CHRONIC HYPOBARIC HYPOXIA INCREASES ISOLATED RAT FAST-TWITCH AND SLOW-TWITCH LIMB MUSCLE FORCE AND FATIGUE

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Short title: Chronic hypoxia and limb muscle function

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Summary

Chronic hypoxia alters respiratory muscle force and fatigue, effects that could be attributed to hypoxia and/or increased activation due to hyperventilation. We hypothesized that chronic hypoxia is associated with phenotypic change in non-respiratory muscles and therefore we tested the hypothesis that chronic hypobaric hypoxia increases limb muscle force and fatigue. Adult male Wistar rats were exposed to normoxia or hypobaric hypoxia (P_B = 450mmHg) for 6 weeks. At the end of the treatment period, soleus (SOL) and extensor digitorum longus (EDL) muscles were removed under pentobarbitone anaesthesia and strips were mounted for isometric force determination in Krebs solution in standard water-jacketed organ baths at 25^{o}C. Isometric twitch and tetanic force, contractile kinetics, force-frequency relationship and fatigue characteristics were determined in response to electrical field stimulation. Chronic hypoxia increased specific force in SOL and EDL compared to age-matched normoxic controls. Furthermore, chronic hypoxia decreased endurance in both limb muscles. We conclude that hypoxia elicits functional plasticity in limb muscles perhaps due to oxidative stress. Our results may have implications for respiratory disorders that are characterized by prolonged hypoxia such as COPD.

Key words: Hypoxia, Skeletal muscle, Fatigue.

Introduction

Chronic hypoxia occurs in humans in a variety of circumstances, including respiratory disease and exposure to altitude. The effects of chronic continuous hypoxia on skeletal muscle structure have been well investigated. In rats, limb muscles generally show a transition from slow to fast phenotype (Sillau and Branchero, 1977; Itoh *et al.* 1990; Bigard *et al.* 1991; Ishihara *et al.* 1995; Mortola and Naso, 1995; Faucher *et al.* 2005), although Shiota *et al.* (2004) observed the opposite shift and no change has been observed in some muscles (Bigard *et al.* 1991; Ishihara *et al.* 1995; Shiota *et al*. 2004). Chronic hypoxia has also been shown to cause angiogenesis in skeletal muscle (Smith and Marshall 1999). In humans, limb muscle fibre diameter is reduced but fibre type is unaffected by altitude exposure (Green *et al.* 1989; MacDougall *et al*. 1991) and recently Edwards et al. (2009) reported atrophy in skeletal muscle with maintained function. There is good evidence that chronic altitude exposure leads to a decrease in mitochondrial function and aerobic metabolism (Green *et al.* 1989; Hoppeler *et al.* 2003; Murray 2009). Patients with chronic obstructive pulmonary disease (COPD) have reduced aerobic metabolism in limb muscles (Gertz *et al.* 1977) and they show evidence of fibre atrophy and a higher proportion of fast fibres (Hildebrand *et al.* 1991; Whittom *et al*. 1998; Debigare *et al*. 2003), which could be influenced by a number of factors including hypokinesis and chronic hypoxia (Man *et al.* 2009).

Functional studies in humans exposed to altitude, examining muscle contraction and endurance have been inconsistent in their findings (Caquelard *et al.* 2000; Fulco *et al.* 1994; Garner *et al.* 1990; Kayser *et al.* 1994) but there is evidence of increased muscle

fatigability after altitude exposure (Caquelard *et al.* 2000) and in COPD (Zattara-Hartmann *et al.* 1995). We previously reported that chronic hypobaric hypoxia alters isolated rat respiratory muscle force and fatigue (El-Khoury *et al.* 2003). We have also demonstrated that chronic intermittent hypoxia – modelling human sleep apnoea – alters respiratory and limb muscle endurance (McGuire *et al.* 2002a,b; McGuire *et al.* 2003; Bradford *et al.* 2005; Dunleavy *et al.* 2008). This suggests a generalized effect of hypoxia on skeletal muscle. Because of the increased activity in the respiratory muscles of rats exposed to chronic continuous and chronic intermittent hypoxia – due to hyperventilation – a 'training' effect i.e. activity-dependent plasticity may at least in part explain functional changes in active muscles exposed to chronic hypoxia. We speculated that 6 weeks of chronic hypobaric hypoxia, sufficient to cause functional plasticity in respiratory muscle (El-Khoury *et al.* 2003), would also elicit widespread effects on skeletal muscle function. Therefore, the present study sought to determine the effects of chronic hypobaric hypoxia on rat slow-twitch (soleus, SOL) and fast-twitch (extensor digitorum longus, EDL) muscle contractile and endurance properties. We hypothesized that chronic hypoxia increases limb muscle fatigue.

Methods

Animal care

All procedures were performed in accordance with National and European legislation under licence from the Irish Government Department of Health and Children with additional institutional approval from the Royal College of Surgeons in Ireland animal research ethics committee. Adult male Wistar rats (~10 weeks old) were randomly

assigned to control (N=12) or hypoxia (N=15) groups. The hypoxia group was placed in a hypobaric chamber at an ambient pressure of 450 mmHg (inspired PO_2 = 85 mmHg). Decompression and recompression were performed gradually over 1-2 hours. Recompression for cage cleaning and food and water replenishment occurred every 2-3 days. Age-matched control rats remained at sea-level pressure in the same room in parallel.

In vitro muscle preparation

After 6 weeks, animals were anaesthetized with an intra-peritoneal injection of sodium pentobarbitone (70mg/kg body weight). A midline cervical incision was made and a tracheal cannula inserted through which the animals could breathe spontaneously. Whole SOL and EDL muscles with tendinous insertions intact were removed. Blood samples were taken for detemination of haematocrit. Animals were euthanized with an overdose of anaesthetic. After removal, the muscles were placed in a bath at room temperature containing continuously gassed (95% O_2/5% CO_2) Krebs solution. The solution contained in mM: NaCl 120, KCl 5, Ca^{2+} gluconate 2.5, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.5. Strips of muscle were prepared and then suspended vertically in Krebs solution in water-jacketed organ baths at 25^{o}C (pH 7.4). The physiological stability of rat skeletal muscle *in vitro* is temperature-dependent and stability for muscle strips of 1-2mm diameter is better at 25^{o}C compared to the *in vivo* temperature of 37^{o}C (Segal and Faulkner 1985). The strips were suspended between a pair of platinum electrodes, with the base fixed to an immobile hook and the other end tied to an isometric force transducer. The position of the force transducer could be adjusted by a micro-positioner,

thus altering preload.

Protocol

After an equilibration period of 30 min, the optimal length (*i.e.* muscle length producing maximal isometric twitch force) was determined. The muscle was held at this length for the remainder of the experiment. The single isometric twitch force, contraction time, half-relaxation time, force-frequency relationship and fatigue characteristics of the muscles were determined in response to electrical field stimulation and were recorded using a commercial data acquisition system and stored for later analysis on a computer. First, a single twitch was elicited (supra-maximal voltage, 1 ms duration). Twitch force, contraction time (time to peak force) and half-relaxation time (time for peak force to decay by 50%) were determined. Next, force-frequency relationship was determined by sequentially stimulating the muscle strips at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 Hz for 300msec at each stimulus frequency allowing a 2 min recovery interval between each stimulus. Ten min following this force-frequency protocol, fatigue was induced by stimulation at 40Hz with 300msec trains at 0.5 Hz for 5 min. Force was measured at 1 min intervals during fatigue.

Data Analysis

Specific force was calculated in N/cm^2 of muscle cross-sectional area. The latter was approximated by weighing the muscle strip 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 weights. The contraction time

and half-relaxation time were measured as indices of isometric twitch kinetics. For the force-frequency relationship, the values were normalized by expressing force at each of the different stimulus frequencies as a percentage of the maximum tetanic force developed during each trial. For the fatigue protocol, values were normalized by expressing the force generated at each 1 min time point, as a percentage of the initial force at the beginning of the fatigue trial. Absolute and normalized values are expressed as mean±SEM. Statistical comparisons between control and hypoxia groups were performed using Student's *t* test or one-way ANOVA and Fischer's least significant difference test as appropriate with $P<0.05$ taken as significant in all tests.

Results

General

The body weight of the hypoxic rats was significantly lower than control animals after 6 weeks (308±8g vs. 275±5g, control vs. hypoxia, $P<0.05$, Student's *t* test). Furthermore, the haematocrit was significantly higher in the hypoxia group (51±2% vs. 64±2%, mean±SEM, control vs. hypoxia, $P<0.05$, Student's *t* test).

Soleus

Chronic hypobaric hypoxia caused a significant increase in twitch and peak tetanic force (Table 1). Contraction time was unaffected by hypoxia, but half-relaxation time was significantly prolonged (Table 1). Chronic hypoxia had no effect on the force-frequency relationship (Fig. 1A) but decreased SOL muscle endurance (Fig. 1B). Fatigue was significantly decreased after 4 and 5 min of the repeated muscle stimulation trial; one can see from Fig. 1B that the fatigue curves for normoxic and chronic hypoxic muscles diverge considerably after 4 min of stimulation. The magnitude of the decline in fatigue tolerance in hypoxic muscles would presumably have continued to increase with extended time.

Extensor Digitorum Longus

Chronic hypoxia caused a significant increase in twitch force with no effect on contractile kinetics (Table 1). Peak tetanic force was increased but this did not achieve statistical significance (Table 1). Chronic hypoxia caused a significant left-shift in the force-frequency relationship (Fig. 2A), which was different to the response seen in SOL (Fig.

1A). Chronic hypoxia significantly decreased EDL endurance (Fig. 2B). This was significant after 3 min of the fatigue trial. Unlike SOL, the increased fatigue in hypoxic muscles appeared to have reached a plateau within the 5 min time trial.

Discussion

The main finding of this study is that chronic hypobaric hypoxia increases specific force and fatigue in rat slow-twitch and fast-twitch limb muscles; however differences in the effects of hypoxia on SOL and EDL were noted. Surprisingly little is known about the effects of chronic hypoxia on skeletal muscle contractile and endurance characteristics. In humans, chronic hypoxia has generally been reported to have little effect on force and fatigue (Garner *et al*. 1990; Fulco *et al*. 1994; Kayser *et al*. 1994), although forearm force and endurance were reduced after 32 days of high altitude exposure (Caquelard *et al*. 2000). Regarding the chronic hypoxia of respiratory disease, chronic hypoxaemic patients are reported to have reduced respiratory and limb muscle force and endurance (Zattara-Hartmann *et al.* 1995; Polkey *et al*. 1996), but this may relate to other factors associated with chronic respiratory disease (*e.g.* hyperinflation/loading, fibre atrophy, inflammation, nutritional status, hypokinesis *etc*) rather than hypoxia *per se* (Man *et al.* 2009). There have been relatively few studies of the effects of chronic hypoxia on isolated skeletal muscle function (Itoh *et al*. 1990; Shiota *et al*. 2004; Faucher *et al*. 2005). Using isolated *in vitro* muscle preparations allows the accurate determination of muscle functional characteristics independent of a number of potentially confounding variables *in vivo* such as: oxygen and nutrient supply, neuromuscular excitability, and muscle cross-sectional area, temperature and initial fibre length. We acknowledge too however that there are significant limitations associated with isolated muscle preparations. Prolonged incubation of muscle bundles in Krebs may have led to tissue swelling; we did not measure tissue mass at the start of the protocol for comparison to mass at the end of the study so we cannot rule out this possibility. Moreover, we assume that functional changes *in vitro* are

reflective of muscle performance *in vivo* but this may not necessarily be the case. Itoh *et al.* (1990) reported that chronic hypobaric hypoxia (10 weeks at a simulated altitude of 4,000m) in rats resulted in a reduction in force and fatigue in the EDL muscle but SOL muscle was unaffected. Faucher *et al*. (2005) reported that chronic hypoxia (FiO_2 = 0.10 for 4 weeks) increased SOL muscle force but decreased EDL force and endurance whilst others reported that chronic hypobaric hypoxia (6 weeks at a simulated altitude of 5,000m) had no effect on limb muscle force or fatigue (Shiota *et al*. 2004). In single fibre studies, SOL muscle force was decreased an effect attributed to decreased myosin content (Degens *et al.* 2010). One plausible explanation for the different findings in these studies may be differences in the duration and intensity of chronic hypoxia as well as different methodological approaches. We have recently shown that respiratory muscle re-modelling during sustained hypoxia is time-dependent (McMorrow *et al.* 2011). Moreover, the effects of chronic hypoxia on respiratory muscle endurance are dependent on the severity of the hypoxic challenge and the intrinsic structural and metabolic properties of the muscles studied (El-Khoury *et al.* 2003; McMorrow *et al.* 2011).

In the present study, chronic hypoxia increased force and fatigue of both SOL and EDL muscles. It is possible that fibre-type transition towards glycolytic metabolism drives this functional change. Chronic hypoxia has been shown to cause an increase in fast fibres in the EDL and plantaris muscle (Bigard *et al*. 1991), in anterior tibialis and gastrocnemius (Sillau and Banchero 1977) and in SOL if hypoxic exposure occurred during development (Ishihara *et al*. 1995) but not during adult life (Sillau and Banchero 1977; Bigard *et al*. 1991; Ishihara *et al*. 1995). Hypoxia-induced inhibition of growth-related

transitions in fibre phenotype in limb muscles has been proposed (Faucher *et al*. 2005). The increase in force observed in the present experiments would be consistent with a transition to fast fibres since fast fibres generate more force than slow fibres. The increase in muscle fatigue would also be consistent with a transition to fast fibres since the latter have low fatigue resistance. However, in a recent study (McMorrow *et al.* 2011) we showed that chronic hypobaric hypoxia does not alter fibre distribution or oxidative capacity in rat limb muscle. At the molecular level, one might speculate that myostatin is implicated in hypoxic adaptation in skeletal muscle (Hayot *et al.* 2011). We suggest that redox modulation of muscle function underlies the functional changes reported herein. Reactive oxygen species (ROS) are important signalling molecules in muscle but oxidative stress is implicated in muscle fatigue. We have shown that respiratory muscle dysfunction in a rat model of chronic intermittent hypoxia is prevented by antioxidant treatment (Skelly *et al.* 2011). It would be interesting to explore the effects of antioxidant supplementation on skeletal muscle function in sustained hypoxia.

The observed changes in muscle function in both slow-twitch and fast-twitch limb muscles suggest a widespread effect of chronic hypoxia on skeletal muscle. Recently, a study of the effect of acute hypoxia on fatigue development in rat limb muscles studied *in vivo* clearly demonstrated that hypoxia induces greater fatigue in hindlimb muscle composed primarily of fibres with low oxidative capacity compared with that of a muscle with a more oxidative phenotype (Howlett and Hogan 2007). Concomitant with this apparent increased hypoxic sensitivity in fast muscle were greater increases in cellular metabolites such as lactate, hydrogen ions, inorganic phosphate, and free ADP and AMP

(Howlett and Hogan 2007), all of which are known to correlate with skeletal muscle fatigue (Allen *et al*. 1995; Westerblad and Allen 2003). However, *in vivo*, differential effects of hypoxia on muscle function may also be related to differences in oxygen delivery and uptake between muscle groups. The results of our study suggest that at the level of the muscle fibre, chronic hypoxia causes phenotypic plasticity that is qualitatively similar in limb muscles of different structural make-up, though it is interesting to note that the magnitude of the hypoxia-induced increase in muscle fatigue was greater in the fast EDL muscle. We conclude that chronic systemic hypoxaemia has widespread effects on skeletal muscle. Our results have relevance to respiratory disorders that are characterized by prolonged hypoxia such as COPD where limb muscle fatigue is known to occur.

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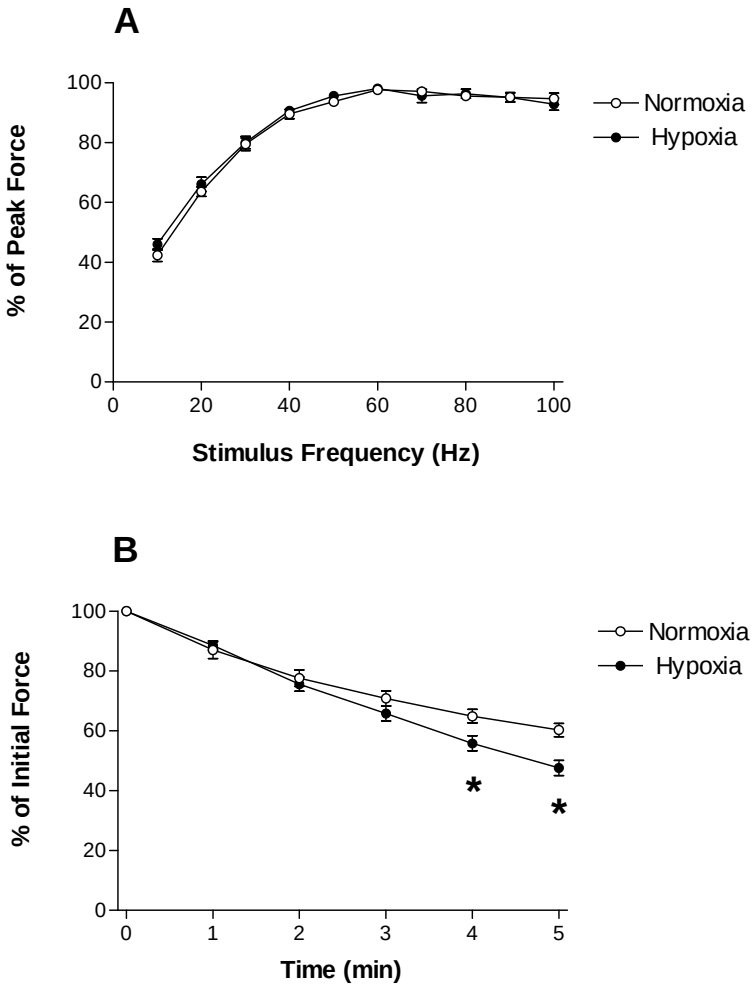

Figure 1.

Force-frequency relationship (A) and fatigue characteristics (B) for soleus muscle in normoxic (N=12) and chronically hypoxic (N=14) rats. Values are mean±SEM. In A, values are expressed at each stimulus frequency as a percentage of the peak tetanic force developed during the trial. In B, values are expressed at each time point as a percentage of the initial force at the beginning of the fatigue trial (time 0). * indicates a significant difference from normoxia (control); $P<0.05$, ANOVA.

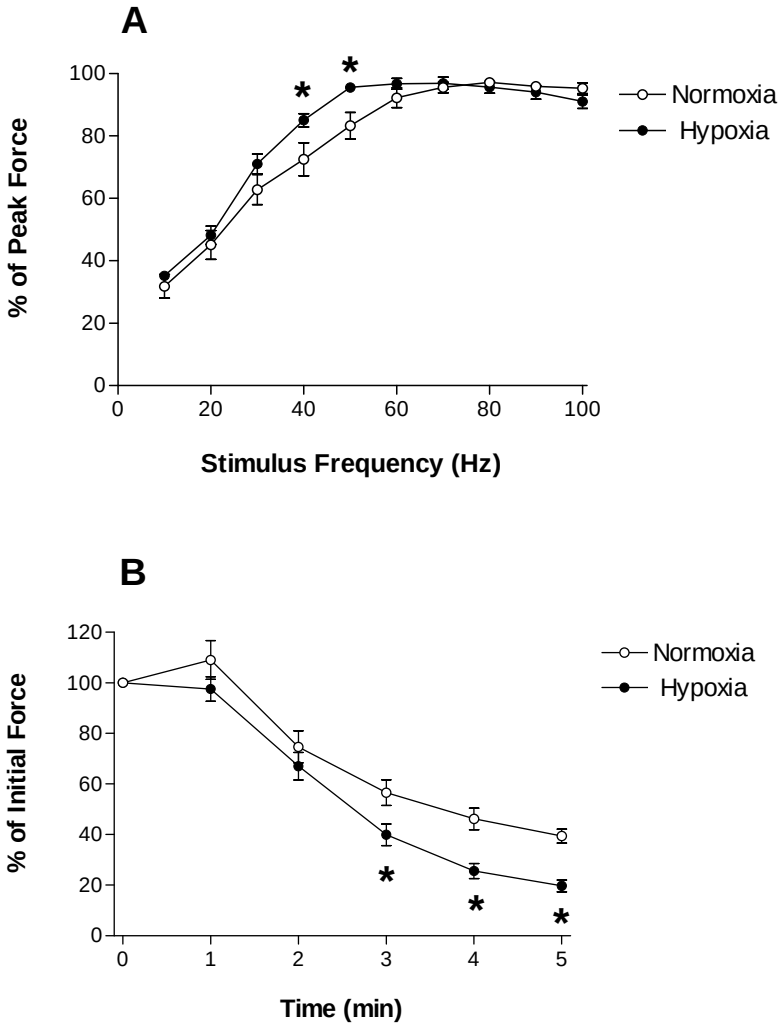

Figure 2.

Force-frequency relationship (A) and fatigue characteristics (B) for extensor digitorum longus muscle in normoxic (N=10) and chronically hypoxic (N=15) rats. Values are mean±SEM. In A, values are expressed at each stimulus frequency as a percentage of the peak tetanic force developed during the trial. In B, values are expressed at each time point as a percentage of the initial force at the beginning of the fatigue trial (time 0).

* indicates a significant difference from normoxia (control); $P<0.05$, ANOVA.

Table 1.

Contractile properties of soleus (SOL) and extensor digitorum longus (EDL) muscles from normoxic and chronically hypoxic rats

	Normoxia	Hypoxia
SOL	(N=12)	(N=14)
Twitch Force (N/cm^2)	2.0±0.2	2.8±0.2*
Contraction time (ms)	72±3	70±3
Half-relaxation time (ms)	70±4	97±4*
Tetanic Force (N/cm^2)	9.0±12.1	12.1±0.9*
Twitch/Tetanus Ratio	0.23±0.03	0.24±0.01
EDL	(N=10)	(N=15)
Twitch Force (N/cm^2)	2.5±0.3	3.8±0.4*
Contraction time (ms)	32±2	33±2
Half-relaxation time (ms)	22±6	25±2
Tetanic Force (N/cm^2)	8.5±1.8	10.9±1.1
Twitch/Tetanus Ratio	0.35±0.04	0.35±0.01

Values are mean ± SEM. * indicates significant difference from corresponding value in normoxic control rats; $P<0.05$, Student's *t* test.