isometric force generation was due solely to direct muscle stimulation. The muscle bundles were positioned between a pair of platinum plate electrodes, with the base fixed to an immobile hook and the other end tied to an isometric force transducer with non-elastic string. The position of the force transducer could be adjusted by a micropositioner thus altering the length of the muscle strips. A fixed graduated scale located behind the muscle strip allowed the accurate measurement of muscle length.

2.3 Protocol

The optimum length (*i.e.* muscle length producing maximal isometric twitch force in response to supra-maximal stimulation) was determined by adjusting muscle length between intermittent stimulations. Once determined the muscle remained at this length for the full protocol. Next, the muscle was allowed a 5 min equilibration period. Peak tetanic force was determined in response to a 300msec train at 100Hz. Tissue baths were then emptied and re-filled with Krebs ± antioxidant and the bundles were exposed to one of four gas treatments (see below). After 30 min, contractile and endurance properties were determined in response to electrical field stimulation delivered via plate electrodes flanking the tissue connected to a square pulse constant current stimulator. Data were recorded using a commercial data acquisition system (PowerLab, AD Instruments) and stored for later analysis on a computer. First, force-frequency relationship was determined by sequentially stimulating the muscle strips at 10, 20, 30, 40, 60, 80 and 100Hz for 300 ms at each stimulus frequency allowing a 2 min recovery interval between each stimulus (Fig. 1A). Two min following the force-frequency protocol, repeated muscle contraction was induced by stimulation at 40 Hz with 300 ms trains every 2 sec for a period of 2.5 min.

2.4 Gas and Antioxidant Treatments

For control studies, muscle bundles remained in Krebs solution gassed with a hyperoxic gas mixture (95% O_2/5% CO_2). We have previously established that sternohyoid muscle bundles incubated at 35^{o}C generate higher forces in hyperoxia compared to a normoxic (21% O_2/5% CO_2) bathing medium (Skelly *et al.* 2010a). For SH, muscle bundles were exposed to an anoxic gas mixture (95% N_2/5%CO_2; producing a tissue bath PO_2 of ~40 mmHg) and contractile and endurance performance was assessed in hypoxia. For HR, muscle bundles were exposed to an

anoxic gas mixture (95% N_2/5% CO_2) for 15 min followed by a hyperoxic gas mixture (95% O_2/5% CO_2) for 15 min. Finally, for IH, bundles were exposed to 3 x 5 min anoxic gas treatment (95% N_2/5% CO_2) interspersed with 5 min intervals of hyperoxia (95% O_2/5% CO_2). Muscle function was assessed under hyperoxic conditions in all groups with the exception of the SH group. Studies were performed in the absence (control) or presence of 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol, 10mM) performed in parallel using a double tissue bath set-up allowing paired comparisons for each of the four gas treatments. All compounds were purchased from Sigma–Aldrich Company, Dublin, Ireland and made up fresh each day.

2.5 Data Analysis

Specific force was calculated in N/cm^2 of muscle cross-sectional area. The latter was approximated by weighing the dry muscle strips at the end of the experimental protocol and dividing this by the product of optimal length and muscle density (assumed to be $1.056 g/cm^3$). The force transducers were calibrated using known gramme weights. Peak tetanic force (Fmax) was determined in response to 100Hz stimulation at the beginning of the protocol. For the force-frequency relationship, data across the range of stimulus frequencies employed in the study were normalized to the initial peak force determined at the beginning of the study. Additionally, non-linear regression (curve-fit) analysis was employed (Graph Pad Prism) for all groups, allowing us to determine minimum, maximum, slope and EF_{50} values (*ie* stimulus frequency producing 50% of peak force) for sternohyoid muscle following each of the gas treatments ± Tempol. Finally, to assess muscle performance in response to repeated activation we performed two sets of analyses. First, each tetanic contraction was measured and specific force was averaged in 10s bins (*ie* 5 consecutive tetanic

contractions) over the initial 1 min and in 30s bins over the remaining 90 sec. Second, a performance index was calculated for the whole fatigue trial according to the following: [average twitch amplitude of all 60 contractions/initial twitch amplitude x 100] modified from Healy et al. (Healy *et al.* 2008). Values are expressed as mean ± SEM. Statistical comparisons between groups were performed using one-way (gas treatment) or two-way ANOVA (drug x gas treatment) and Bonferroni post-hoc tests. $P<0.05$ was the criterion for significance in all tests.

3. Results

3.1 Effect of gas treatment on sternohyoid muscle force

Prior to commencing gas treatments, peak tetanic force in response to 100Hz stimulation (Fmax) was similar in all four groups (P=0.9705, one-way ANOVA; Fig. 1B). The effect of gas treatment on rat upper airway muscle force is illustrated in Fig. 1C. For each preparation, data are normalized to the initial peak force (Fmax) determined before gas treatments. Both IH and HR had a significant depressor effect on muscle force compared to control (Figs. 1C and 1D). The negative inotropic effect of the two gas treatments was equivalent (Figs. 1C and 1D). As expected, SH significantly depressed sternohyoid muscle force (Figs. 1C and 1D).

3.2 Effect of Tempol on sternohyoid muscle force

Fmax was equivalent in all four groups prior to commencing gas+Tempol treatments (P=0.7722, one-way ANOVA). Tempol partially ameliorated sternohyoid muscle force decline following IH, HR and SH causing a significant leftward shift in the force-frequency relationship (Fig. 2). However, two-way ANOVA comparing control and individual gas treatments indicated that there was no significant gas x Tempol interaction for force-frequency relationships. Peak force decline in IH, HR and SH groups was unaffected by Tempol treatment (Fig. 3). EF_{50} was significantly decreased by Tempol independent of gas treatment (Fig. 4).

3.3 Sternohyoid muscle performance during repeated stimulation

Data illustrating sternohyoid muscle performance during repeated stimulation in each of the four gas treatments in the absence and presence of Tempol are shown in Figs. 5A and 5B respectively. The performance index was significantly decreased by SH,

but not by IH or HR treatments (Figs. 5C and 5D). Tempol failed to recover SH-induced decrease in sternohyoid muscle performance (Figs. 5C and 5D).

4. Discussion

The main findings of the present study are: 1) IH and HR have an equivalent negative inotropic effect on isolated rat sternohyoid muscle force; 2) Tempol increases sternohyoid muscle sensitivity to electrical stimulation and was modestly effective in preventing muscle weakness following gas treatments; 3) SH, but not IH or HR treatments, decreased sternohyoid muscle endurance properties. Tempol, however, failed to recover decreased upper airway muscle performance during SH.

OSAS is now recognized as an oxidative stress disorder (Lavie *et al.* 2003, 2004, 2009) and upper airway muscle dysfunction in OSAS patients may be ROS-dependent. Free radicals are vital for a number of physiological functions, including optimum muscle contraction (Reid *et al.* 1993). Excitation-contraction coupling in skeletal muscle involves alterations in the redox state of several key proteins central to muscle contraction. Cellular redox balance is dynamic, and when free radicals are produced in excess of a cell's capacity to scavenge them, oxidative stress ensues leading to cellular dysfunction. ROS-mediated skeletal muscle dysfunction is well described (Anzueto *et al.* 1994, Supinski 1998, Dunleavy *et al.* 2008). The mechanical performance of the pharyngeal dilator muscles is a critical determinant of pharyngeal airway stability and hence respiratory homeostasis. We observed that IH and HR both depressed sternohyoid muscle force. Our experimental design was such that we measured peak specific force in all groups prior to commencing experimental protocols, and we established that forces were not different when groups were compared. Therefore differences across groups can be attributed solely to gas treatment and are not likely related to group differences *per se*. Of interest, the two gas protocols decreased pharyngeal dilator muscle force to a similar extent. Cumulatively, both protocols exposed the sternohyoid muscle bundles to 15 min of

hypoxia and 15 min of hyperoxia. That is, the duration and intensity of the stimuli were identical in the two protocols, but the pattern of exposure was (marginally) different. It would be interesting to assess if further variations in the pattern of IH enhanced the negative inotropic effect. The decrement in muscle force following IH and HR can be attributed to a number of mechanisms. Oxidative modification of regulatory proteins such as sarcolemmal potassium channels (Sen *et al.* 1995, Dudley *et al.* 2006), sarcoplasmic reticulum calcium release (RYR) and re-uptake (SERCA) channels (Donoso *et al.* 2011), troponin (Putkey *et al.* 1993), tropomyosin (Williams & Swenson 1982), actin (Hinshaw *et al.* 1991) myosin light (Sweeney *et al.* 1993) and heavy chains (Brooke & Kaiser 1970, Ajtai & Burghardt 1989) could account for free radical-mediated decreases in skeletal muscle force.

Previous studies point to ROS-mediated modification of myofibrillary calcium sensitivity (Bruton *et al.* 2008), but in the present study EF_{50} (a measure of sensitivity to stimulation) was minimally affected by gas treatments suggesting that sensitivity to electrical stimulation was unchanged by gas treatment. Force decline was observed in response to stimulus frequencies ranging 60-100Hz, which one could argue is in the supra-physiological range as motor neuronal firing and muscle fibre recruitment occurs *in vivo* over 10-50Hz in a typical skeletal muscle. However, instantaneous firing frequencies in excess of 100Hz have been observed in human upper airway muscles (Farina & Falla 2009). Thus, we argue that the depressant effect of IH and HR is not only physiologically relevant but may be especially relevant to upper airway dilator muscles and OSAS, where recruitment of these muscles to recover airway collapse can occur hundreds of times over the course of the night in some patients (American *et al.* 1999). One could speculate that impaired force-generating capacity in upper airway muscles following IH increases the vulnerability of the upper

airway to collapse and/or impairs the ability to restore airway calibre following an occlusive event, given that recruitment of the pharyngeal dilator muscles is essential in this regard. We suggest that IH may contribute to upper airway muscle dysfunction in OSAS patients and may, at least in part, explain why the severity of occlusive events increases with time over the course of the night (Charbonneau *et al.* 1994).

As expected, sternohyoid muscle force was significantly depressed in SH. Hypoxia is a known depressor of force generation through actions on ion channel membrane conductance and reductions in sarcolemmal excitability leading to impaired excitation-contraction coupling. Also, under hypoxic conditions, several changes in the internal milieu such as acidosis and alterations in the concentration of inorganic phosphate and adenine nucleotides are capable of directly depressing the function of the contractile apparatus (Godt & Nosek 1989). The SH group was included as a reference benchmark of maximum hypoxic depression in muscle. However, comparison of the magnitude of force decline observed in SH with that seen in the IH and HR groups should be made with caution as contractile and endurance parameters were assessed in hyperoxia (control conditions) in the latter groups. Therefore, the negative inotropic effect of SH may be more representative of maximal force depression. It is plausible that hypoxic preconditioning during the 30 min gas exposure period may have altered the magnitude of hypoxic depression in sternohyoid muscle determined during the muscle function study. Indeed, muscle bundles incubated in hyperoxic conditions and then switched to hypoxia show ~30% reduction in peak isometric force compared to the ~50% reduction in peak force shown in Fig. 3. Thus, as expected, longer durations of hypoxia are associated with decreased muscle function. Of note, however, EF_{50} values and performance index are equivalent in the above two hypoxic groups.

ROS are implicated in skeletal muscle fatigue and increased fatigue is reported in upper airway muscles from OSAS patients (Sériès *et al.* 1996a). Thus, we hypothesized that hypoxia/re-oxygenation protocols would compromise pharyngeal dilator muscle endurance. However, sternohyoid muscle performance index, which assesses all components of the fatigue trial (potentiation and run down) was similar in control and treatment groups, though specific forces were lower in treatment groups compared to control. It is conceivable that *chronic* exposure to HR cycles is necessary for muscle re-modelling leading to the development of a more fatigable phenotype such as that seen in OSAS and rat models of chronic IH (McGuire *et al.* 2002b). Sternohyoid muscle performance index was significantly decreased during SH exposure.

Pharmacological therapy has been suggested as a clinical strategy in the treatment of OSAS (Hudgel & Thanakitcharu 1998, Veasey 2003, Hedner *et al.* 2008) and agents that improve upper airway muscle performance could serve as viable adjunct therapies in patients with sleep-disordered breathing. Previous work has characterized the action of a number of pharmacological agents on isolated upper airway muscle function with some encouraging results (Van Lunteren *et al.* 1995, Van Lunteren *et al.* 1996, Cantillon & Bradford 2001, O'Halloran 2006, Skelly *et al.* 2010a). Since reactive oxygen species (ROS) are implicated in skeletal muscle function and antioxidants have been shown to improve skeletal muscle performance (Reid *et al.* 1992, Reid 2008, Skelly *et al.* 2010a) including diaphragm (Wright *et al.* 2005) and pharyngeal dilator muscle function (Skelly *et al.* 2010a) we sought to test the efficacy of an antioxidant on sternohyoid muscle function in acute models manipulating tissue oxygenation. Tempol increased muscle sensitivity to stimulation (*ie* caused a decrease

in the EF_{50}) in all groups, but only modestly ameliorated force decline in the IH and SH treatment groups, and Tempol had no effect on sternohyoid muscle peak isometric force. Our findings are incongruent with observations that superoxide scavengers protect diaphragm (Mohanraj *et al.* 1998, Wright *et al.* 2005) and upper airway (Skelly *et al.* 2010a) muscle contractile function in severe hypoxia. Our findings do not support our original hypothesis that Tempol would prove more effective in preventing muscle dysfunction in IH and HR treated muscles due to its free radical scavenging properties. In addition, Tempol was ineffective in preventing decreased sternohyoid muscle performance during SH, suggesting that muscle fatigue in hypoxia is not primarily driven by oxidative stress.

It is important to recognize, however, that we may not have employed the optimum concentration of Tempol in our study. We chose one concentration of Tempol in this study based on our previous report (Skelly *et al.* 2010a). It has been suggested that at high concentrations Tempol loses its protective action most likely because of an increased pro-oxidative action when superoxide levels are low (Edwards *et al.* 2007). Also, since ROS are required for optimal muscle function excessive scavenging of ROS could adversely affect muscle performance. The *in vitro* preparation described in this study could prove useful in the screening of established and novel antioxidant compounds with potential application in the treatment of respiratory muscle disorders. However, it should be noted that a plausible, if unexpected, conclusion of our study is that muscle weakness following IH and HR may relate, for the most part, to factors unrelated to oxidative stress.

In summary, we examined the effects of acute exposures to IH, HR and SH ± Tempol (a SOD-mimetic) on pharyngeal dilator muscle contractile and endurance properties

in male rats. IH and HR both depressed sternohyoid muscle force but not fatigue compared to control. Tempol increased sensitivity to stimulation but failed to rescue pharyngeal dilator muscle force during gas challenges. Our *in vitro* model could prove useful in the screening of the effects of novel antioxidant compounds on upper airway muscle performance.

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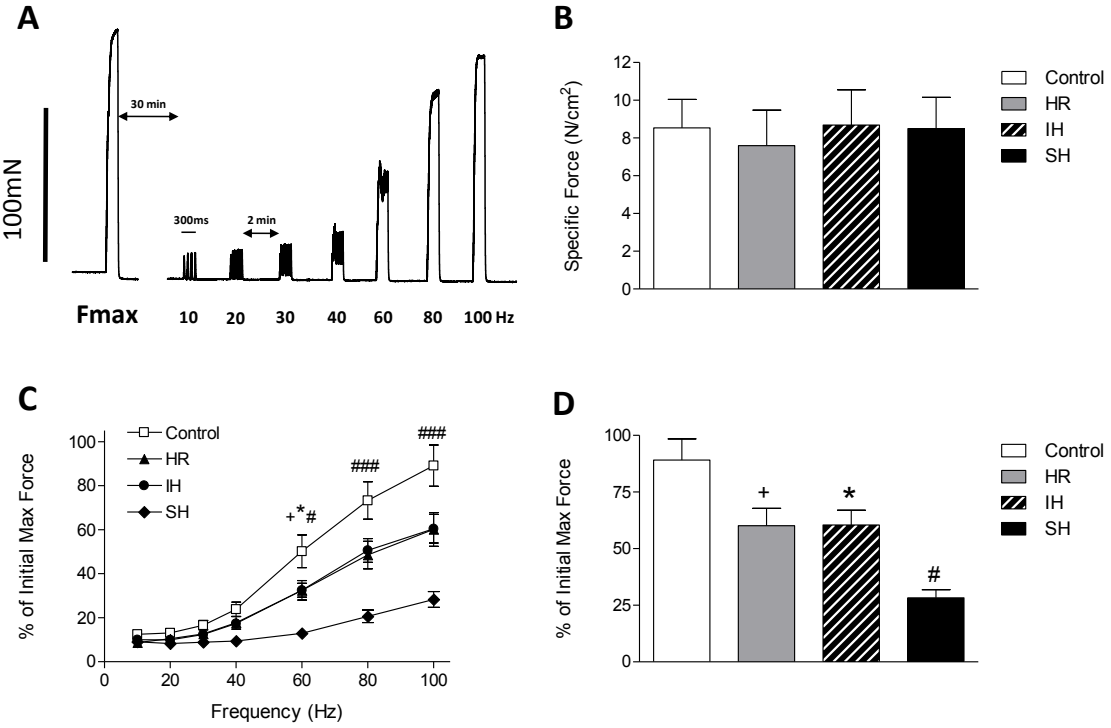
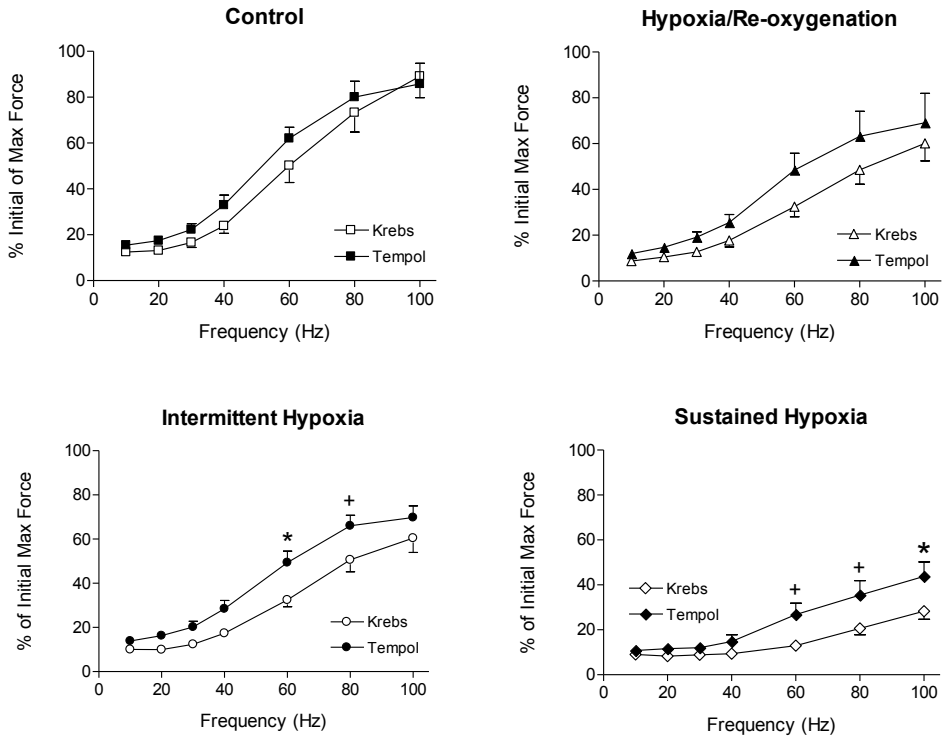
Figures and Legends:

Fig. 1.



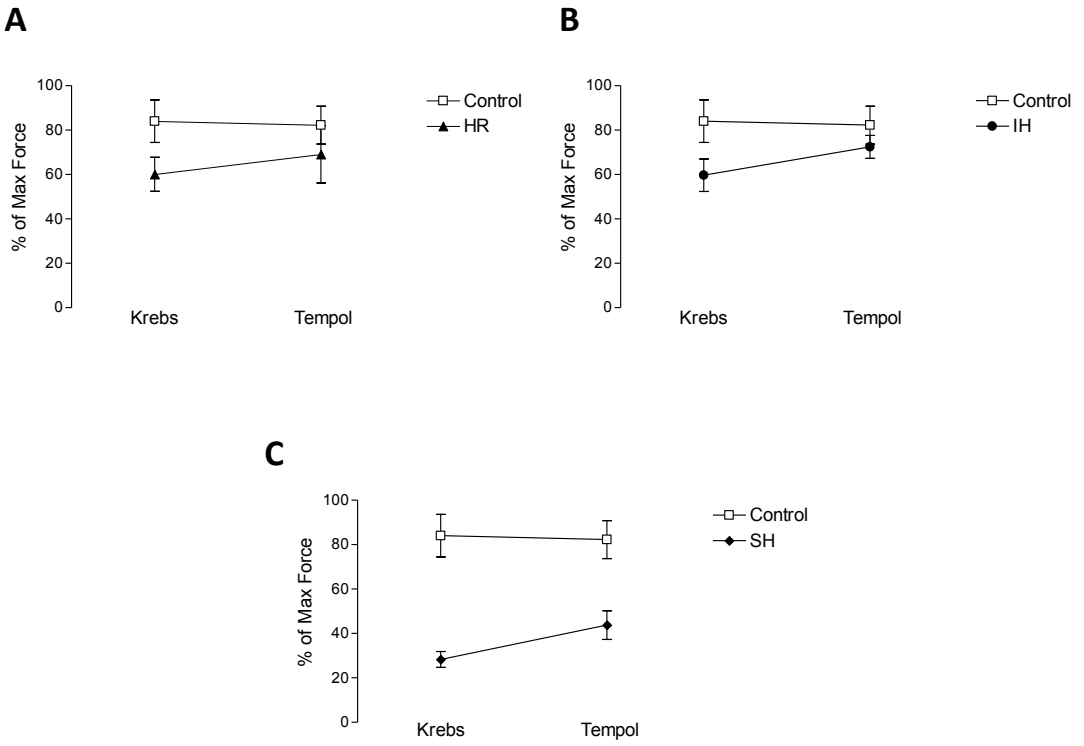
(A) Original traces of peak isometric force (Fmax) and force-frequency protocol (incremental stimulus frequencies of 10-20Hz up to 100Hz) in drug-free Krebs under control (hyperoxic) gas conditions. **(B)** Values (mean ± S.E.M) for Fmax in all four gas conditions. **(C)** Values for force-frequency relationship in all groups normalized to initial Fmax. Data show that hypoxia/re-oxygenation (HR), intermittent hypoxia (IH) and sustained hypoxia (SH) caused a significant decrease in isometric force compared to control muscles [HR, IH, and SH at100Hz (#), $p<0.001$; HR, IH, and SH at 80Hz (#), $p<0.001$; SH at 60Hz (#), $p<0.001$; IH at 60Hz (*), $p<0.01$; HR at 60Hz (+), $p<0.05$; one-way ANOVA]. **(D)** Values (mean ± S.E.M) for peak force in each gas group normalized to initial Fmax. Note the significantly lower force in HR, IH and SH compared to control muscles (HR (+), $p<0.05$; IH (*), $p<0.01$; SH (#), $p<0.001$; one-way ANOVA).

Fig. 2.



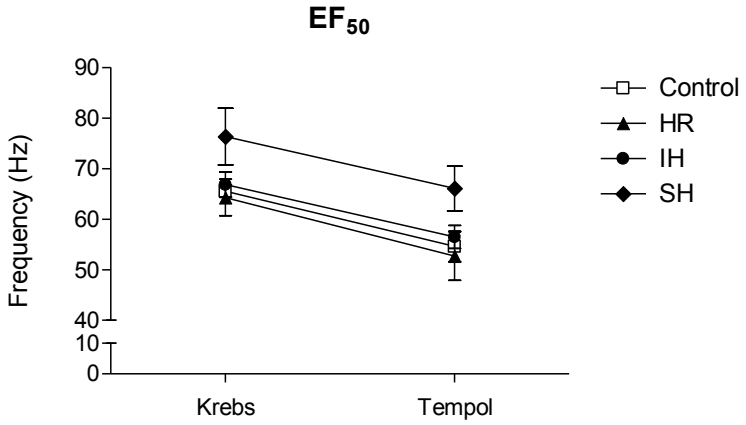
Values (mean ± S.E.M) for force-frequency relationship normalized to Fmax in all four gas states ± Tempol. Tempol increased force in Control ($P=0.0715$), HR ($P=0.0068$), IH ($P<0.0001$) and SH ($P<0.0001$); two-way ANOVA. Post-hoc analysis revealed: IH at 60Hz (*), $p<0.01$ and 80Hz (+), $p<0.05$; SH at 60Hz (+), $p<0.05$; 80Hz (+), $p<0.05$ and 100Hz (*), $p<0.01$.

Fig. 3.



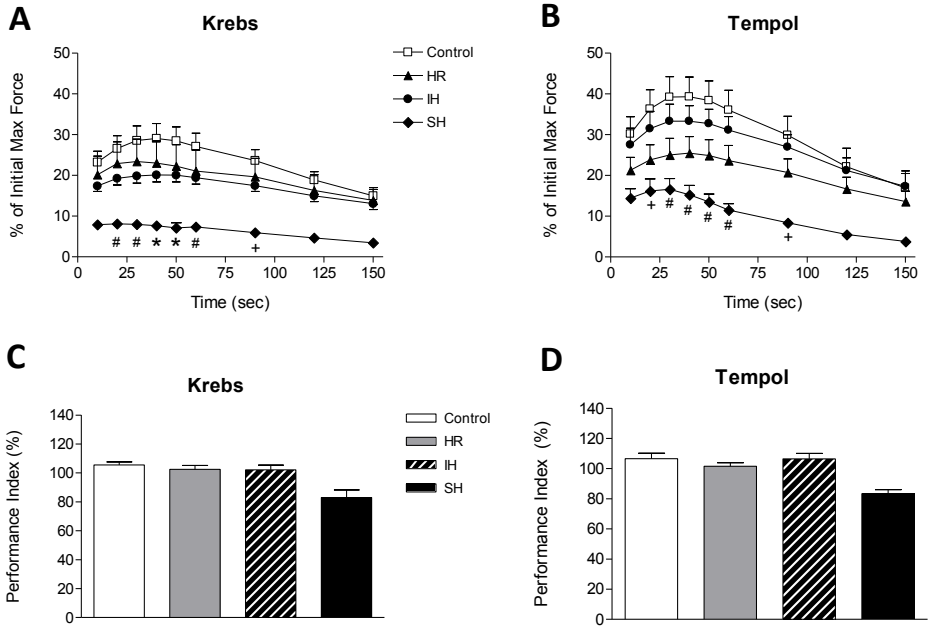
Values (mean ± S.E.M) for peak isometric force normalized to Fmax in muscle bundles incubated in Krebs ± Tempol following hypoxia-reoxygenation (HR), intermittent hypoxia (IH), and sustained hypoxia (SH). **(A)** Two-way ANOVA revealed that there was no significant effect of gas treatment ($p=0.0711$) or Tempol ($p=0.7172$). The interaction term (HR x Tempol) was also not significant ($p=0.5901$). **(B)** There was a significant effect of gas treatment ($p=0.0226$) but not Tempol ($p=0.6195$). The interaction term (IH x Tempol) was not significant ($p=0.4666$). **(C)** There was a significant effect of gas treatment ($p<0.0001$) but not Tempol ($p=0.3640$). The interaction term (SH x Tempol) was not significant ($p=0.2555$).

Fig. 4.



Values for EF_{50} for all four gas conditions ± Tempol. Tempol significantly decreased EF_{50} independent of gas treatment (p=0.0002, two-way ANOVA).

Fig. 5.



Values (mean ± S.E.M) for sternohyoid muscle force (normalized to Fmax) during repeated stimulation in all four gas conditions in Krebs (A) and Tempol-treated (B) muscle bundles. Gas treatments significantly decreased force during repeated muscle stimulation (HR: P=0.0326; IH: P<0.0001; SH: P<0.0001, two-way ANOVA). Post-hoc analysis for A: SH at 20s (#), p<0.01; at 30s (#), p<0.01; at 40s (*), p<0.001; at 50s (*), p<0.001; at 60s (#), p<0.01, and at 90s (+), p<0.05. Post-hoc analysis for B: SH at 20s (+), p<0.05; at 30s (#), p<0.01; at 40s (#), p<0.01; at 50s (#), p<0.01; at 60s (#), p<0.01, and at 90s (+), p<0.05. Performance index was significantly decreased by SH (p<0.0001, two-way ANOVA) (5C and 5D). There was no significant effect of Tempol and no significant interaction (SH x Tempol).