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ORIGINAL PAPER

Improved tolerance of acute severe hypoxic stress in chronic hypoxic diaphragm is nitric oxide-dependent

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Abstract The effects of chronic hypoxia (CH) on respiratory muscle performance have hardly been investigated, despite clinical relevance. Results from recent studies are indicative of unique adaptive strategies in hypoxic diaphragm. Respiratory muscle tolerance of acute severe hypoxic stress was examined in normoxic and CH diaphragm in the presence and absence of a nitric oxide (NO) synthase inhibitor. We tested the hypothesis that improved tolerance of severe hypoxic stress in CH diaphragm is NO-dependent. Wistar rats were exposed to normoxia (sea-level, $n = 6$) or CH (ambient pressure = 380 mmHg, $n = 6$) for 6 weeks. Diaphragm muscle functional properties were determined ex vivo under severe hypoxic conditions (gassed with 95 %N_2/5 % CO_2) with and without 1 mM L-N^G-nitroarginine (L-NNA, nNOS inhibitor). Fatigue tolerance, but not force, was significantly improved in CH diaphragm ($p = 0.008$). CH exposure did not affect diaphragm muscle fibre oxidative capacity determined from cluster analysis of area–density plots of muscle fibre succinate dehydrogenase activity. Acute NOS inhibition reduced diaphragm peak tetanic force ($p = 0.018$), irrespective of gas treatment, and completely reversed improved fatigue tolerance of the CH diaphragm. We conclude that CH exposure improves fatigue tolerance during acute severe hypoxic stress in an NO-dependent manner, independent of muscle fibre oxidative capacity.

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Introduction

Chronic hypoxia (CH) induces differential structural and functional adaptation in respiratory and limb muscles of animal models [1–7] (e.g. weakness and increased fatigue tolerance of the diaphragm but not of the soleus muscle [4, 6, 7], and reduced fibre cross-sectional areas in the diaphragm but no change in the soleus muscles [6]). Similar changes have been observed in the diaphragm muscle of patients with respiratory-related diseases characterised by CH such as chronic obstructive pulmonary disease (COPD). COPD patients have lower trans-diaphragmatic pressure-generating capacity and improved diaphragm muscle fatigue tolerance [8–11], or at least fatigue tolerance is not reduced even though the muscle is at a mechanical disadvantage [12]. Diaphragm fatigue tolerance is potentially important in the context of CH-induced hyperventilation but muscle weakness is associated with poor clinical outcome. The effects of CH on respiratory muscle performance have hardly been investigated, despite the clinical relevance, although results from recent studies are indicative of unique adaptive strategies in hypoxic diaphragm [4–6].

Nitric oxide (NO) is a potent modulator of skeletal muscle function and homeostasis [13–17]. Physiological levels of NO have an inhibitory effect on Ca^{2+}-release channels [17]. Moreover, an NO donor reduced myofibril Ca^{2+} sensitivity [13], and an NO inhibitor reduced markers of tissue damage after eccentric contractions [16]. Also, NO can modulate PGC-1α or Akt signalling to affect metabolic components and muscle growth, respectively

[18, 19]. Neuronal NO synthase (nNOS) and endothelial (e)NOS expression, but not inducible-(i)NOS expression, undergo significant alterations in rat diaphragm after CH exposure from birth [20]. NOS activity is potentially linked to changes in oxygen tension [21], and we have previously reported that chronic NOS blockade reverses CH-induced increase in diaphragm Na^+–K^+ ATPase pump content and prevents CH-induced functional remodelling in rat diaphragm [4].

We sought to examine the effects of CH exposure on respiratory muscle tolerance of acute severe hypoxic stress and to determine if NO is implicated in altered respiratory muscle tolerance of severe hypoxia after CH. We tested the hypothesis that improved tolerance of severe hypoxic stress in CH diaphragm is NO-dependent.

Materials and methods

Animal model

All procedures described in this study were approved by the local ethics committee and were performed under licence from the Irish Government Department of Health and Children. The animals were an unreported subset from a previously published study [4]. Age and weight-matched adult male Wistar rats (Harlan, UK) were exposed to normoxia (sea-level, $n = 6$) or CH (ambient pressure = 380 mmHg, $n = 6$) for 6 weeks in purpose-built hypobaric chambers in which ambient pressure was regulated. Decompression to a target pressure of 380 mmHg was achieved over a 2–3 h period. Ambient PO_2 was ~80 mmHg, equivalent to an F_IO_2 of 10.5 %. Food and water were available ad libitum. Chamber pressure was measured continuously by use of a digital manometer (model C9505; Comark, UK). Pressure fluctuations as a result of drift were ±2 % of the target pressure. Ambient CO_2 was measured periodically and was <1 %. After the treatment periods, animals were euthanised by cervical dislocation. Blood was collected in microcapillary tubes, in triplicate, for determination of haematocrit as a marker of CH exposure.

Respiratory muscle function

The diaphragm muscle was excised with the lower rib and central tendon intact and placed in gassed Krebs solution (NaCl 120 mM, KCl 5 mM, Ca^{2+} gluconate 2.5 mM, $MgSO_4$ 1.2 mM, NaH_2PO_4 1.2 mM, $NaHCO_3$ 25 mM, and glucose 11.5 mM; pH 7.4) at room temperature before transfer to a tissue bath for functional assessment. Longitudinal strips of muscle were mounted vertically in custom tissue baths of Krebs solution at 30 °C, gassed with 95 %N_2/5 % CO_2 (ambient PO_2 ~40 mmHg), and containing the neuromuscular blocking agent D-tubocurarine (25 μM). The muscle strips were positioned between a pair of platinum electrodes, with the rib fixed to an immobile hook and the tendon tied to an isometric force transducer with non-elastic string. The position of the force transducer was adjusted by use of a micro-positioner, thus altering the length of the muscle strips. Diaphragm bundles were set to the optimum length (L_o—the length at which peak twitch force occurs) by incrementally adjusting the position of the force transducer and sequentially stimulating with a single pulse until peak twitch force was reached. The muscle remained at L_o for the duration of the experiment. The single isometric twitch force, contraction time, half-relaxation time, force–frequency relationship, and fatigue were then determined in response to electrical field stimulation. First, a single twitch was elicited (supramaximum voltage, 1 ms duration). Twitch force, time to peak force, and half-relaxation time (time for peak force to decay by 50 %) were determined. Next, the force–frequency relationship was determined by sequentially stimulating the muscle strips at 10, 20, 30, 40, 60, 80, and 100 Hz for 300 ms at each stimulus frequency, allowing a 2-min recovery interval between each stimulus. Five minutes after the force–frequency procedure, repeated muscle contraction was induced by stimulation at 40 Hz with 300-ms trains every 2 s for a period of 5 min. Acute pharmacological blockade of NO synthase (NOS) was used (1 mM L-N^G-nitroarginine, L-NNA) to determine whether or not NO is implicated in diaphragm muscle adaptation to CH. Thus there were four groups: normoxia, CH, normoxia + L-NNA, and CH + L-NNA, all studied under acute severe hypoxic conditions.

Succinate dehydrogenase activity

After chronic gas treatment, diaphragm muscles were quickly excised and snap frozen. Transverse 10 μm cryosections were obtained and histochemically stained to determine succinate dehydrogenase (SDH) activity as described elsewhere [4]. Briefly, slides were stained with sodium succinate and nitro blue tetrazolium chloride in phosphate buffer (pH 7.4), dehydrated in a graded series of rinses with acetone and methanol, and imaged by use of a BX51 Olympus microscope (Olympus Life Science Microscopes, Munchen, Germany) and an Olympus DP71 camera. Control and CH samples were processed in parallel. Optical densities of individual muscle fibres and fibre cross-sectional area (CSA) were calculated by use of Scion Image™ software (Maryland, USA). Area–density plots were constructed for the normoxic and CH groups.

Data Analysis

Peak specific force (F_{max}) was calculated in N/cm^2 of muscle CSA calculated as the blotted dry muscle bundle weight divided by the product of L_o and the specific density, assumed to be 1.056 g/cm^3. Fatigue index, force expressed as a percentage of initial force, was calculated after 5 min of repeated isometric contractions.

K-means cluster analysis was performed on SDH area–density plots of muscles from normoxic and CH animals. *K*-means clustering partitions *x* observations into *k* clusters by iterative fine-tuning until all observations are grouped into the cluster of the nearest mean. Three means/clusters ($k = 3$) were chosen, representing small, medium, and large fibre CSAs. Cluster centroids of normoxic and CH diaphragm muscles were determined.

Statistical analysis

All values are expressed as mean ± SEM. After testing for normality and equal variance in the data sets, statistical comparisons were performed between groups by use of Student *t* tests, one-way ANOVA (hypoxia), or two-way ANOVA (hypoxia × drug), with Bonferroni post-hoc tests as appropriate, by use of Graph-Pad Prism (USA). $P < 0.05$ was the criterion for statistical significance in all tests.

Results

Body mass and haematocrit

CH exposure significantly reduced body mass gain compared with age-matched normoxic controls. CH significantly increased haematocrit (43 ± 1 vs. 75 ± 2 %, normoxic vs. CH, $n = 6$ both groups; Student unpaired *t* test, $P < 0.001$).

Effects of CH on muscle physiology during acute severe hypoxia

The effects of CH on diaphragm twitch force, time to peak force, half-relaxation time, and peak tetanic force are shown in Table 1. CH had no significant effect on the force–frequency relationship of the diaphragm. However, CH significantly increased fatigue tolerance during acute severe hypoxia (Figs. 1, 2).

Table 1 Force and contractile kinetics of normoxic and chronic hypoxic diaphragm during severe acute hypoxic stress ± NOS blockade

	Pt (N/cm^2)	*CT* (ms)	*HRT* (ms)	*Po* (N/cm^2)
Control (drug-free)				
Normoxia	3.0 ± 0.4	27 ± 2	25 ± 2	10.0 ± 1.8
CH	3.6 ± 0.4	23 ± 1	25 ± 2	12.4 ± 1.8
L-NNA				
Normoxia	3.7 ± 0.5	24 ± 1	21 ± 1	7.3 ± 2.3*
CH	3.1 ± 0.5	20 ± 1	20 ± 1	7.9 ± 1.9*

Values are mean ± SEM

Pt single twitch tension, *CT* contraction time, *HRT* half-relaxation time, *Po* peak tetanic tension, *CH* 6 weeks of hypoxia

* Two-way ANOVA; drug: $p = 0.018$

Effects of CH on diaphragm muscle fibre SDH activity

A representative area–density plot for diaphragm SDH-stained fibres is shown in Fig. 3a. CH did not affect diaphragm muscle fibre oxidative capacity determined by cluster analysis of area–density plots (Fig. 3b).

Effects of nNOS inhibition on diaphragm function during acute severe hypoxia

Acute NOS inhibition with 1 mM L-NNA reduced diaphragm muscle force irrespective of chronic gas treatment (Table 1). L-NNA did not affect fatigue tolerance of normoxic diaphragm. Conversely, NOS blockade significantly reduced the CH diaphragm fatigue index, reversing the increased tolerance of severe hypoxia in CH diaphragm (Fig. 1).

Discussion

The main findings of this study are:

1 CH exposure improves diaphragm muscle fatigue tolerance during acute severe hypoxic stress (independent from muscle fibre oxidative capacity);
2 NO facilitates diaphragm force during severe hypoxia (irrespective of gas treatment); and
3 CH-induced increase in diaphragm fatigue tolerance during severe hypoxia is NO-dependent.

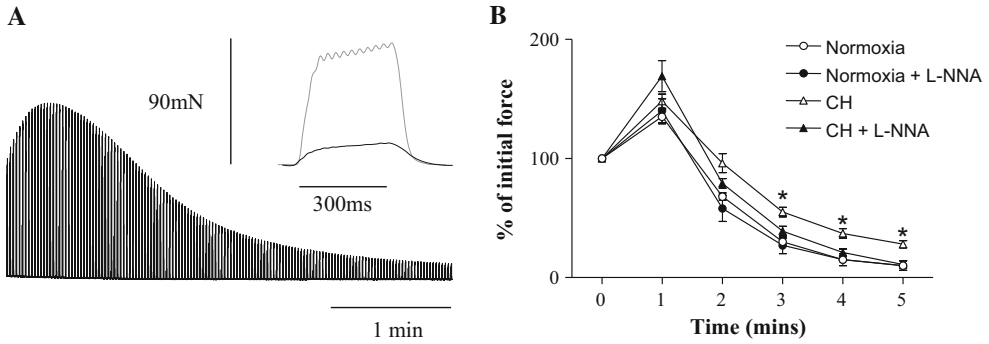

Fig. 1 **a** Original representative trace of diaphragm fatigue obtained during repeated muscle stimulation (40 Hz every 2 s for 5 min) during severe hypoxic stress. *Inset* shows the first and last muscle contraction superimposed to illustrate the altered amplitude and kinetics characteristic of muscle fatigue. **b** Group data illustrating force potentiation and fatigue in each group over the 5 min of repeated muscle stimulation. Values are mean ± SEM ($n = 6$ per group). *$p < 0.05$ one-way ANOVA followed by Newman–Keuls post-hoc test. *CH* chronic hypoxia, *L-NNA* N^G-nitro-L-arginine

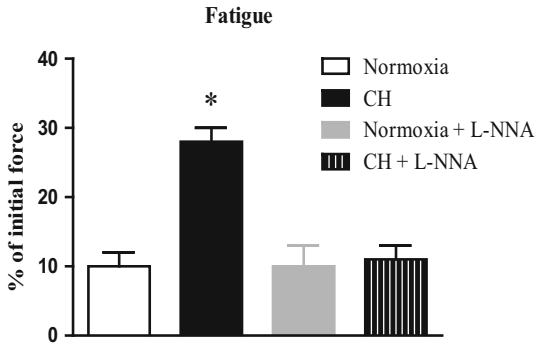

Fig. 2 Data for diaphragm muscle fatigue index. Values are mean ± SEM ($n = 6$ per group). Two-way ANOVA revealed a significant effect of CH ($p = 0.008$) and drug ($p = 0.027$) treatment. *Asterisk* indicates significant difference from normoxia; $p < 0.05$. *CH* chronic hypoxia, *L-NNA* N^G-nitro-L-arginine

There is a general paucity of data concerning the effects of CH on respiratory muscle function, notwithstanding the potential clinical value of such studies. This study extends our previous report [4] illustrating that CH exposure serves to pre-condition the diaphragm muscle resulting in substantial improvement in fatigue tolerance during acute severe hypoxic stress exposure ex vivo. The result is analogous with ischaemic pre-conditioning of the heart [22] and other tissues [23, 24] and may involve similar mechanisms including modulation of NO signalling and/or protein sensitivity to NO, and redox regulation of proteins [25–29]. NO is an important modulator of skeletal muscle function [13] including rat diaphragm [4, 30]. Acute blockade of nNOS reduced diaphragm force-generating capacity, revealing that NO facilitates diaphragm force

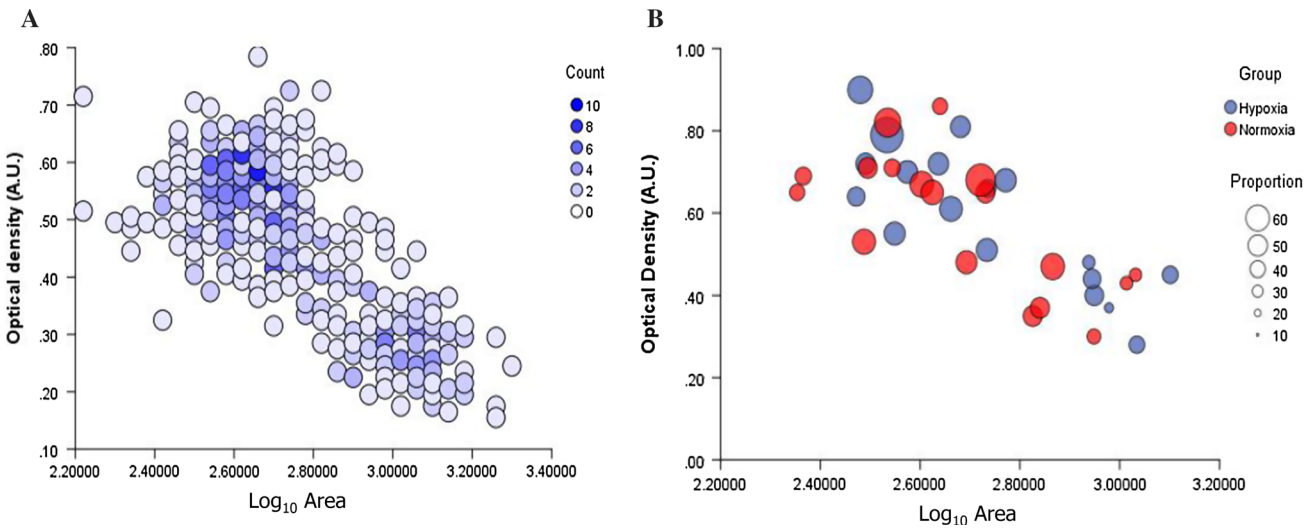

Fig. 3 **a** A representative area–density plot for diaphragm fibres histochemically stained for the oxidative enzyme succinate dehydrogenase. **b** Results from cluster analysis for normoxic and CH diaphragm ($n = 6$ per group)

under conditions of severe hypoxic stress. Several proteins of the excitation–contraction coupling mechanism in muscle (including the ryanodine receptors and SERCA pumps, actin and troponins of the sarcomere) are implicated given the evidence that they are targets for NO [13, 15, 17, 31–33]. Moreover, NO, at low concentrations in cells, is an antioxidant serving to scavenge superoxide anions [34–36] which may be increased under conditions of severe hypoxia [37–39]. Acute nNOS blockade presumably disrupts NO signalling in muscle and would seem to be especially detrimental to diaphragm function in severe hypoxia. Interestingly, this effect was equivalent for normoxic and CH diaphragms.

The experimental approach used in our study, employing acute pharmacological intervention after gas treatment, indicates that a dynamic, persistent, reversible, NO-dependent facilitatory process is underpinning improved CH diaphragm fatigue tolerance in severe hypoxia. The observation strongly implicates a direct NO-dependent process in diaphragm muscle fibres, whereas chronic NOS inhibition such as that used in our previous study [4], could relate to direct or indirect NO-dependent remodelling. The lack of effect of NOS blockade on normoxic diaphragm fatigue during severe hypoxia in this study illustrates a CH-specific effect. Our study suggests that it is either a change in the CH diaphragm's ability to produce NO (e.g. through altered NOS isoform expression/structural remodelling) [20, 40] or a change in the sensitivity of downstream targets of NO, including the contractile apparatus and mitochondria, that is required to induce the CH-dependent functional adaptation (e.g. structural remodelling of a target protein by means of carbonylation and/or thiol oxidation; direct or indirect alterations in mitochondrial respiration). Both nNOS and eNOS expression are down-regulated in rat diaphragm after CH exposure from birth [20] and no change in protein nitrosylation is observed after CH exposure of adult rat diaphragm muscle [7]. These findings are suggestive of reduced or unaltered diaphragm capability to produce NO after CH. Since we demonstrate that blockade of NO production acutely reverses the CH-induced improvement in diaphragm fatigue tolerance without effect on diaphragm preparations from normoxic animals, our findings suggest that altered sensitivity to NO in CH diaphragm (e.g. structural modification of an NO target protein) is required for CH-induced improved fatigue tolerance. Furthermore, as CH does not affect diaphragm muscle contractile kinetics or an index of diaphragm oxidative metabolism (i.e. SDH activity), the changes are potentially occurring at the level of the cross-bridge [41, 42]. Chronic heart failure in rats induces oxidation of diaphragm contractile apparatus proteins and disrupts actin–myosin interactions similar to direct exposure of the contractile apparatus proteins to peroxynitrite [43]. We postulate that redox remodelling of proteins key to muscle endurance, induced by CH, alters the sensitivity to NO. If hypoxia per se limits the production of NO in muscle because of reduced oxygen availability, further increases in NOS enzyme content to increase NO production in CH would serve to further deplete oxygen availability and be detrimental to metabolic processes. One potential physiological outcome would be an increase in the sensitivity to NO of downstream targets relevant to function.

Of interest, there are no differences in nNOS or iNOS expression between control and COPD patient diaphragm muscles, but eNOS expression is reduced in the COPD diaphragm [44]. Protein nitrotyrosine levels are also unaffected [44], but protein carbonylation and redox remodelling of proteins is observed in the COPD diaphragm [44, 45]. Potentially, a change in the redox status of the diaphragm after CH exposure elicits structural changes in proteins affecting intrinsic fatigue, a process that is NO sensitive.

A limitation of the study is that we did not assess NO concentrations in normoxic and CH diaphragm muscle before and after exposure to acute severe hypoxic stress. Acute severe hypoxia may have affected intracellular NO concentrations in isolated muscle. However, the finding that diaphragm force was significantly reduced after acute NOS blockade in severe hypoxia (equivalent in normoxic and CH diaphragm preparations) indicates that a substantial basal NO "tone" was maintained in the muscle under these conditions, and our findings strongly suggest that this was no different for normoxic and CH diaphragms. The NO concentration in muscle may have been reduced by acute severe hypoxic stress and as such we may have underestimated the magnitude of the effect of the NOS blocker, L-NNA.

Summary and conclusion

In summary, CH exposure causes diaphragm muscle remodelling in rats with resulting increased tolerance ex vivo of severe hypoxic stress, independent of changes in oxidative metabolism. CH-induced adaptation is NO-dependent, given that acute pharmacological blockade of NOS reverses improved fatigue tolerance in the CH diaphragm but has no effect on fatigue tolerance in the normoxic diaphragm. We conclude that CH induces NO-dependent functional plasticity in rat respiratory muscle. Our results may have relevance to human respiratory disorders characterised by CH, such as COPD.

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Conflict of interest None of the authors has any conflict of interest to report.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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