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Evidence of hypoxic tolerance in weak upper airway muscle from young *mdx* mice

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ABSTRACT

Duchenne muscular dystrophy (DMD) is a genetic disease characterised by deficiency in the protein dystrophin. The respiratory system is weakened and patients suffer from sleep disordered breathing and hypoventilation culminating in periods of hypoxaemia. We examined the effects of an acute (6 h) hypoxic stress on sternohyoid muscle function (representative pharyngeal dilator). 8 week old male, wild-type (WT; C57BL/10ScSnJ; $n = 18$) and *mdx* (C57BL/10ScSn-Dmd^{mdx}/J; $n = 16$) mice were exposed to sustained hypoxia ($F_IO_2 = 0.10$) or normoxia. Muscle functional properties were examined *ex vivo*. Additional WT ($n = 5$) and *mdx* ($n = 5$) sternohyoid muscle was exposed to an anoxic challenge. Sternohyoid dysfunction was observed in *mdx* mice with significant reductions in force and power. Following exposure to the acute *in vivo* hypoxic stress, WT sternohyoid muscle showed evidence of functional impairment (reduced force, work and power). Conversely, *mdx* sternohyoid showed an apparent tolerance to the acute hypoxic stress. This tolerance was not maintained for *mdx* following a severe hypoxic stress. A dysfunctional upper airway muscle phenotype is present at 8 weeks of age in the *mdx* mouse, which may have implications for the control of airway patency in DMD. Hypoxic tolerance in *mdx* respiratory muscle is suggestive of adaptation to chronic hypoxia, which could be present due to respiratory morbidity. We speculate a role for hypoxia in *mdx* respiratory muscle morbidity.

1. Introduction

Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy and presents with the most devastating phenotype (Wicklund, 2013). DMD is an X-chromosome linked recessive disorder, which results in patients lacking the structural protein dystrophin (427 kDa) due to alteration or deletion of the dystrophin gene. Dystrophin is primarily expressed in skeletal and cardiac muscle where its physiological role is to link the internal actin cytoskeleton (F-actin) to the sarcolemma (Nowak and Davies, 2004). Absence of dystrophin predisposes muscle to contraction-induced injury, causing damage to muscle fibres and the sarcolemma by the formation of stretch-induced tears (Petrof et al., 1993). As the disease progresses and the regenerative capacity of muscle fibres becomes exhausted, there is a resultant increase in fibrosis and adipose deposition in muscle (De Bruin et al., 1997). Premature death usually occurs due to respiratory or cardiac failure (Mosqueira et al., 2013b).

The adequate performance of respiratory muscles such as the diaphragm and upper airway muscles is essential for optimal ventilation (White, 2005). The respiratory muscles are adversely affected in DMD patients (Phillips et al., 2001) and scoliosis develops in many patients impacting on respiratory vital capacity (Smith et al., 1989a). In the first decade of life, many patients suffer from sleep disordered breathing (SDB) (Barbé et al., 1994) resulting in oxygen desaturations culminating in periods of nocturnal hypoxaemia and hypercapnia (Bersanini et al., 2012). Due to a reduction in the force-generating capacity of the diaphragm muscle (Beck et al., 2006), patients have impaired ventilation (hypoventilation) which is highly prevalent in the second decade of life (Hukins and Hillman, 2000). This chronic hypoventilation can cause diurnal hypercapnia in patients (Katz et al., 2004). Due to the nature of the striated dilator muscles of the upper airway, it is likely that they are predisposed to contraction-induced injury and weakness similar to other skeletal muscles in DMD. Moreover, reports of patients experiencing airway obstructions (hypopnoea/apnoea) during sleep further supports their implication in DMD pathology.

The *mdx* mouse is a widely studied animal model of DMD which has a genetic mutation resulting in dystrophin deficiency (Bulfield et al., 1984). *Mdx* mouse diaphragm shows signs of muscle dysfunction (Coirault et al., 2003) and undergoes progressive degeneration

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similar to that seen in the human condition. The extent of knowledge of the consequences of dystrophin deficiency from the skeletal muscles of the upper airway in comparison to the diaphragm is limited for both human patients and the *mdx* mouse. Sternohyoid muscle, a representative pharyngeal dilator phasically active during inspiration and involved in the control and maintenance of upper airway patency (Van de Graaff et al., 1984; van Lunteren et al., 1987), shows signs of mechanical dysfunction in the *mdx* mouse at 6 months of age and an alteration in the muscle fibre type composition compared to age-matched wild-type (WT) controls (Attal et al., 2000). Interestingly, normoxic ventilation is impaired in *mdx* mice (6–7 months) and associated with reduced arterial PO_2—both features of the human condition (Mosqueira et al., 2013a). We have recently shown that impaired normoxic ventilation is also present in young adult (8 weeks) *mdx* mice (Burns et al., 2015). Previously we demonstrated that hypoxia causes respiratory muscle dysfunction in animal models of respiratory disease, with evidence of respiratory muscle weakness in diaphragm (Shortt et al., 2014, 2013; McMorrow et al., 2011; Dunleavy et al., 2008; Carberry et al., 2014) and sternohyoid muscle (Dunleavy et al., 2008; Skelly et al., 2010b, 2012). Farkas et al. (2007) have shown that intermittent hypoxia (central feature of SDB) causes further exacerbation of the *mdx* diaphragm muscle phenotype. Although hypoxia is a feature of DMD, there is a paucity of information within the current literature concerning the putative role of hypoxia in DMD pathophysiology.

Given that dystrophin deficiency impairs skeletal muscle function, we sought to characterise sternohyoid muscle function (pharyngeal dilator involved in the maintenance of upper airway calibre) in 8 week old *mdx* mice and WT controls. Since hypoxia is a feature of DMD, we examined the effects of an acute (6 h) sustained hypoxic stress on *mdx* and WT sternohyoid muscle function. We hypothesised that dystrophin deficiency would impair sternohyoid muscle function and reveal enhanced susceptibility to hypoxic stress compared with WT controls.

2. Methods

2.1. Ethical approval

All procedures were performed under licence from the Irish Government Department of Health and Children in accordance with National and European guidelines following local research ethics committee approval.

2.2. Animals

Male and female WT (C57BL/10ScSnJ) and *mdx* (C57BL/10ScSn-Dmd^{mdx}/J) mice were purchased from the Jackson Laboratory (Jackson Laboratory, Bar Harbor, ME) and were bred in our institution's animal housing facility. Animals were housed conventionally in a temperature- and humidity-controlled facility, operating on a 12 h light: 12 h dark cycle with food and water available *ad libitum*. 8 week old male WT and *mdx* mice were studied. In the first set of experiments, animals were exposed to 6 h of a premixed hypoxic gas mixture (10% O_2, balance N_2; Irish Oxygen Ltd.) or normoxia (control) in plethysmography chambers (BUXCO Ltd., Europe). Animals were assigned at random into four groups: WT normoxia ($n = 8$; 23.2 ± 0.3 g), WT hypoxia ($n = 10$; 22.3 ± 0.7 g), *mdx* normoxia ($n = 8$; 25.1 ± 0.6 g) and *mdx* hypoxia ($n = 8$; 24.1 ± 0.6 g). In the second series, naïve (untreated) WT ($n = 5$; 23.8 ± 0.4 g) and *mdx* ($n = 5$; 27.4 ± 0.4 g) mice of the same age were used for the assessment of the effects of severe hypoxia on sternohyoid muscle force *ex vivo*.

2.3. Experimental protocol

2.3.1. In vitro *muscle preparation*

Animals were anaesthetised with 5% isoflurane by inhalation in oxygen and euthanized by cervical transection of the spinal cord. The sternohyoid muscles were immediately excised and placed in a tissue bath at room temperature containing continuously gassed hyperoxic (95% O_2/5% CO_2) 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). The paired sternohyoid muscles were carefully separated along a natural division in the midline. A single longitudinal muscle strip (1–2 mm in diameter) from each animal was studied in a water-jacketed muscle bath maintained at 35 °C gassed with 95% O_2/5% CO_2. Each muscle strip was placed between a pair of platinum plate electrodes, with the caudal end fixed to an immobile hook and the rostral end attached to a dual-mode lever transducer system by non-elastic string. Muscle preparations were allowed a 5 min equilibration period.

2.3.2. *Isometric protocol*

Following equilibration, the optimum length (L_o) was determined by adjusting the position of the force transducer by use of a micro-positioner as previously described (Skelly et al., 2011). The L_o was taken as the muscle length associated with maximal isometric twitch force in response to single isometric twitch stimulation (supramaximal stimulation, 1 ms duration). Once L_o was determined, the muscle stayed at this length for the duration of the protocol. A single isometric twitch was measured. Peak isometric twitch force (P_t), contraction time (CT; time to peak force) and half relaxation time (½ RT; time for peak force to decay by 50%) were determined. Similarly, an isometric tetanic contraction was elicited by stimulating muscle strips with supramaximal voltage at 100 Hz for 300 ms duration (Shortt et al., 2014; Skelly et al., 2010a, 2012). Peak isometric tetanic force (P_o) was determined.

2.3.3. *Isotonic protocol*

Following the isometric protocol, concentric contractions were elicited in incremental steps with varying load (0.001%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 60%, 80%, 100%; % of P_o) with 30 s rest between each contraction. Muscle length returned to L_o following each contraction. Total shortening was determined as the maximum distance shortened during contraction. Shortening velocity was determined as the distance shortened during the initial 30 ms of shortening. Mechanical work (force × total shortening) and power (force × shortening velocity) were determined at each step of the incremental load step test.

2.3.4. *Repeated stimulation (fatigue)*

Five minutes following the isotonic protocol, repeated muscle contraction was induced by stimulation at 33% load with 300 ms trains every 2 s for a period of 250 s to produce isotonic fatigue. Total shortening and shortening velocity were measured as described above allowing calculation of mechanical work and power over the duration of the fatigue test. Following completion of the fatigue test, muscle bundles were blotted dry and weighed.

2.3.5. *Muscle bath hypoxia*

Sternohyoid muscle was dissected and mounted in the muscle bath as described above (Section 2.3.1). Muscle strips were studied under isometric conditions following a 5 min equilibration period under hyperoxic (control) conditions (Section 2.3.2). Following this assessment, muscle bundles were exposed to an anoxic gas mixture (95% N_2/5% CO_2) for 15 min, producing a tissue bath PO_2 of ~45 mmHg (Skelly et al., 2010a). The isometric protocol described above was then performed on the same muscle strips. In order to