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Research Paper

Manipulation of environmental oxygen modifies reactive oxygen and nitrogen species generation during myogenesis

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ABSTRACT

Regulated changes in reactive oxygen and nitrogen species (RONS) activities are important in maintaining the normal sequence and development of myogenesis. Both excessive formation and reduction in RONS have been shown to affect muscle differentiation in a negative way. Cultured cells are typically grown in 20% O_2 but this is not an appropriate physiological concentration for a number of cell types, including skeletal muscle. The aim was to examine the generation of RONS in cultured skeletal muscle cells under a physiological oxygen concentration condition (6% O_2) and determine the effect on muscle myogenesis.

Primary mouse satellite cells were grown in 20% or 6% O_2 environments and RONS activity was measured at different stages of myogenesis by real-time fluorescent microscopy using fluorescent probes with different specificities i.e. dihydroethidium (DHE), 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) and 5-(and-6)-chloromethyl-2′,7′ -dichlorodihydrofluorescein diacetate (CM-DCFH-DA).

Data demonstrate that satellite cell proliferation increased when cells were grown in 6% O_2 compared with 20% O_2. Myoblasts grown in 20% O_2 showed an increase in DCF fluorescence and DHE oxidation compared with myoblasts grown at 6% O_2. Myotubes grown in 20% O_2 also showed an increase in DCF and DAF-FM fluorescence and DHE oxidation compared with myotubes grown in 6% O_2. The catalase and MnSOD contents were also increased in myoblasts and myotubes that were maintained in 20% O_2 compared with myoblasts and myotubes grown in 6% O_2. These data indicate that intracellular RONS activities in myoblasts and myotubes at rest are influenced by changes in environmental oxygen concentration and that the increased ROS may influence myogenesis in a negative manner.

1. Introduction

Skeletal muscle satellite cells are ubiquitous mononuclear cells, classically identified by their location between the fibre plasmalemma and basal lamina [1]. Satellite cells play an important role during skeletal muscle injury and regeneration. When skeletal muscle damage occurs, satellite cells within the muscle bulk are activated and proliferate to become myogenic precursor cells (mpc's otherwise known as myoblasts; [2]). Proliferating myoblasts migrate to the damaged region of the muscle, fuse to form myotubes and differentiate to form skeletal muscle. Regeneration of skeletal muscle *in vivo* involves similar processes to those occurring during myogenesis and can be studied in well-characterised cell culture models.

The environmental O_2 concentration used for satellite cell cultivation *in vitro* is almost always 20%, whereas normal adult skeletal muscle tissue O_2 levels are significantly lower, potentially between 1.8 and 10.5% [3,4]. Environmental oxygen concentration has been previously shown to modify satellite cell behaviour [3] in a process that has been linked to reactive oxygen species (ROS) generation [5]. The mechanisms by which ROS mediate myogenesis are unclear but are likely due to changes in gene expression via redox-sensitive transcription factor activation [5]. However, the pattern of generation of specific ROS in skeletal muscle cells during the processes of myogenesis under different oxygen concentrations is currently unknown.

The aim was therefore to examine the activities of RONS in cultured skeletal muscle cells under approximately physiological

Abbreviations: CuZnSOD, copper, zinc superoxide dismutase; MnSOD, manganese superoxide dismutase; mpc's, myogenic precursor cells; NFκB, nuclear transcription factor κappa B; NO, nitric oxide; ROS, reactive oxygen species; RONS, reactive oxygen and nitrogen species; SOD, superoxide dismutase; SOD1, copper, zinc superoxide dismutase; WT, wild type; H_2O_2, hydrogen peroxide

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conditions (6% oxygen) compared with 20% O_2 and also determine the effect of the different O_2 concentrations on muscle myogenesis. Primary skeletal muscle cultures were grown in 20% or 6% oxygen environments and RONS were assessed at different stages of myogenesis using RONS-sensitive fluorescent probes [6–8]. Use of these probes allows the assessment of specific RONS in single cells in real time. The fluorescent probes dihydroethidium (DHE), 5-(and-6)-chloromethyl-2′,7′ -dichlorodihydrofluorescein diacetate (CM-DCFH-DA) and 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) were used in this study. DCFH reacts with hydrogen peroxide (H_2O_2) in the presence of peroxidases and less rapidly with some other ROS, DAF-FM reacts with NO and peroxynitrite, and DHE is primarily oxidised by superoxide. Changes in fluorescence in skeletal muscle myoblasts and myotubes were measured using fluorescence microscopy.

Our hypothesis was that myoblasts and myotubes grown in 20% O_2 would have increased superoxide content leading to an increase in intracellular DCF and DHE oxidation but no effect on DAF-FM fluorescence compared with cells grown in 6% O_2 and that this would be associated with reduced myogenesis in the myoblasts grown in 20% O_2.

2. Materials and methods

2.1. Cultures of skeletal muscle myoblasts and myotubes

Myoblasts were derived from adult (4–8 months old) male wild-type (WT) mice. Primary mouse myoblasts were prepared from hind leg muscles as previously described [6]. Briefly, muscles were digested in 0.1% pronase solution. Cells were cultured in 35 mm gelatin coated tissue culture plates in DMEM containing 20% (v/v) FCS. Cells were incubated at 37 °C in a water saturated atmosphere containing 5% (v/v) CO_2 in either 20% or 6% oxygen environments. To induce myotube formation the medium was replaced with DMEM containing 2% horse serum containing 2% horse serum (HS) with 0.45% (*w*/*v*) glucose with 2 mM glutamine, 50 I.U./ml penicillin and 50 μg/ml streptomycin.

2.2. Loading of cells with fluorophores

To detect intracellular ROS and nitric oxide, myoblasts and myotubes at 5–7 days following differentiation (multinuclear fused) were loaded with different fluorophore probes; 5-(and-6)-chloromethyl-2′,7′ -dichlorodihydrofluorescein diacetate (CM-DCFH-DA) (Molecular Probes™, Invitrogen) was used as a general probe for ROS, 4-amino-5-methylamino-2′,7′ -difluorofluorescein diacetate (DAF-FM-DA) as an indicator of nitric oxide and dihydroethidium (DHE) as an indicator of superoxide activity (all purchased from Molecular Probes™, Invitrogen). Cells were loaded with CM-DCFH-DA (10 μM) DHE (5 μM) or DAF-FM-DA (10 μM) in D-PBS for 30 min at 37°. Cells were then washed with D-PBS and the media replaced with D-PBS alone.

2.3. Microscopy and fluorescent imaging

Images were obtained using a C1 confocal laser-scanning microscope (Nikon Instruments Europe BV, Surrey, UK) equipped with a 405 nm excitation diode laser, a 488 nm excitation argon laser, and a 543 nm excitation helium-neon laser. Emission fluorescence was detected through a set of 450/35, 515/30 and 605/15-emission filters. Fluorescence images were captured and analysed with the EZC1 V.3.9 (12 bit) acquisition software. For the cultures grown at 6% oxygen, oxygen concentration was maintained at 6% for the duration of the experiments. All experiments were carried out at 25 °C.

2.4. Analysis of MnSOD, Cu/ZnSOD and catalase content of myoblasts and myotubes

Myoblasts and myotubes at 5–7 days following differentiation were harvested and sonicated in 1% SDS containing 1 mM iodoacetimide, 1 mM benzithonium chloride, and 5.7 mM phenylmethylsulfonyl fluoride and 5 mM EGTA (Sigma Co.). Following sonication, cellular debris was removed by centrifugation, and samples were stored at −70 °C until analysis. Protein content of samples was determined by using the bicinchoninic acid method (Sigma Co.). Twenty micrograms of total cellular protein was separated on SDS-PAGE followed by Western blotting. Ponceau S staining (Po-S; Sigma Co.) was used to visualise the protein loading during western blot analysis. The contents of manganese superoxide dismutase (MnSOD) and copper/zinc superoxide dismutase (Cu/ZnSOD) were analysed by using rabbit polyclonal antibodies obtained from Enzo scientific (Cat. no. ADI-SOD-111F and ADI-SOD-100F respectively). The content of catalase was analysed using a mouse monoclonal anti-catalase antibody obtained from Sigma (Cat. no C0979). Bands were visualised and analysed using a Biorad Chemi-Doc System (Bio-Rad, Hercules, CA).

2.5. Statistics

Data are presented as mean + SE of values for 6–8 wells for each experiment. Data were initially analysed by analysis of variance followed by modified Student's *t* test. Data were considered significant at $p < 0.05$.

3. Results

3.1. Cell morphology

Myoblasts grown either in 6% or 20% oxygen proliferated in culture and, following addition of differentiation medium, formed myotubes. There were some variations in the efficiency of proliferation and fusion; proliferation of satellite cells was clearly increased when cells were grown in 6% O_2 compared with cells grown in 20% O_2 (Fig. 1). Analyses of RONS activities in myoblasts and myotubes were undertaken at an approximately equivalent stage of maturation rather than at precisely the same time point following differentiation.

3.2. Effect of a 20% or 6% O_2 environment on DHE, DCF and DAF-FM fluorescence in myoblasts

DHE, DCF and DAF-FM fluorescence was measured from 4–6 myoblasts in each culture well (6–8 wells in total). Example images of myoblasts loaded with the 3 fluorophores are shown in Fig. 2A–C. Background measurements of fluorescence from areas of the well where myoblasts were not present were also undertaken. DHE, DCF and DAF-FM fluorescence (minus background readings) was measured over three 10-min periods for a total of 30 min. Myoblasts grown in 20% O_2 showed a significant increase in DHE (Fig. 3A) and DCF (Fig. 3B) fluorescence at each time point compared with myoblasts grown in 6% O_2. No significant differences were seen in DAF-FM fluorescence between myoblasts grown in 6% and myoblasts grown in 20% O_2 concentrations (Fig. 3C).

3.3. Effect of a 20% or 6% O_2 environment on DHE, DCF and DAF-FM fluorescence in myotubes

DHE oxidation and DCF and DAF-FM fluorescence were measured from at least 4 myotubes in each culture well as previously