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Diaphragm muscle weakness and increased UCP-3 gene expression following acute hypoxic stress in the mouse

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ABSTRACT

The effects of acute hypoxia on the diaphragm are largely unknown despite the clinical relevance to respiratory conditions such as acute respiratory distress syndrome and ventilator-induced lung injury.

Adult male C57BL6/J mice were exposed to 1, 4 or 8 h of hypoxia (F_iO_2 = 0.10) or normoxia. Ventilation was assessed by whole-body plethysmography during gas exposures. Diaphragm isotonic contractile parameters were assessed *ex vivo*. Diaphragm gene expression was determined using qRT-PCR.

Acute hypoxic stress resulted in significant diaphragm muscle weakness. Gene expression data revealed that hypoxia results in temporal changes in various transcriptional genes regulating mitochondrial function and a time-dependent progressive increase in the expression of the mitochondrial uncoupling protein 3 (UCP-3) with concomitant changes in genes encoding sarcoplasmic reticulum calcium release proteins.

Altered gene expression and muscle weakness are likely due to direct effects of hypoxic stress *per se*, and not related to increased diaphragm muscle activity, as there was no persistent change in ventilation during hypoxic exposure. These findings suggest a potentially critical role for hypoxia in diaphragm muscle remodeling in acute respiratory-related disorders.

1. Introduction

The diaphragm is the primary pump muscle of inspiration and, as such, it is active throughout life—due to breathing, as well as activities such as clearing of the airway. The diaphragm has a mixed muscle fiber type composition (McMorrow et al., 2011) and it is an extremely adaptable and malleable muscle. Indeed, when a hypoxemic respiratory patient in the intensive care unit (ICU) is placed on a mechanical ventilator due to respiratory failure, diaphragm atrophy and dysfunction (ventilator-induced diaphragmatic dysfunction, VIDD) occurs in a matter of hours (Bruells et al., 2013), and there is recent evidence to suggest that the ubiquitin-proteasome pathway, as well as mitochondrial abnormalities, may be involved (Hooijman et al., 2015; Picard et al., 2015). Whilst inactivity of the diaphragm, due to the mechanical ventilator performing the work of breathing, is widely accepted as a major cause of this weakness (Jaber et al., 2011; Supinski and Callahan, 2013), often compounded by infection (Petrof et al., 2010; Supinski and Callahan, 2013), the potential role, if any, that hypoxia plays in the development and/or exacerbation of this weakness is unclear. Indeed, there exists some controversy as to whether inactivity *per se* induces diaphragm muscle atrophy, and it has been suggested that other factors such as neurone-derived trophic factors may be at play (Sieck and Mantilla, 2013), which warrants further exploration.

The effects of acute hypoxia on diaphragm form and function are largely unknown despite the fact that hypoxia is a prominent feature of respiratory conditions such as acute hypoxemic respiratory failure or acute respiratory distress syndrome (ARDS) and ventilator-associated/induced lung injury (VALI/VILI), conditions that can lead to a recognized phenomenon—ICU acquired weakness (ICUAW), including respiratory muscle weakness, which is a strong predictor of poor outcome in patients(Batt et al., 2013; Sieck and Mantilla, 2015; Supinski and Callahan, 2013). Hypoxia also occurs during exposure to high altitude. High altitude/hypobaric hypoxia is known to affect skeletal muscle, including the diaphragm, and the effects of exercise on the diaphragm differ when performed at high altitude or at sea level (Bigard et al., 1992; Levett et al., 2012). Thus, insight into the acute adaptations of the respiratory muscles to hypoxia may have wide-ranging relevance. Respiratory muscle plasticity is reported in animal models of chronic hypoxia (Carberry et al., 2014; Gamboa and Andrade, 2012, 2010; Lewis et al., 2015a,b; McMorrow et al., 2011); however, very little is known about the effects of an acute hypoxic stress on diaphragm muscle performance.

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With this in mind, we sought to determine the effects of acute exposure to sustained hypoxia on: (1) diaphragm muscle function *ex vivo*, allowing us to examine effects intrinsic to the muscle; (2) ventilation *in vivo*, to determine if the activity of the diaphragm was likely different under hypoxic conditions; and (3) the transcriptional response of the diaphragm, exploring gene expression, with a particular focus on mitochondrial metabolism and calcium release from the sarcoplasmic reticulum (SR).

2. Materials and methods

2.1. Ethical approval

All protocols involving animals described in this study were approved by local ethics committee and were performed under licence from the Irish Government Department of Health and Children in accordance with EU legislation.

2.2. Animal model and plethysmography

Thirty two adult male C57BL6/J mice (Harlan, UK) were exposed to 1, 4 or 8 h of hypoxia ($F_iO2 = 0.10$) or normoxia ($F_iO2 = 0.21$) ($n = 8$ per group, 4 groups) in environmental chambers at room temperature. An additional 16 freely behaving, unrestrained mice were exposed individually to 8 h of hypoxia ($F_iO2 = 0.10$, $n = 8$) or normoxia ($F_iO2 = 0.21$, $n = 8$) in environmental plethysmography chambers (Buxco Ltd., St. Paul, Minneapolis, USA), allowing breathing parameters to be measured on a breath-by-breath basis in real time. Exposures to normoxia and hypoxia were performed in parallel using a two-chamber set-up. Following gas exposures, animals were immediately euthanized by a rising concentration of CO_2 until narcosis (in order to avoid re-exposure to ambient O_2 while the animals were still breathing), followed immediately by cervical dislocation to confirm euthanasia. Diaphragm muscles were excised *post-mortem* for functional analysis or immediately snap frozen in liquid N_2 and stored at -80 °C for gene expression analysis.

2.3. Muscle function

2.3.1. Ex-vivo *muscle preparation*

Experiments were performed on diaphragm muscle preparations from the 16 mice used in the plethysmography experiments above. Animals were euthanized (as above) and the diaphragm muscle was excised and placed in a storage bath containing continuously aerated (95% O_2/5% CO_2) Krebs solution (containing 25 μM D-tubocurarine) at room temperature as described in Lewis et al. (2015a). A longitudinal strip of diaphragm muscle was mounted vertically in a tissue bath at 35 °C, gassed with 95% O_2/5% CO_2, and connected to a dual-mode force transducer as previously described in Lewis et al. (2015b) for the assessment of contractile properties.

2.3.2. Protocol

The contractile performance of the diaphragm muscle was determined, and functional data analysed as previously described (Lewis et al., 2015b). In brief, muscle preparations were set to optimal length. Isometric twitch force and contractile kinetics were determined. Next, under isotonic conditions, mechanical work and power were determined across a range of loads (0–100% of maximal force), allowing assessment of work-load and power-load relationships, and allowing determination of peak work and peak power. Maximum velocity of shortening and maximum shortening were also determined.

Table 1
PCR assay details.

Gene title	Gene symbol	Assay ID
PGC-1α	Ppargc1a	313427
NRF1	Nrf1	314790
NF-κB1	Nfkb1	300085
UCP-3	Ucp3	313410
Selenoprotein N1	Sepn1	316860
Junctophilin 1	Jph1	316620
Junctophilin 2	Jph2	316843
Ryanodine receptor 3	Ryr3	316841
Dihydropyridine receptor	Cacna1s	301252
Calsequestrin 1	Casq1	316619
HPRT1	Hprt1	307879

Assay details for PGC-1α, NRF1, NF-κB1, UCP-3, selenoprotein N1, junctophilin 1, junctophilin 2, ryanodine receptor 3, dihydropyridine receptor, and calsequestrin 1.

2.4. Gene expression

2.4.1. RNA extraction and reverse transcription

Total RNA was extracted, using Tripure Isolation Reagent (Roche Diagnostics Ltd., West Sussex, UK), from 20 to 70 mg of frozen muscle tissue using a standard laboratory homogeniser (Omni-Inc., Kennesaw, Georgia, USA) as per the manufacturer's instructions, with an additional chloroform wash step during phase separation. Following isolation, RNA was treated with TURBO DNA-free Kit (Life Technologies, Bio-Sciences, Dun Laoghaire, Ireland). RNA quantity and purity was assessed by spectrophotometry with a Nanodrop 1000 (Thermo Scientific, Wilmington, Delaware, USA). RNA integrity was assessed using an agarose gel electrophoresis system (E-gel, Life Technologies) and visualisation of clear 18S and 28S ribosomal RNA bands. RNA was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Ltd.) as per the manufacturer's instructions.

2.4.2. qPCR

cDNA was amplified using Realtime ready Catalog or Custom Assays (Table 1, Roche Diagnostics Ltd.) and Fast Start Essential DNA Probe Master (Roche Diagnostics Ltd.) in 20 μl reactions (5 μl cDNA and 15 μl master mix) as per the manufacturer's instructions, using the LightCycler 96 (Roche Diagnostics Ltd.) on 96-well plates. All reactions were performed in duplicate. Data were normalized to a reference gene, hprt1, to compensate for variations in input RNA/cDNA amounts and efficiency of reverse transcription. Several candidate reference genes were screened and hprt1 was found to be most stable considering time and gas exposures. Relative expression was calculated using the ΔΔCT method to normalized expression to the reference gene with changes in expression displayed as a fold change over the control group.

2.5. Statistical analysis

Data were statistically compared using Prism (GraphPad Software, Inc., La Jolla, California, USA). Statistical comparisons between groups were performed using one-way ANOVA with Tukey's *post hoc* test, two-way ANOVA with Bonferroni *post hoc* test or unpaired *t*-test, where appropriate. $p < 0.05$ was taken to be significant in all comparisons. Values are expressed as mean ± SEM.

3. Results

Peak force-generating capacity of the diaphragm was significantly reduced by ~30% (30 ± 3 *vs.* 21 ± 2 N/cm^2, $p = 0.033$, unpaired *t*-test) following 8 h of hypoxia compared with normoxia (Fig. 1(A)). Power-generating capacity of the diaphragm over a range of loads on the muscle, from 0 to 100% of maximal force-generating capacity of the muscle, was significantly reduced ($p = 0.0011$ (gas), two-way