Chronic hypoxia increases rat diaphragm muscle endurance and Na^+-K^+ ATPase pump content

Online supplement

METHODS

Animals

Experiments were performed on 72 adult (3 month old) male Wistar rats (Harlan, UK). All protocols described in this study were approved by local ethics committee and performed under licence from the Irish Government Department of Health and Children. In each series of experiments, animals were randomly separated into control (normoxic) and chronic hypoxia (CH) groups.

Hypobaric hypoxia

CH rats were placed in a hypobaric chamber for 1-6 weeks. Decompression to a target pressure of 380 mmHg was achieved over a 2-3 hour period. Ambient PO_2 was ~ 80mmHg equivalent to an F_IO_2 of 10.5%. Food and water were available *ad libitum*. Chamber pressure was measured continuously using a digital manometer (model C9505, Comark Ltd, UK). Pressure fluctuations due to drift were ±2% of the target pressure. Ambient CO_2 was measured periodically and was <1%. Age- and weight-matched control animals were held at ambient atmospheric pressure (~760 mmHg) in parallel.

Effects of CH on respiratory muscle function

In the first set of studies, adult male rats were exposed to normoxia or CH for 1 (n=12), 2 (n=12), 3 (n=12) and 6 (n=24) weeks. At the end of the treatment periods,

sternohyoid and diaphragm muscle contractile and endurance properties were determined *in vitro*. Sternohyoid, right hemi-diaphragm, soleus and extensor digitorum longus (EDL) from normoxic and CH animals were snap frozen in isopentane, cooled in liquid nitrogen, and stored at –80^{o}C until later use.

In vitro muscle preparation

After the treatment periods, animals were euthanized by a stunning blow and cervical spinal cord transection. Blood was sampled in heparanised capillary tubes in triplicate for haematocrit determination. The heart was removed for determination of right and left (plus septum) ventricular mass. The paired sternohyoid muscles and left costal hemidiaphragm with central tendon and lower rib intact were excised and immediately placed in continuously aerated (95% O_2/5% CO_2) physiological salt solution (PSS). The solution contained the following components: NaCl 120mM, KCl 5mM, Ca^{2+} gluconate 2.5mM, $MgSO_4$ 1.2mM, NaH_2PO_4 1.2mM, $NaHCO_3$ 25mM and glucose 11.5mM (pH 7.4). Longitudinal strips of muscle (~1.5mm diameter) were prepared and placed vertically in Plexiglas tissue holders in PSS in water-jacketed organ baths at 30°C aerated with either 95% O_2/5% CO_2 for control studies or 95% N_2/5% CO_2 to create tissue hypoxia. The neuromuscular paralyzing agent, d-tubocurarine (25μM), was added to the bathing medium to eliminate activation of intramuscular nerve branches thus ensuring that isometric force generation was due solely to direct muscle stimulation. Sternohyoid muscle strips were positioned between a pair of platinum plate electrodes, with the base fixed to an immobile hook and the other end tied to an isometric force transducer with non-elastic string. For the diaphragm, rectangular strips dissected parallel to the long axis of muscle fibres were used. The rib margin was anchored to the hook and the central tendon was attached to

the transducer. The position of the force transducer could be adjusted by a micro-positioner thus altering the length of the muscle strips. A fixed graduated scale located behind the muscle strip allowed the accurate measurement of muscle length.

Protocol

The optimum length (L_O, muscle length producing maximal isometric twitch force in response to supra-maximal stimulation) was determined by incrementally adjusting the micro-positioner between intermittent stimulations. Once determined the muscles remained at this length for the full protocol. Next, the muscle was allowed a 15 min equilibration period. The single isometric twitch force, contraction time (CT), half-relaxation time (HRT), stress-frequency relationship and fatigue tolerance were then determined in response to electrical field stimulation delivered via the plate electrodes flanking the tissue connected to a square pulse constant current stimulator (Model S44, Grass Instruments, Quincy, Massachusetts). Data were recorded using a commercial data acquisition system (PowerLab, AD Instruments, UK) and stored for later analysis on a computer. First, a single twitch was elicited (supra-maximal voltage, 1 ms duration). Twitch force, CT (time to peak force) and HRT (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, 60, 80 and 100Hz for 300 ms at each stimulus frequency allowing a 2 min recovery interval between each stimulus. Five min following the force–frequency protocol, fatigue was induced by stimulation at40 Hz with 300 ms trains every 2 sec for a period of 5 min. Studies of contractile properties of sternohyoid and diaphragm muscle bundles from normoxic and CH animals were performed under *in vitro* hyperoxic (control)

conditions. All compounds were purchased from Sigma–Aldrich Company, Dublin, Ireland and made up fresh each day.

SDH and NADPH-diaphorase histochemistry in respiratory and limb muscles

Serial transverse 10μm sections were cryosectioned (Model CM30505, Leica Microsystems, Nussloch, Germany) at –22^{o}C and were mounted on polysine-coated glass slides. To determine oxidative capacity muscle sections were stained histochemically for the mitochondrial enzyme succinate dehydrogenase (SDH). SDH activity was determined using an incubation solution containing sodium succinate and nitro blue tetrazolium chloride (NBT) in phosphate buffer brought to pH 7.4 using drops of NaOH (1M). The incubation period was 20 mins at 37^{o}C. NADPH-diaphorase activity was determined using an incubation solution containing β-NADPH and NBT in phosphate buffer (pH 7.4). Incubation was for 40 mins at 37^{o}C. Normoxic and CH muscle sections were processed in parallel. Control reactions were performed on serial sections using solutions that lacked substrate to ensure that non-specific redox reactions did not contribute to the blue histochemical staining. All slides were then dehydrated in a graded series of acetone and methanol rinses. Slides were then passed through xylene and cover-slipped.

MHC immunohistochemistry in respiratory and limb muscles

Indirect immunofluorescence for MHC isoform composition was performed on serial unfixed muscle sections. Serial transverse muscle sections (10μm thick) from normoxic and CH rats were cryosectioned and placed on polysine-coated glass slides. Sections were incubated for 30 mins in PBS, 1% bovine serum albumin and 5% goat serum to block non-specific binding of antibodies. Sections were incubated with one

of two antibody cocktails. Primary antibodies, developed by S. Schiaffino, were obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by University of Iowa, Department of Biology, Iowa City, USA. One half of the sections in normoxic and CH rats were incubated with a cocktail of primary antibodies which targeted MHC type 1, 2A and 2B fibres. The cocktail consisted of mouse anti-myosin antibodies: type 1 IgG2b (1:100), type 2A IgG1 (1:100) and type 2B IgM (1:25). The remaining sections were incubated with a mouse IgG1 primary antibody (1:10) that targeted all but myosin type 2X fibres. Muscle sections were incubated with the primary antibodies in a humidity chamber overnight at 4^{o}C. The sections were then washed three times in PBS before application of specific secondary antibodies. All fibre types apart from type 2X were highlighted with a Dylight594-conjugated anti-mouse IgG1 secondary antibody (1:500). For the other sections, secondary antibodies included an AlexaFluor350-conjugated goat anti-mouse IgG2b (1:500, Invitrogen), a Dylight594-conjugated goat anti-mouse IgG1 (1:500, Jackson) and an AlexaFluor488-conjugated goat anti-mouse IgM (1:250, Invitrogen). Secondary antibodies were applied for 1 hour in a dark humidity chamber at room temperature. Negative control experiments in which the primary antibodies were omitted were also performed.

SERCA immunohistochemistry in diaphragm muscle

Diaphragm sections were incubated overnight at 4^{o}C with a primary mouse IgG1 anti-SERCA2 antibody (catalogue number MA3-919; 1:500; Affinity Bioreagents Inc., USA) followed by a Dylight594-conjugated goat anti-mouse IgG1 (1:500, Jackson) secondary for 1 hour in a dark humidity chamber at room temperature. Negative control experiments in which the primary antibodies were omitted were also performed. Following MHC and SERCA immunohistochemistry protocols, slides

were rinsed in PBS, mounted with Vectashield (H-1000 Vector Laboratories, Peterborough, UK), cover-slipped and stored at 4^{o}C.

Measurements of the contents of [^{3}H]oubain binding sites, and Na^{+}, K^{+} and Ca^{2+} in muscle

Muscle content of the Na^{+}–K^{+} pump α_2 isoform was determined using the vanadate-facilitated [^{3}H]ouabain binding method, in which muscles are sectioned before incubation (1). Previously, it has been reported that the values for [^{3}H]ouabain binding obtained using this method are not significantly different from those obtained in intact muscles (1, 2). For the assay, approximately 20 mg of muscle was divided into four samples (each ~5 mg), and washed 2 x 10 min at 37°C in Tris–vanadate buffer (250mM sucrose, 10 mM Tris-HCl, 3mM $MgSO_4$ and 1mM $NaVO_4$; pH 7.25–7.30). Muscle samples were then incubated for 120 min at 37°C in the same buffer with the addition of [^{3}H]ouabain (10^{-6}M, 2 μCi.ml^{-1}) and were then washed 4 x 30 min in ice-cold vanadate buffer to remove any unbound [^{3}H]ouabain. Following washout, samples were blotted, the four pieces from each muscle sample were combined, muscle wet weight was determined, and the samples were soaked overnight in 0.5 ml 0.3 M TCA containing 0.1mM ouabain as carrier. After overnight soaking, 2.5 ml of scintillation cocktail (Opti-Fluor, Packard, Perkin Elmer, Boston, MA, USA) was added to 0.5 ml of the TCA extract for counting in the β-counter. The content of [^{3}H]ouabain binding sites was calculated on the basis of both the sample wet weights and the specific activity of the incubation medium. The final [^{3}H]ouabain binding site content was then calculated by subtracting the non-specific [^{3}H]ouabain uptake measured using vanadate buffer containing an excess of unlabelled ouabain.

To determine muscle ion contents, muscle samples were weighed to determine wet weight (ww) and the samples were soaked overnight in 3 ml 0.3M TCA to extract Na^+, K^+ and Ca^{2+}. Ca^{2+} content was determined by atomic absorption spectrophotometry (Solaar AAS Thermo, UK) using 1.5 ml of the TCA extract mixed with 150 µl of 0.27 M KCl. The muscle extracts were measured against a blank and standards containing 12.5 or 25 µM Ca^{2+} and the same amount of TCA and KCl as the muscle extracts. Na^+ and K^+ contents of the TCA extracts were determined using a Radiometer FLM3 flame photometer (Copenhagen, Denmark) with lithium as internal standard. For each 0.5 ml sample of the TCA extract, 1.5 ml 5 mM LiCl and 0.5 ml 0.3 M TCA were added.

Effect of chronic NOS inhibition on diaphragm muscle structure and function

In a separate series of experiments, adult male rats were treated chronically with L-NNA (2mM in the drinking water) commencing 3 days before normoxia (n=6) or CH (n=6) treatments and continuing throughout a 6-week treatment period. Fresh solutions were made every 1-2 days. At the end of the chronic drug study, structural (MHC isoform, fibre morphology, oxidative capacity and Na^+-K^+ pump content) and functional assessments of diaphragm muscle were performed.

Data Analysis

Specific force was calculated in N/cm^2 of muscle cross-sectional area (CSA). The latter was approximated by weighing the dry muscle strips 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 CT and HRT were measured as indices of isometric twitch kinetics. For the force-frequency relationship, data were plotted as

absolute stress across the range of stimulus frequencies employed in the study. Fatigue was assessed by determining the amplitude of tetanic contractions at 1, 2, 3, 4 and 5 mins of the fatigue trial, expressing force at each data point as a percentage of the initial contraction; the average of these 5 data points across the fatigue trial was calculated to generate a single fatigue index. Values are expressed as mean ± S.E.M. Three-way ANOVA (frequency x hypoxia x drug) with $P<0.05$ taken as significant was used to statistically compare the force-frequency relationships. Two-way ANOVA (hypoxia x drug) and Student's *t* tests were used elsewhere as appropriate with $P<0.05$ taken as significant. To determine SDH and NADPH-diaphorase activity, low power, bright-field images of muscle sections were captured under controlled lighting conditions using a BX51 Olympus microscope (Olympus Life Science Microscopes, Munchen, Germany) and an Olympus DP71 camera. Optical density of muscle sections was calculated using Scion Image™ software (Scion Corportion, Maryland, USA) according to the following equation $y = \log_{10}(255/(255-X))$ for a 256 grayscale image, where X equals the gray value of the image. Multiple sections were analyzed per animal and an average optical density was calculated. Normoxic and CH muscles were compared using a Student's *t* test with $P<0.05$ taken as significant. For immunofluorescence analysis, Cell A™ (Olympus) software was used to digitally analyze images and calculate numerical and areal density and CSA for each MHC fibre type. A stereological method of a square test frame (640,000μm^2) with inclusion and exclusion boundaries randomly placed over muscle sections (three per animal) was employed. Areal density was calculated as the sum of the CSAs for a positively-labelled fibre divided by the area of the square test frame x 100. Relative area of fibres containing SERCA2 was calculated in the same fashion. This approach was considered more physiologically relevant (3) than simple fibre type counts (i.e.

numerical density). All data per animal were first averaged before computing group means.

1. Norgaard A, Kjeldsen K, Hansen O, Clausen T. 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 Comm* 1983; 111: 319-325.
2. McKenna MJ, Gissel H, Clausen T. Effects of electrical stimulation and insulin on Na^{+}-K^{+}-ATPase ([3H]ouabain binding) in rat skeletal muscle. *J Physiol* 2003; 547: 567-580.
3. Egginton S. Numerical and areal density estimates of fibre type composition in a skeletal muscle (rat extensor digitorum longus). J Anat 1990; 168: 73-80.