Free Radical Biology and Medicine 78 (2015) 82–88

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Nitric oxide availability is increased in contracting skeletal muscle from aged mice, but does not differentially decrease muscle superoxide

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ARTICLE INFO

Article history:
Received 22 July 2014
Received in revised form
26 September 2014
Accepted 13 October 2014
Available online 22 October 2014

Keywords:
NO
ROS
Aging
Skeletal muscle
Fluorescent confocal microscopy

ABSTRACT

Reactive oxygen and nitrogen species have been implicated in the loss of skeletal muscle mass and function that occurs during aging. Nitric oxide (NO) and superoxide are generated by skeletal muscle and where these are generated in proximity their chemical reaction to form peroxynitrite can compete with the superoxide dismutation to hydrogen peroxide. Changes in NO availability may therefore theoretically modify superoxide and peroxynitrite activities in tissues, but published data are contradictory regarding aging effects on muscle NO availability. We hypothesised that an age-related increase in NO generation might increase peroxynitrite generation in muscles from old mice, leading to an increased nitration of muscle proteins and decreased superoxide availability. This was examined using fluorescent probes and an isolated fiber preparation to examine NO content and superoxide in the cytosol and mitochondria of muscle fibers from adult and old mice both at rest and following contractile activity. We also examined the 3-nitrotyrosine (3-NT) and peroxiredoxin 5 (Prx5) content of muscles from mice as markers of peroxynitrite activity. Data indicate that a substantial age-related increase in NO levels occurred in muscle fibers during contractile activity and this was associated with an increase in muscle eNOS. Muscle proteins from old mice also showed an increased 3-NT content. Inhibition of NOS indicated that NO decreased superoxide bioavailability in muscle mitochondria, although this effect was not age related. Thus increased NO in muscles of old mice was associated with an increased 3-NT content that may potentially contribute to age-related degenerative changes in skeletal muscle.

Introduction

Aging results in a loss of physical capacity and increased frailty through a reduction in skeletal muscle mass and function (sarcopenia) [1] through a substantial reduction in muscle cross-sectional area and reduced force generation [2,3]. In man such changes result in an increased risk of a fall [4] and need for residential care [5] that can have a significant impact upon an individual's quality of life and personal and financial independence.

During aging, the skeletal muscles of old rodents contained increased amounts of oxidised lipid, DNA, and proteins in comparison with young or adult rodents (e.g., [6–8]). Increased superoxide generation has been implicated in the process of aging in skeletal muscle and other tissues [7,9]. Superoxide and nitric oxide (NO) are the primary radical species generated in skeletal muscle and their generation is increased during contractile activity [10–13]. Superoxide and NO are the precursors for the generation of a number of secondary species and muscle has enzymatic systems to control the cellular activities of these species. When superoxide and NO are both present, their chemical reaction to form peroxynitrite is likely and competes with the dismutation of superoxide to hydrogen peroxide by SOD [14].

Previous studies have examined the activity of ROS in skeletal muscle during aging using the nonspecific fluorescent probe DCFH [15]. These data indicated that ROS activities were increased in isolated muscle fibers from old mice at rest in comparison with fibers from adult mice, but that the increase in ROS following contractile activity normally seen in fibers from adult mice was not seen in those from old mice. Unfortunately these studies do not allow the specific species involved to be determined since the DCFH probe has been reported to be oxidised by hydrogen peroxide, NO, hydroxyl radical, and peroxynitrite [16]. In a mouse model showing an accelerated skeletal muscle aging phenotype (SOD1null mice), we have previously obtained data to indicate that peroxynitrite is formed in excess in skeletal muscle and appears to play a role in the muscle loss [17]. Detection of peroxynitrite is difficult in biological processes and is usually inferred from monitoring and modification of cellular NO and superoxide that

Abbreviations: DHE, dihydroethidium; MEM, minimum essential medium Eagle; NO, nitric oxide; NOS, nitric oxide synthases; 3-NT, 3-nitrotyrosine; Prx5, peroxiredoxin 5

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http://dx.doi.org/10.1016/j.freeradbiomed.2014.10.505

combine to form peroxynitrite and from examining the content of 3-nitrotyrosines (3-NT) of muscle proteins [8,13,17,18], since these posttranslational modifications are usually formed following the reaction of tyrosine residues with peroxynitrite [14].

A number of studies have examined the effect of age on superoxide generation from mitochondria or mitochondrial particles extracted from muscles of different species [19–22] and concluded that aging increases mitochondrial superoxide generation, but studies of NO synthesis and bioavailability in muscle during aging have provided contradictory data. NO is generated by the activity of the nitric oxide synthases (NOS) and studies have reported an age-related increase [23] or decrease [24] in the muscle protein content of nNOS (NOS1), increased iNOS (NOS3) protein content [25,26] in rodent muscle, and an increase in both nNOS and eNOS (NOS2) in muscle from older humans [27]. This latter study also examined the bioavailability of NO in muscle and conversely found an age-related decrease in interstitial NO in the presence of the increased muscle content of the 2 NOS enzymes [27].

Our hypothesis was that that an age-related increase in NO generation might increase peroxynitrite generation in muscles from old mice, leading to an increased nitration of muscle proteins and decreased superoxide availability. The aim of this study was therefore to examine NO in muscle fibers from adult and old mice both at rest and following contractile activity and to determine the effect of any age-related changes in NO on the levels of superoxide detected in cytosol and mitochondria. We also examined 3-NT and the peroxiredoxin 5 (Prx5) content of muscles from old mice as markers of increased peroxynitrite activity.

Materials and methods

Mice

These studies used adult (5–7 months) and old (26–28 months) male C57Bl/6 mice. All experiments were performed in accordance with UK Home Office guidelines and under the UK Animals (Scientific Procedures) Act 1986. Mice were killed by Schedule 1 and the flexor digitorum brevis (FDB) muscle was rapidly removed (see below). The gastrocnemius muscles were also rapidly removed and snap-frozen in liquid nitrogen for future analysis.

Isolation of single mature skeletal muscle fibers

Single fibers were isolated from the FDB muscles of mice [28]. Briefly, mice were killed and the FDB muscles were rapidly dissected. Muscles were incubated for 1.5 h at 37 °C in 0.4% (w/v) sterile type I collagenase (EC 3.4.24.3, Sigma Chemical Co., Poole, Dorset, UK) in minimum essential medium Eagle (MEM) media containing 2 mM glutamine, 50 IU penicillin, 50 μg ml^{-1} streptomycin and 10% fetal bovine serum (FBS, Sigma Chemical Co., Poole, Dorset, UK). The muscles were agitated every 30 min during the digestion period. Single myofibers were released by gentle trituration with a wide-bore pipette and fibers were washed three times in MEM media containing 10% FBS. Fibers were plated onto precooled 35-mm glass-bottomed cell culture dishes (MatTek, MA, USA) precoated with Matrigel (BD Biosciences, Oxford, UK) and were allowed to attach before adding 2 ml MEM media containing 10% FBS. Fibers were incubated for 20 h at 37 °C in a 5% CO_2 tissue culture incubator. Fibers prepared and cultured in this manner are viable for up to 6 days in culture though in this study all fibers were used within 30 h [15]. Experiments were only performed on fibers that displayed excellent morphology and exhibited prominent cross-striations.

Chemicals

MitoSox Red, DHE, and DAF-FM DA (Invitrogen, Paisley, UK) were all made in DMSO, vehicle equivalent to 0.0125, 0.1, and 0.2%, respectively (no effect of the vehicle was found). MEM-eagles, D-PBS, and L-NAME were from Sigma Chemical Co. MEM solution consisted of (in mM) $MgSO_4 \cdot H_2O$ 0.8, KCl 5.4, NaCl 116.4, $NaH_2PO_4 \cdot H_2O$ 1, D-glucose 5.5, $NaHCO_3$ 26.2, Hepes 10, $CaCl_2 \cdot 2H_2O$ 1.9 and pH 7.4; salts were acquired from Sigma Chemical Co.

Use of DAF-FM DA to monitor nitric oxide in isolated fibers

NO availability was examined using the NO-specific probe DAF-FM essentially as described by Pye et al. [29]. In brief, fibers were loaded by incubation in 2 ml Dulbecco's phosphate-buffered saline (D-PBS) containing 10 μM DAF-FM DA for 30 min at 37 °C in a tissue culture incubator. Cells were washed twice with D-PBS and two further washes using MEM solution; the fibers were then maintained in 2 ml MEM solution during the experimental protocol. DAF-FM DA readily diffuses into cells and within the cytoplasm releases DAF-FM by the action of intracellular esterases. DAF-FM is essentially nonfluorescent until it is nitrosylated by products of oxidation of NO, resulting in DAF-FM triazole that exhibits about a 160-fold greater fluorescence efficiency [30].

Use of dihydroethidium to monitor cytoplasmic superoxide in isolated fibers

Cytoplasmic superoxide was examined using dihydroethidium (DHE, hydroethidine) as described by Pearson et al. [31]. In brief, fibers were loaded by incubation in 2 ml D-PBS containing 5 μM DHE for 20 min at 37 °C in a tissue culture incubator. Cells were then washed twice with D-PBS and two further washes using MEM. The fibers were maintained in 2 ml MEM solution during the experimental protocol. Additionally, fibers were either untreated or incubated with 100 μM L-NAME to block nitric oxide synthase for a minimum of 1 h prior to loading with DHE. These fibers were maintained in L-NAME throughout the experiment.

Use of MitoSox Red to monitor mitochondrial superoxide in isolated fibers

Mitochondrial superoxide was examined using MitoSox red as described by Pearson et al. [31]. Fibers were loaded by incubation in 2 ml D-PBS containing 125 nM MitoSox Red for 10 min at 37 °C in a tissue culture incubator. Cells were then washed twice with D-PBS and two further washes using MEM; the fibers were maintained in 2 ml MEM during the experimental protocol. Fibers were either untreated or incubated for a minimum of 1 h in the presence of 100 μM L-NAME as described above.

Confocal microscopy

A Nikon E-Ti inverted microscope with a motorised stage (TI-S-EJOY, Nikon) for a 35-mm petri dish was used. A C1 confocal microscope (Nikon Instruments Europe BV, Surrey, UK) comprising a diode (UV) 405 nm excitation, argon laser with 488 nm excitation, and a helium–neon laser with 543 nm excitation were used for live cell imaging. Acquisition software was EZC1 V.3.9 (12 bit). DHE and MitoSox Red were excited sequentially at 405 nm using a diode laser (25% intensity) and 488 nm (3% intensity) using an argon laser, each passing through a main dichroic and secondary beam splitter with the emission collected through a 605/15 filter to a detector. DAF-FM was excited at 488 nm and emission recorded between 515 and 30 nm. Bright-field images were acquired using the 543 nm laser to a CCD. The objective was a