Eur Respir J 2011; 37: 1474–1481
DOI: 10.1183/09031936.00079810

Chronic hypoxia increases rat diaphragm muscle endurance and sodium–potassium ATPase pump content

C. McMorrow*, A. Fredsted#, J. Carberry*, R.A. O'Connell*, A. Bradford¶, J.F.X. Jones* and K.D. O'Halloran*

ABSTRACT: The effects of chronic hypoxia (CH) on respiratory muscle are poorly understood. The aim of the present study was to examine the effects of CH on respiratory muscle structure and function, and to determine whether nitric oxide is implicated in respiratory muscle adaptation to CH.

Male Wistar rats were exposed to CH for 1–6 weeks. Sternohyoid and diaphragm muscle contractile properties, muscle fibre type and size, the density of fibres expressing sarco/endoplasmic reticulum calcium-ATPase (SERCA) 2 and sodium–potassium ATPase (Na^+,K^+-ATPase) pump content were determined. Muscle succinate dehydrogenase (SDH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) dehydrogenase activities were also assessed. Acute and chronic blockade of nitric oxide synthase (NOS) was employed to determine whether or not NO is critically involved in functional remodelling in CH muscles.

CH improved diaphragm, but not sternohyoid, fatigue tolerance in a time-dependent fashion. This adaptation was not attributable to increased SDH or NADPH dehydrogenase activities. The areal density of muscle fibres and relative area of fibres expressing SERCA2 were unchanged. Na^+,K^+-ATPase pump content was significantly increased in CH diaphragm. Chronic NOS inhibition decreased diaphragm Na^+,K^+-ATPase pump content and prevented CH-induced increase in muscle endurance.

This study provides novel insight into the mechanisms involved in CH-induced muscle plasticity. The results may be of relevance to respiratory disorders characterised by CH, such as chronic obstructive pulmonary disease.

KEYWORDS: Chronic obstructive pulmonary disease, fatigue, myosin heavy chain isoforms, nitric oxide synthase, sarco/endoplasmic reticulum calcium-ATPase 2

Skeletal muscle has enormous capacity for remodelling, as evident in various physiological and pathophysiological settings. Chronic hypoxia (CH), a feature of respiratory disease, is known to affect skeletal muscle structure and function. Alterations include changes in capillarity [1, 2], fibre size and distribution [3–10], oxidative capacity [5, 6, 11, 12] and contractile performance [5, 9, 13–16]. CH induces reflex hyperventilation. Thus respiratory muscles are unique in that they must increase their workload in the face of a reduction in oxygen availability, necessary for aerobic metabolism. Respiratory muscle remodelling is a feature of chronic obstructive pulmonary disease (COPD) [17–27], which may be the result of hypoxic adaptation. Surprisingly, there is a general paucity of information concerning the effects of CH on respiratory muscle structure and function despite the clinical relevance. Translational animal models permit examination of the effects of CH on skeletal muscle independent of other confounding factors that are present in disease. Furthermore, they permit a thorough exploration of the molecular mechanisms that underpin muscle adaptation. As such, the major aim of the present study was to conduct a comprehensive assessment of respiratory muscle properties in an animal model of CH.

We sought to examine the effects of CH on rat respiratory pump and upper airway muscle contractile and endurance properties, fibre type and size, oxidative enzyme activity, relative area of

AFFILIATIONS
*University College Dublin School of Medicine and Medical Science, Health Sciences Centre, University College Dublin, and
¶Dept of Physiology and Medical Physics, Royal College of Surgeons in Ireland, Dublin, Ireland.
#Dept of Physiology and Biophysics, Aarhus University, Aarhus, Denmark.

CORRESPONDENCE
K.D. O'Halloran
University College Dublin School of Medicine and Medical Science,
C228 Health Sciences Centre
University College Dublin
Belfield
Dublin 4
Ireland
E-mail: ken.ohalloran@ucd.ie

Received:
May 21 2010
Accepted after revision:
Nov 01 2010
First published online:
Dec 09 2010

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

This article has supplementary material accessible from www.erj.ersjournals.com

fibres expressing sarco/endoplasmic reticulum calcium ATPase (SERCA) 2 and sodium–potassium ATPase (Na^+,K^+-ATPase) pump content. SERCAs are responsible for a significant proportion of energy consumption in skeletal muscle, second only to myosin ATPase. Muscle endurance correlates with SERCA function, especially that of the SERCA2 isoform [28], and a fast-to-slow SERCA transformation has been reported in COPD diaphragm [29]. The Na^+,K^+-ATPase pump plays a dynamic role in the maintenance of myocyte excitability during contractile activity [30, 31]. Na^+,K^+-ATPase pump function is extremely malleable and readily adapts to a variety of stimuli, including hormones, electrolytes, diet, contractile activity and hypoxia [32]. Skeletal muscles express all three isoforms of nitric oxide synthase (NOS), and nitric oxide is implicated in skeletal muscle adaptation in health and disease. Therefore, it was explored whether NO is critically involved in CH-induced respiratory muscle plasticity. It was hypothesised that: 1) CH affects respiratory muscle function in a time-dependent fashion; 2) CH causes structural and metabolic adjustments in respiratory muscle leading to functional remodelling; 3) CH alters respiratory muscle Na^+,K^+-ATPase content; and 4) NOS inhibition prevents CH-induced functional plasticity in respiratory muscle.

METHODS

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

Animals

Experiments were performed on 72 adult male Wistar rats. CH groups were placed in a hypobaric chamber for 1–6 weeks at 380 mmHg (ambient oxygen tension ~80 mmHg, equivalent to an inspiratory oxygen fraction of 10.5%). Age- and weight-matched control animals were held in parallel at ambient atmospheric pressure (~760 mmHg).

Effects of CH on respiratory muscle function

Rats were exposed to normoxia or CH for 1, 2, 3 and 6 weeks. Sternohyoid and diaphragm muscle contractile and endurance properties were determined *in vitro*. Respiratory and limb muscles from normoxic and CH animals were snap frozen and stored at -80°C.

SDH and reduced NADPH dehydrogenase histochemistry in respiratory and limb muscles

The succinate dehydrogenase (SDH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) dehydrogenase activity of respiratory and limb muscles were determined. Normoxic and CH muscle sections were processed in parallel.

Myosin heavy chain immunohistochemistry in respiratory and limb muscles

Indirect immunofluorescence was performed to determine myosin heavy chain (MHC) isoform composition. Normoxic and CH muscle sections were incubated with a cocktail of primary antibodies that targeted MHC type 1, 2A and 2B fibres or a primary antibody that targeted all of the isoforms but MHC type 2X.

SERCA immunohistochemistry in diaphragm muscle

Indirect immunofluorescence determination of the SERCA2 isoform was performed on normoxic and CH diaphragms.

Measurement of the content of [^{3}H]ouabain binding sites, and Na^+, K^+ and Ca^{2+} in muscle

Respiratory and limb muscle content of the Na^+,K^+-ATPase pump α_2-isoform was determined using the vanadate-facilitated [^{3}H]ouabain binding method [33, 34]. Additionally, muscle Na^+, K^+ (flame photometry) and Ca^{2+} (atomic absorption spectrophotometry) content were determined.

Effect of chronic NOS inhibition on diaphragm muscle structure and function

In a separate series of experiments, structural and functional assessments were performed on diaphragm muscle from rats treated chronically with N^{ω}-nitro-L-arginine (L-NNA; 2 mM in the drinking water), commencing 3 days before normoxia or CH treatment and continuing throughout a 6-week treatment period.

Data analysis

Specific force was calculated in newtons per square centimetre of muscle cross-sectional area (CSA). Fatigue tolerance was determined. In order to determine SDH and NADPH dehydrogenase activity, the optical density of muscle sections was calculated using Scion Image™ software (Scion Corporation, Frederick, MD, USA). For immunofluorescence analysis, Cell A™ software (Olympus Life Science Microscopes, Munich, Germany) was used to digitally analyse images and calculate numerical and areal density and CSA for each MHC fibre type. The relative area of diaphragm fibres containing SERCA2 was calculated. All data were averaged per animal before computing group means. Data are expressed as mean ± SEM. Data were compared across groups by one-way (hypoxia), two-way (hypoxia × drug) or three-way (hypoxia × drug × frequency or time) ANOVA as appropriate. Some datasets were compared using unpaired t-tests when appropriate. In all tests, a p-value of <0.05 was taken as significant.

RESULTS

Body mass, haematocrit and right ventricular mass

CH exposure for 1, 2, 3 and 6 weeks significantly decreased body mass compared to that of age-matched normoxic controls (table 1 of online supplementary material). Haematocrit and right ventricular mass were significantly elevated in all hypoxic groups (table 1 of online supplementary material). Left ventricular mass (normalised to body mass) was unaffected by CH (data not shown).

Muscle physiology

The effects of CH (1–6 weeks) on sternohyoid and diaphragm twitch and peak tetanic force, contraction time and half-relaxation time are shown in table 2 of the online supplementary material. Data for the 6-week exposure are shown in table 1. CH had no significant effect on the force–frequency relationship in the sternohyoid, but CH decreased diaphragm muscle force (table 1 of the online supplementary material; fig. 1 of the online supplementary material). CH had no effect on sternohyoid muscle endurance (fig. 1) (fig. 2 of the online supplementary material). Conversely, CH increased diaphragm

TABLE 1 Respiratory muscle force

	P_t N·cm^{-2}	P_0 N·cm^{-2}
Sternohyoid		
Normoxia	3.3±0.4	14.4±1.2
CH	2.5±0.3	15.6±0.7
Diaphragm		
Normoxia	4.0±0.7	20.0±2.3
CH	2.8±0.4	14.2±1.8$^{\#}$

Data are presented as mean±SEM (n=6 for all groups). P_t: single-twitch tension; P_0: peak tetanic tension; CH: chronic hypoxia (6 weeks). $^{\#}$: p=0.08 (unpaired t-test).

endurance (fig. 1) (figs. 2 and 3 of the online supplementary material). The magnitude of the improved fatigue tolerance was greatest after 6 weeks of CH (fig. 2 of the online supplementary material).

MHC isoform composition and fibre morphometry

Representative MHC immunofluorescence images are shown in figure 2. CH increased the numerical density of MHC type 1 fibres in diaphragm, but this failed to reach statistical significance (33±2 *versus* 39±3%; p=0.09 (unpaired t-test)). CH decreased diaphragm MHC type 1, type 2X and type 2B CSA (table 2). CH generally decreased or had no effect on fibre CSAs in the other muscles analysed, although hypertrophy of type 2A and type 2X fibres was observed in the sternohyoid (table 2). Areal density for MHC protein-determined fibre types are shown in table 2. There was no significant effect of CH on areal density of fibre types (table 2), and little or no change in the sternohyoid (table 2), soleus and extensor digitorum longus (EDL) muscles (data not shown).

SERCA2 immunohistochemistry

There was no significant difference in the areal density of fibers expressing SERCA2 between control and CH diaphragms (fig. 3).

SDH and NADPH dehydrogenase activities

There was no significant difference in SDH or NADPH dehydrogenase activity between control and CH respiratory muscles (table 3). Similarly, CH did not affect limb muscle enzyme activities (data not shown). Optical densities were highest in the diaphragm and lowest in the sternohyoid (diaphragm>soleus>EDL>sternohyoid).

Na^+,K^+-ATPase pump content and diaphragm muscle ionic content

CH caused a significant increase in diaphragm (fig. 4) and EDL Na^+,K^+-ATPase pump content, but had no effect on pump content in the sternohyoid and the soleus (table 3 of online supplementary material). CH significantly increased K^+ content in the diaphragm (table 4).

Effects of chronic NOS inhibition on diaphragm structure and function

Chronic inhibition of NOS decreased peak force in normoxic, but not CH, diaphragms (fig. 4 of online supplementary material). Chronic NOS blockade had no effect on diaphragm oxidative capacity. There were no major effects of chronic NOS inhibition on diaphragm MHC areal density measurements. Chronic NOS inhibition did not affect CH-induced atrophy of diaphragm type 1 and type 2X fibres (table 4 of online supplementary material). However, chronic NOS inhibition decreased diaphragm Na^+,K^+-ATPase pump content (fig. 4) and prevented the CH-induced increase in fatigue tolerance (fig. 5).

DISCUSSION

The major findings of the present study are: 1) CH improves diaphragm muscle endurance; 2) CH-induced muscle plasticity is time-dependent and differentially expressed in respiratory muscles; 3) CH does not increase diaphragm SDH or NADPH dehydrogenase activities; 4) CH does not alter respiratory or limb MHC areal density; 5) CH does not increase the relative area of diaphragmatic fibres expressing SERCA2; 6) CH increases Na^+,K^+-ATPase pump content in diaphragm; and 7) chronic NOS blockade decreases diaphragm Na^+,K^+-ATPase content and prevents CH-induced functional remodelling in the diaphragm.

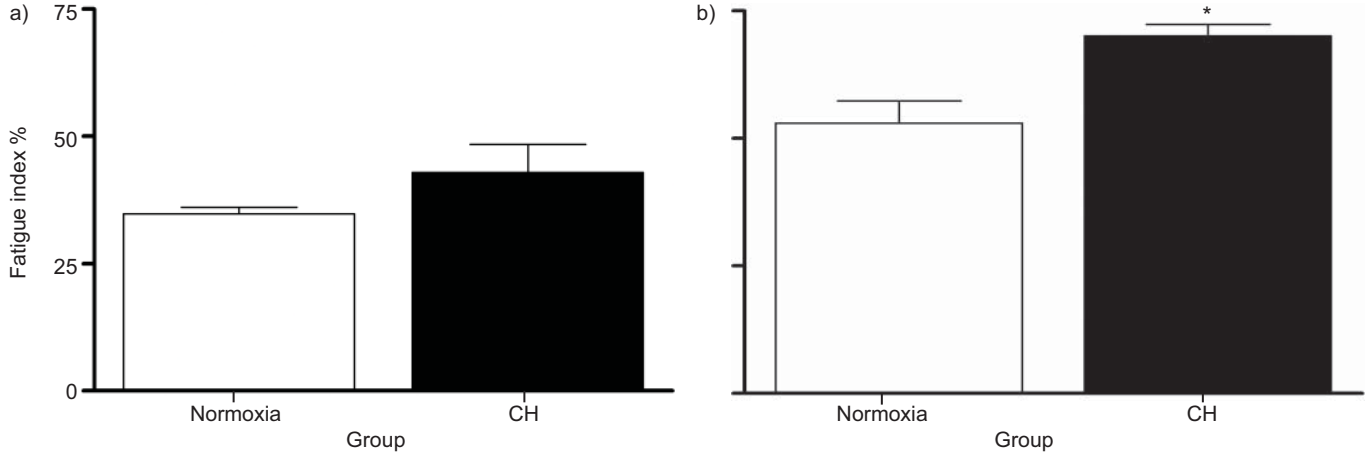

FIGURE 1. Effect of chronic hypoxia (CH) on respiratory muscle fatigue, based on initial force, in a) sternohyoid and b) diaphragm. Data are presented as mean±SEM (n=6 for all groups) in adult rats exposed to 6 weeks of normoxia or CH. *: p<0.05 *versus* normoxia (unpaired t-test).

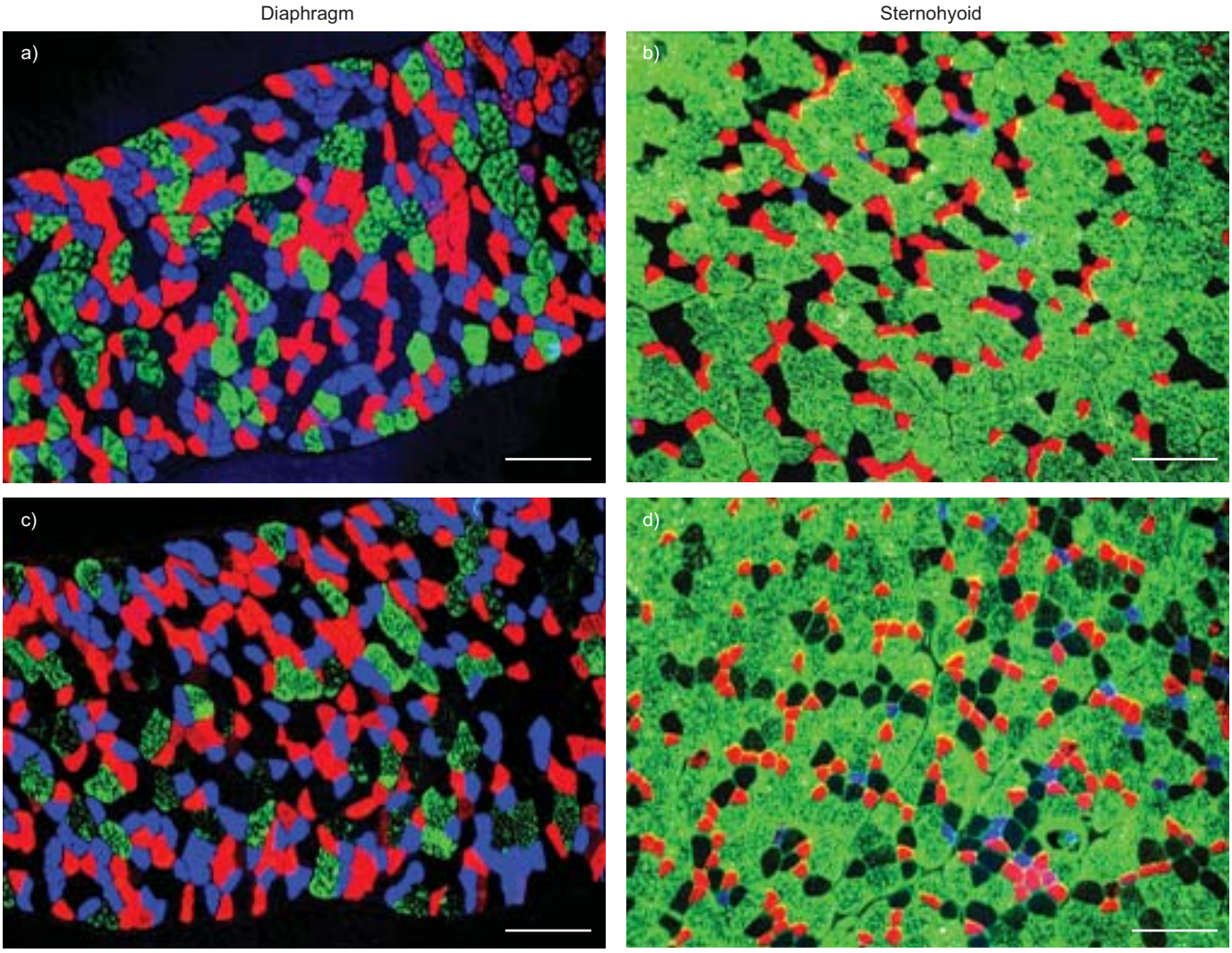

FIGURE 2. Myosin heavy chain (MHC) immunohistochemistry in rat respiratory muscles. Triple-labelling of muscle fibres in a, b) normoxic and c, d) chronic hypoxic rat; a, c) diaphragm; and b, d) sternohyoid. Using indirect immunohistochemistry, MHC type 1 (blue), type 2A (red) and type 2B (green) are tagged (merged images). Note that the sternohyoid muscle shows a higher complement of MHC type 2B fibres than the diaphragm. Scale bars=200 μm.

CH-induced functional remodelling

Few have studied the effects of CH on the respiratory muscles [13–16], which is surprising since alterations to respiratory muscle function at altitude or in diseases characterised by hypoxia could have consequences for respiratory homeostasis. CH decreased diaphragm force, a finding consistent with some [13, 14] but not other reports [15, 16]. CH improved diaphragm endurance, which contrasts with the findings of previous studies [13, 15, 16]. It could be speculated that differences in the intensity of the hypoxic challenges, as well as differences in the experimental paradigms employed when studying muscle function, contribute, at least in part, to this apparent discrepancy.

CH had differential effects on sternohyoid and diaphragm function. The different structural and metabolic profiles of these muscles may explain why endurance was enhanced in the diaphragm and not in the sternohyoid. It is plausible that reduced oxygen availability triggers adaptive mechanisms in skeletal muscle of high oxidative capacity since these muscles are heavily reliant on oxygen to generate energy. In contrast, perturbations in oxygen supply to muscles with a lower oxidative capacity may not trigger such adaptive mechanisms. It is interesting to note that differential effects of CH have also been reported in limb muscles of varying structural phenotype [5, 15]. This suggests that the effects of CH on muscle function may be dependent upon the intrinsic structure, metabolism and physiological function of individual muscle types.

It was hypothesised that functional remodelling would proceed gradually in a time-dependent manner during CH exposure, and the results of the present study support this notion. The reason for the differential effect of CH on sternohyoid and diaphragm muscle endurance remains unclear, but, in light of the present findings with chronic NOS blockade (see below), fibre-specific differences in NOS expression and activity may have been a contributing factor [35–37]. NOS activity is highest in fast oxidative fibres, which are considerably more abundant in diaphragm than in sternohyoid muscle. As such, the diaphragm may possess greater capacity for NO-dependent functional remodelling than other muscles.

TABLE 2 Respiratory muscle myosin heavy chain (MHC) areal density and fibre cross-sectional area (CSA)

	Type 1	Type 2A	Type 2X	Type 2B
Sternohyoid				
MHC isoform areal density %				
Normoxia	0.52±0.10	14.1±1.4	7.3±1.0	77.2±2.2
CH	0.49±0.20	13.6±1.2	11.0±1.2	77.1±1.5
Fibre CSA μm^2				
Normoxia	993±64	1192±31	1999±43	3792±64
CH	891±44	1489±52*	2235±70*	3834±53
Diaphragm				
MHC isoform areal density %				
Normoxia	21.8±1.7	24.3±2.1	27.8±4.0	19.1±4.4
CH	25.3±2.7	22.0±1.3	25.5±3.1	26.9±6.1
Fibre CSA μm^2				
Normoxia	1410±38	1422±26	2565±80	5024±177
CH	1232±25*	1384±23	2040±75*	3424±85*

Data are presented as mean±SEM (n=8–11 per group). CH: chronic hypoxia (6 weeks). *: $p<0.05$ *versus* normoxia (unpaired t-test).

CH-induced structural remodelling

There is a paucity of information concerning the effects of CH on respiratory muscle structure. MORTOLA and NASO [38] reported fibre transitions in diaphragm and limb muscle of rats after 9 months, but not 60 days, of CH. Others have documented no effect of CH on adult diaphragm muscle fibres [15]. In the present study, the numerical density of MHC type 1 and type 2B fibres increased. However, owing to changes in fibre CSA, CH had no effect on MHC areal density in the diaphragm.

CH caused atrophy in fibres of the diaphragm. Atrophy or no change was noted in limb muscle fibres, but CH caused hypertrophy of fast 2A and 2X fibres of the sternohyoid. In general, the literature from animal and human studies examining the effects of CH on fibre typology and morphology is quite unsettled. The mechanisms underlying the differential response of muscle fibres to CH are complex and largely unknown, and were not a major focus of the present study.

SDH activity was quantified as an index of oxidative capacity. It was found that CH had no effect on the SDH activity of diaphragm (or any muscle studied), suggesting that factors other than oxidative potential contribute to improved fatigue tolerance in the present model. However, it should be noted that the activity of other key oxidative enzymes that may have increased in the CH diaphragm were not measured.

It was speculated that the relative area of fibres expressing SERCA2 would increase in CH diaphragm. The present data indicate, however, that there was no significant difference between normoxic and CH muscles. This is consistent with findings in a rat model of emphysema [39]. SERCA2 activity was not measured, and this may have increased in CH diaphragm. However, half-relaxation time, which is determined by SERCA activity, was unchanged in CH diaphragm. Thus SERCA pump content and activity are most probably unchanged.

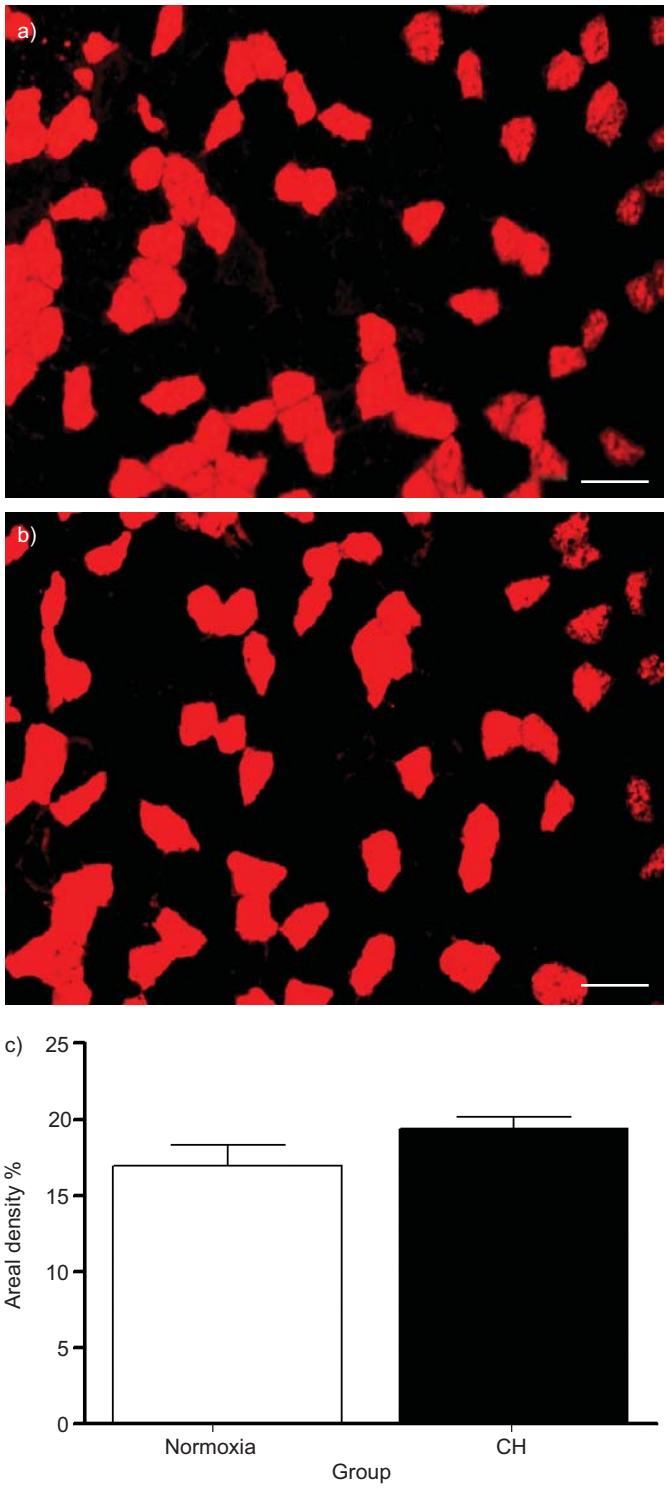

FIGURE 3. Diaphragm sarco/endoplasmic reticulum calcium-ATPase (SERCA) 2 immunohistochemistry. Representative images showing SERCA2-immunolabelled muscle fibres in rat diaphragm a) normoxic and b) chronic hypoxic (CH). Scale bars=100 μm. c) Group data (n=6 for both groups) indicate that CH has no significant effect on the areal density of fibres expressing the SERCA2 isoform.

TABLE 3 Succinate dehydrogenase (SDH) and reduced nicotinamide adenine dinucleotide phosphate dehydrogenase (NADPHDH) histochemistry

	Optical density AU	
	SDH	NADPHDH
Sternohyoid		
Normoxia	0.20±0.01	0.09±0.01
CH	0.20±0.01	0.08±0.01
Diaphragm		
Normoxia	0.59±0.04	0.18±0.01
CH	0.60±0.02	0.18±0.01

Data are presented as mean±SEM (n=6–11 per group). AU: arbitrary unit; CH: chronic hypoxia.

TABLE 4 Effect of chronic hypoxia (CH)# on diaphragm ionic content

	Na^+ μmol·g^{-1}	K^+ μmol·g^{-1}	Ca^{2+} μmol·g^{-1}
Normoxia	31±5	95±3	1.5±0.1
CH	31±2	106±1*	1.7±0.3

Data are presented as mean±SEM (based on wet weight; n=5–6). #: 6 weeks of hypoxia. *: p<0.05 *versus* normoxia (unpaired t-test).

Na^+,K^+-ATPase pump

The present study is the first to show that CH increases Na^+,K^+-ATPase pump content in diaphragm muscle. It was found that Na^+,K^+-ATPase pump content increased by ~24% in the CH diaphragm. CH also elevated pump content in the EDL, but elicited no change in the sternohyoid and soleus muscles of the same animals. A previous study reported a decrease in Na^+,K^+-ATPase pump content in human vastus lateralis on exposure to altitude for 3 weeks [40]. Interestingly, the same group found that, when exercise is performed in a hypoxic environment, pump content is also depressed [41]. It is difficult to reconcile why pump content is increased in some CH rat muscles but is decreased in hypoxic human muscle. This may relate to species differences or is perhaps dependent upon differences in the duration and severity of hypoxic exposure, in addition to differences in the intrinsic structure of these muscles. A comprehensive assessment of structure in respiratory and limb muscles was performed for comparative purposes in order to determine whether CH-induced structural remodelling were influenced by muscle activity. It could be argued that the increase in Na^+,K^+-ATPase pump content in CH diaphragm is secondary to the augmented activity of this muscle as a result of CH-induced hyperventilation. This is in keeping with the repeated reports of training-induced upregulation of Na^+,K^+-ATPase pumps in skeletal muscle [30, 31]. However, pump content was significantly elevated in EDL in the present study, suggesting that the increase was a result of a direct effect of hypoxia *per se*.

Since most fibres of the CH diaphragm atrophied, it could be argued that the increase in muscle surface area-to-volume ratio accounts for the increase in Na^+,K^+-ATPase pump content. In order to explore this further, whole-muscle and fibre-specific surface area-to-volume ratios were calculated and it was determined that CH causes a 13% increase (from a mean of 71,487 to 80,955 μm^2·mm^{-3}) in the area-to-volume ratio for whole diaphragm. Thus the increase in Na^+,K^+-ATPase pump

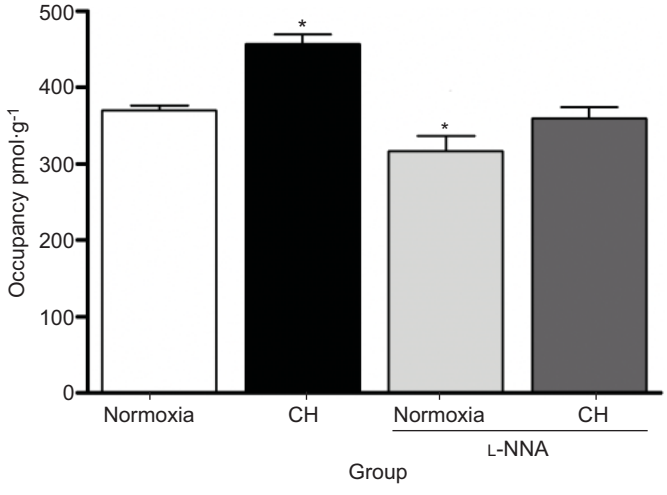

FIGURE 4. Diaphragm muscle sodium–potassium ATPase (Na^+,K^+-ATPase) pump content. Data are presented as mean±SEM ouabain binding site occupancy (based on wet weight) in rats exposed to 6 weeks of normoxia or chronic hypoxia (CH) with and without chronic nitric oxide synthase inhibition with N^{ω}-nitro-L-arginine (L-NNA; 2 mM in drinking water throughout normoxia or CH treatment). Two-way ANOVA (hypoxia × drug) revealed significant effects of hypoxia and drug treatment. There was no significant interaction (p=0.1). *: p<0.05 *versus* normoxia (Bonferroni *post-hoc* test; n=5–6 per group).

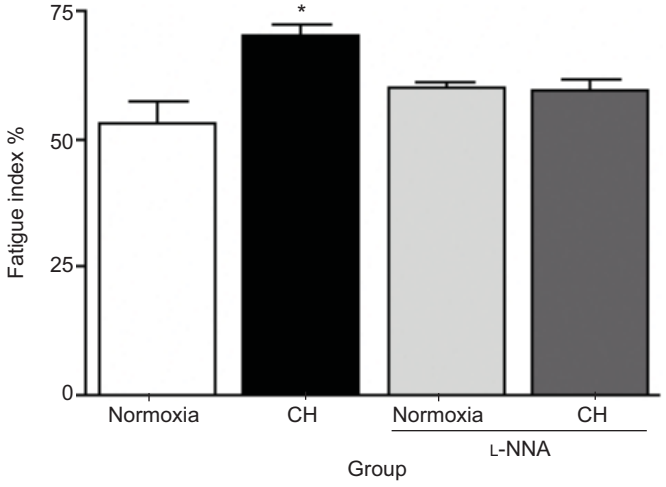

FIGURE 5. Effect of chronic hypoxia (CH) on diaphragm muscle fatigue index. Data are presented as mean±SEM in rats exposed to 6 weeks of normoxia or CH with and without chronic nitric oxide synthase inhibition with N^{ω}-nitro-L-arginine (L-NNA; 2 mM in drinking water throughout normoxia or CH treatment). Two-way ANOVA (hypoxia × drug) revealed a significant effect of hypoxia, but not drug, treatment. The interaction was significant. *: p<0.05 *versus* normoxia (Bonferroni *post-hoc* test; n=5–6 per group).

content in CH diaphragm (24%) may be partly due to a concomitant increase in sarcolemmal area-to-volume ratio. It is interesting to note that the pump content increased by 11% in CH EDL, with no change in total muscle surface area-to-volume ratio (-1.6%). Thus, when surface area-to-volume adjustments are accounted for, the effect of CH on respiratory and limb muscle Na^+,K^+-ATPase pump content is equivalent.

Increased Na^+,K^+-ATPase pump content may facilitate adaptation to a low oxygen environment by supporting the optimal muscle function essential for respiratory homeostasis. It would be most interesting to determine Na^+,K^+-ATPase pump content and activity in respiratory muscles from COPD patients.

Role of NO in CH-induced respiratory muscle remodelling

NOS activity is regulated by muscle stimulation, mechanical activity, exercise and age, and can be up- or downregulated under a range of physiological and pathophysiological conditions. Skeletal muscle NOS activity undergoes significant upregulation during chronic exercise training in the rat and human. Exposure to hypobaric hypoxia for nearly 3 months in newborn rats was associated with an upregulation of endothelial NOS and neuronal (nNOS) levels and an increase in diaphragmatic NOS activity [42]. Chronic NOS blockade inhibits skeletal muscle adaptation to chronic endurance exercise, and results in a severe loss of walking speed in rats [43]. Furthermore, $nNOS^{-/-}$ mice show decreased muscle endurance [44], and diaphragm dysfunction is exacerbated following lipopolysaccharide challenge in $nNOS^{-/-}$ mice compared to wild-type controls [45]. Studies of various tissues have shown that NOS expression and activity are regulated by hypoxia, but the effects of chronic NOS blockade on CH muscle function are not known. It was speculated that chronic NOS inhibition would attenuate or prevent CH diaphragm remodelling. That is, we reasoned that CH-induced respiratory muscle adaptation has a structural basis and that NO is critically involved. It was found that chronic NOS inhibition significantly decreased diaphragm Na^+,K^+-ATPase pump content in normoxic and CH rats. Of interest, CH+L-NNA diaphragm surface area-to-volume ratio (81,096 $\mu m^2 \cdot mm^{-3}$) was equivalent to that of CH diaphragm, but Na^+,K^+-ATPase pump content was decreased to control levels (fig. 4). It is, therefore, apparent that NOS inhibition downregulates respiratory muscle Na^+,K^+-ATPase pump content independent of surface area-to-volume adjustments. NOS inhibition completely suppressed increased fatigue tolerance in CH diaphragm, implicating NO in functional remodelling of CH diaphragm. The present study provides novel data indicating that NO has a regulatory role in modulating Na^+,K^+-ATPase pump content in rat respiratory muscles.

Summary and conclusion

Skeletal muscles, including the striated muscles of breathing, demonstrate a remarkable capacity to respond to physiological and environmental challenges. During hypoxic adaptation, the respiratory muscles face unique challenges that must be overcome in order to ensure maintenance of homeostasis. Our translational model highlights the fact that hypoxic signalling can serve as a trigger of molecular and cellular adjustments that ultimately shape respiratory muscle performance. The present study demonstrates that CH is associated with increased Na^+,K^+-ATPase pump content in diaphragm concomitant with increased muscle endurance. Chronic NOS inhibition reduced diaphragm Na^+,K^+-ATPase pump content and prevented a CH-induced increase in diaphragm endurance. This study provides novel insight into mechanisms involved in CH-induced muscle remodelling. The results may be of relevance to respiratory disorders characterised by CH, such as COPD, in which respiratory muscle remodelling is known to occur.

SUPPORT STATEMENT

This study was funded by the Health Research Board (Dublin, Ireland; grant RP/2006/140) and University College Dublin (UCD) School of Medicine and Medical Science (Health Sciences Centre, UCD, Dublin, Ireland) Translational Medicine PhD Programme. J. Carberry was in receipt of a UCD School of Medicine and Medical Science scholarship. R.A. O'Connell is funded by the Irish Research Council for Science, Engineering and Technology (Dublin, Ireland).

STATEMENT OF INTEREST

None declared.

ACKNOWLEDGEMENTS

We are grateful to T. Clausen (University of Aarhus, Aarhus, Denmark) for critical appraisal of an early draft of the present manuscript. We also thank the anonymous reviewers of the manuscript for several informed suggestions, and are especially grateful to one reviewer for drawing our attention to the issue of surface area-to-volume ratio adjustment in remodelled muscle.

REFERENCES

1 Deveci D, Marshall JM, Egginton S. Chronic hypoxia induces prolonged angiogenesis in skeletal muscles of rat. *Exp Physiol* 2002; 87: 287–291.
2 Snyder GK, Wilcox EE, Burnham EW. Effects of hypoxia on muscle capillarity in rats. *Respir Physiol* 1985; 62: 135–140.
3 Abdelmalki A, Fimbel S, Mayet-Sornay MH, *et al.* Aerobic capacity and skeletal muscle properties of normoxic and hypoxic rats in response to training. *Pflugers Arch* 1996; 431: 671–679.
4 Bigard AX, Sanchez H, Birot O, *et al.* Myosin heavy chain composition of skeletal muscles in young rats growing under hypobaric hypoxia conditions. *J Appl Physiol* 2000; 88: 479–486.
5 Faucher M, Guillot C, Marqueste T, *et al.* Matched adaptations of electrophysiological, physiological, and histological properties of skeletal muscles in response to chronic hypoxia. *Pflugers Arch* 2005; 450: 45–52.
6 Green HJ, Sutton JR, Cymerman A, *et al.* Operation Everest II: adaptations in human skeletal muscle. *J Appl Physiol* 1989; 66: 2454–2461.
7 Hirofuji C, Ishihara A, Itoh K, *et al.* Fibre type composition of the soleus muscle in hypoxia-acclimatised rats. *J Anat* 1992; 181: 327–333.
8 Ishihara A, Itoh K, Oishi Y, *et al.* Effects of hypobaric hypoxia on histochemical fibre-type composition and myosin heavy chain isoform component in the rat soleus muscle. *Pflugers Arch* 1995; 429: 601–606.
9 Itoh K, Itoh M, Ishihara A, *et al.* Influence of 12 weeks of hypobaric hypoxia on fibre type composition of the rat soleus muscle. *Acta Physiol Scand* 1995; 154: 417–418.
10 MacDougall JD, Green HJ, Sutton JR, *et al.* Operation Everest II: structural adaptations in skeletal muscle in response to extreme simulated altitude. *Acta Physiol Scand* 1991; 142: 421–427.
11 Desplanches D, Hoppeler H, Tuscher L, *et al.* Muscle tissue adaptations of high-altitude natives to training in chronic hypoxia or acute normoxia. *J Appl Physiol* 1996; 81: 1946–1951.

12 Howald H, Pette D, Simoneau JA, *et al.* Effect of chronic hypoxia on muscle enzyme activities. *Int J Sports Med* 1990; 11: S10–S14.
13 Jammes Y, Zattara-Hartmann MC, Badier M. Functional consequences of acute and chronic hypoxia on respiratory and skeletal muscles in mammals. *Comp Biochem Physiol A Physiol* 1997; 118: 15–22.
14 Kass LJ, Bazzy AR. Chronic hypoxia modulates diaphragm function in the developing rat. *J Appl Physiol* 2001; 90: 2325–2329.
15 El-Khoury R, O'Halloran KD, Bradford A. Effects of chronic hypobaric hypoxia on contractile properties of rat sternohyoid and diaphragm muscles. *Clin Exp Pharmacol Physiol* 2003; 30: 551–554.
16 Shiota S, Okada T, Naitoh H, *et al.* Hypoxia and hypercapnia affect contractile and histological properties of rat diaphragm and hind limb muscles. *Pathophysiology* 2004; 11: 23–30.
17 Doucet M, Debigare R, Joanisse DR, *et al.* Adaptation of the diaphragm and the vastus lateralis in mild-to-moderate COPD. *Eur Respir J* 2004; 24: 971–979.
18 Levine S, Gregory C, Nguyen T, *et al.* Bioenergetic adaptation of individual human diaphragmatic myofibers to severe COPD. *J Appl Physiol* 2002; 92: 1205–1213.
19 Levine S, Kaiser L, Leferovich J, *et al.* Cellular adaptations in the diaphragm in chronic obstructive pulmonary disease. *N Engl J Med* 1997; 337: 1799–1806.
20 Levine S, Nguyen T, Kaiser LR, *et al.* Human diaphragm remodeling associated with chronic obstructive pulmonary disease: clinical implications. *Am J Respir Crit Care Med* 2003; 168: 706–713.
21 Mercadier JJ, Schwartz K, Schiaffino S, *et al.* Myosin heavy chain gene expression changes in the diaphragm of patients with chronic lung hyperinflation. *Am J Physiol* 1998; 274: L527–L534.
22 Stubbings AK, Moore AJ, Dusmet M, *et al.* Physiological properties of human diaphragm muscle fibres and the effect of chronic obstructive pulmonary disease. *J Physiol* 2008; 586: 2637–2650.
23 Mador MJ, Kufel TJ, Pineda LA, *et al.* Diaphragmatic fatigue and high-intensity exercise in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000; 161: 118–123.
24 Polkey MI, Kyroussis D, Hamnegard CH, *et al.* Diaphragm performance during maximal voluntary ventilation in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1997; 155: 642–648.
25 Polkey MI, Kyroussis D, Keilty SE, *et al.* Exhaustive treadmill exercise does not reduce twitch transdiaphragmatic pressure in patients with COPD. *Am J Respir Crit Care Med* 1995; 152: 959–964.
26 Levine S, Nguyen T, Friscia M, *et al.* Parasternal intercostal muscle remodeling in severe chronic obstructive pulmonary disease. *J Appl Physiol* 2006; 101: 1297–1302.
27 Sanchez J, Brunet A, Medrano G, *et al.* Metabolic enzymatic activities in the intercostal and serratus muscles and in the latissimus dorsi of middle-aged normal men and patients with moderate obstructive pulmonary disease. *Eur Respir J* 1988; 1: 376–383.
28 Aubier M, Viires N. Calcium ATPase and respiratory muscle function. *Eur Respir J* 1998; 11: 758–766.
29 Nguyen T, Rubinstein NA, Vijayasarathy C, *et al.* Effect of chronic obstructive pulmonary disease on calcium pump ATPase expression in human diaphragm. *J Appl Physiol* 2005; 98: 2004–2010.
30 Clausen T, van Hardeveld C, Everts ME. Significance of cation transport in control of energy metabolism and thermogenesis. *Physiol Rev* 1991; 71: 733–744.
31 Clausen T. Na^+-K^+ pump regulation and skeletal muscle contractility. *Physiol Rev* 2003; 83: 1269–1324.
32 Nielsen OB, Clausen T. Regulation of Na^+-K^+ pump activity in contracting rat muscle. *J Physiol* 1997; 503: 571–581.
33 Nørgaard A, Kjeldsen K, Hansen O, *et al.* A simple and rapid method for the determination of the number of ^{3}H-ouabain binding sites in biopsies of skeletal muscle. *Biochem Biophys Res Commun* 1983; 111: 319–325.
34 McKenna MJ, Gissel H, Clausen T. Effects of electrical stimulation and insulin on Na^+-K^+-ATPase ([^{3}H]ouabain binding) in rat skeletal muscle. *J Physiol* 2003; 547: 567–580.
35 Punkt K, Naupert A, Wellner M, *et al.* Nitric oxide synthase II in rat skeletal muscles. *Histochem Cell Biol* 2002; 118: 371–379.
36 Punkt K, Fritzsche M, Stockmar C, *et al.* Nitric oxide synthase in human skeletal muscles related to defined fibre types. *Histochem Cell Biol* 2006; 125: 567–573.
37 Yu Z, Li P, Zhang M, *et al.* Fiber type-specific nitric oxide protects oxidative myofibers against cachectic stimuli. *PLoS One* 2008; 3: e2086.
38 Mortola JP, Naso L. Electrophoretic analysis of contractile proteins of the diaphragm in chronically hypoxic rats. *Am J Physiol* 1995; 269: L371–L376.
39 Kim DK, Zhu J, Kozyak BW, *et al.* Myosin heavy chain and physiological adaptation of the rat diaphragm in elastase-induced emphysema. *Respir Res* 2003; 4: 1–10.
40 Green H, Roy B, Grant S, *et al.* Downregulation in muscle Na^+-K^+-ATPase following a 21-day expedition to 6,194 m. *J Appl Physiol* 2000; 88: 634–640.
41 Green H, MacDougall J, Tarnopolsky M, *et al.* Downregulation of Na^+-K^+-ATPase pumps in skeletal muscle with training in normobaric hypoxia. *J Appl Physiol* 1999; 86: 1745–1748.
42 Javeshghani D, Sakkal D, Mori M, *et al.* Regulation of diaphragmatic nitric oxide synthase expression during hypobaric hypoxia. *Am J Physiol Lung Cell Mol Physiol* 2000; 279: L520–L527.
43 Wang MX, Murrell DF, Szabo C, *et al.* Nitric oxide in skeletal muscle: inhibition of nitric oxide synthase inhibits walking speed in rats. *Nitric Oxide* 2001; 5: 219–232.
44 Percival JM, Anderson KN, Gregorevic P, *et al.* Functional deficits in nNOSμ-deficient skeletal muscle: myopathy in nNOS knockout mice. *PLoS One* 2008; 3: e3387.
45 Comtois AS, Barreiro E, Huang PL, *et al.* Lipopolysaccharide-induced diaphragmatic contractile dysfunction and sarcolemmal injury in mice lacking the neuronal nitric oxide synthase. *Am J Respir Crit Care Med* 2001; 163: 977–982.