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Antioxidant treatment of hindlimb-unloaded mouse counteracts fiber type transition but not atrophy of disused muscles

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ABSTRACT

Oxidative stress was proposed as a trigger of muscle impairment in various muscle diseases. The hindlimb-unloaded (HU) rodent is a model of disuse inducing atrophy and slow-to-fast transition of postural muscles. Here, mice unloaded for 14 days were chronically treated with the selective antioxidant trolox. After HU, atrophy was more pronounced in the slow-twitch soleus muscle (Sol) than in the fast-twitch gastrocnemius and *tibialis anterior* muscles, and was absent in *extensor digitorum longus* muscle. In accord with the phenotype transition, HU Sol showed a reduced expression of myosin heavy chain type 2A (MHC-2A) and increase in MHC-2X and MHC-2B isoforms. In parallel, HU Sol displayed an increased sarcolemma chloride conductance related to an increased expression of ClC-1 channels, changes in excitability parameters, a positive shift of the mechanical threshold, and a decrease of the resting cytosolic calcium concentration. Moreover, the level of lipoperoxidation increased proportionally to the degree of atrophy of each muscle type. As expected, trolox treatment fully prevented oxidative stress in HU mice. Atrophy was not prevented but the drug significantly attenuated Sol phenotypic transition and excitability changes. Trolox treatment had no effect on control mice. These results suggest possible benefits of antioxidants in protecting muscle against disuse.

1. Introduction

Adult skeletal muscles contain slow- and fast-twitch fibers according to their speed of contraction. Slow-twitch myofibers express the slow type-1 isoform of myosin heavy chain (MHC-1), whereas fast-twitch myofibers may express three types of fast MHC (MHC-2A, 2B or 2X) [1]. Differential expression of MHC isoforms affects specific force and is a major determinant of the maximal velocity of shortening of individual muscle fiber. Noteworthy, adult muscle fibers can adapt their phenotype to modified functional requests by expressing different levels or forms of proteins involved in the control of muscle excitability, excitation–contraction coupling, energy metabolism, and contractile function. For instance a slow-to-fast phenotype transition is a hallmark of muscle adaptation to reduced neuromuscular activity [1]. A slow-to-fast transition was also observed in hindlimb muscles of rats treated with a β2-adrenoceptor agonist, and was associated with muscle function impairment [2]. Thus such phenotypic functional changes likely contribute to disuse-induced muscle impairment, and consequently represent a target for prevention. Importantly, the promotion of slow, oxidative fibers has been shown to mitigate the progression of muscular dystrophy and proposed as a possible countermeasure to overcome muscle dysfunction associated with insulin-resistant states [3].

In the hindlimb-unloaded (HU) rat, a model for muscle disuse that mimics the effects of microgravity, a partial slow-to-fast fiber type transition occurs in the postural, slow-twitch soleus (Sol) muscle. After 14 days of HU, the proportion of fast MHC-positive fibers may reach ~40% in the Sol muscle compared to <15% in control rats [4]. These effects are slowly reversed after several weeks of reloading [5]. Little is known about the triggers and molecular mechanisms responsible for disuse-induced fiber type transition. We previously described the effects of HU on ion channels that are critical for phenotype-specific sarcolemma excitability

Abbreviations: HU, hindlimb unloading; Sol, soleus muscle; Gas, gastrocnemius muscle; EDL, *extensor digitorum longus* muscle; TA, *tibialis anterior* muscle; MHC, myosin heavy chain; MDA, malondialdehyde; gCl, chloride conductance of sarcolemma at rest; gK, potassium conductance of sarcolemma at rest; restCa, resting cytosolic calcium concentration; MT, mechanical threshold; CSA, cross-sectional area.

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and contractile function, and may contribute to modulation of gene transcription [4,6–9]. For example, the macroscopic chloride conductance (gCl), which is by far the largest component conductance of adult muscle at resting potential and consequently a key determinant of muscle excitability, is higher in fast-twitch compared to slow-twitch muscle fibers. In accord with fiber type transition, the gCl increases in HU Sol muscle toward a value more similar to that measured in fast-twitch muscles [4]. Such an effect alters sarcolemma excitability in Sol muscle fibers and may contribute to impair resistance to fatigue and to trigger fiber type transition [8].

Besides phenotype transition, HU Sol muscles undergo a significant atrophy due mainly to an increased protein degradation. Muscle atrophy is a severe clinical symptom encountered in various diseases, which has thus deserved much more attention than fiber type transition. Oxidative stress has been observed in many of these diseases and has been recently pinpointed as a possible causative factor for muscle atrophy [10,11]. Whether oxidative stress may influence fiber type transition is however completely unknown.

In the present study, we studied the effects of 14 days HU on hindlimb muscles of 6-month-old mice as well as the effects of concomitant treatment with the potent and selective antioxidant trolox. Muscle atrophy, phenotype, and function were assessed in Sol and gastrocnemius (Gas) muscles using multidisciplinary approaches. Some parameters were also measured in the extensor digitorum longus (EDL) and tibialis anterior (TA) muscles. By measuring lipoperoxidation, we also verified whether oxidative stress occurs in HU mouse muscles and whether it is prevented by trolox treatment. Indeed administration of trolox was previously shown to attenuate diaphragm muscle atrophy induced by mechanical ventilation in rats [12].

The main results showed that the degree of atrophy in each muscle was parallel to the level of oxidative stress, but no beneficial effect of trolox treatment was found on muscle atrophy. Nevertheless trolox, which neutralized oxidative stress in all muscles, balanced the phenotype transition. These results suggest the possibility to use antioxidants to ameliorate phenotype-dependent, disuse-induced muscle functional impairment.

2. Material and methods

2.1. Animal care and hindlimb unloading

Experiments were approved by the Italian Health Department and complied to the Italian guidelines for the use of laboratory animals, which conform with the European Community Directive published in 1986 (86/609/ECC). Six-month-old male C57BL mice weighting 27–32 g (Charles River Laboratories, Calco, Italy) were randomly assigned to CTRL (control mice), HU (hindlimb-unloaded for 14 days), TRO (treated with trolox for 3 weeks) and HUTRO (treated with trolox for 3 weeks and hindlimb unloaded for the last two 14 days) groups. The CTRL mice were maintained free in single cages for 14 days. To induce muscle unloading, the animals of HU group were suspended individually in special cages for 2 weeks using a method similar to that used previously for HU rats [8]. A thin string was linked at one extremity to the tail by sticking plaster and at the other extremity to the top of the cage. The length of the string was adjusted to allow the animals moving freely on the forelimbs, while the body was inclined at 30–40° from the horizontal plane. All mice had water *ad libitum* and received 8 g a day of standard rodent chow. The food remaining on the day after was weighted to calculate daily food consumption. The TRO and HUTRO mice received daily, 6 days a week, an intraperitoneal injection of 0.25 ml of a 1 M $NaHCO_3$ solution containing 5 g/L trolox, corresponding to ~45 mg/kg/day dose (The dose was double on the sixth day to maintain drug level along the seventh day). Trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Sigma, Milan, Italy] is a water-soluble vitamin E analogue that freely crosses cell membranes [13]. The drug displays a very high antioxidant capacity and may lack antioxidant-unrelated side effects, so that it is widely used as a standard compound in antioxidant capacity assays. Accordingly, such a treatment was able to prevent lipid perodixation (see results). At the end of suspension, the mice were unfastened from the string and deeply anesthetized by intraperitoneal injection of urethane (1.2 g/kg body weight) to allow removing of soleus (Sol), gastrocnemius (Gas), tibialis anterior (TA), and extensor digitorum longus (EDL) muscles. Muscles were used immediately for the electrophysiological experiments or frozen in liquid nitrogen and stored at −80 °C for other studies. After surgery, animals were euthanized by an overdose of urethane. For measuring the markers of oxidative stress, the mice were sacrificed by cervical dislocation and the muscles removed and frozen promptly.

2.2. Ex vivo electrophysiological studies

Soleus, EDL, or Gas muscles were fixed by tendons to a glass rod immersed in normal (NP) or chloride-free physiological solution maintained at 30 °C and perfused with 95% O_2/5% CO_2 [4]. The NP solution contained (in mM): NaCl 148, KCl 4.5, $CaCl_2$ 2.0, $MgCl_2$ 1.0, $NaHCO_3$ 12.0, NaH_2PO_4 0.44, glucose 5.5, and pH 7.2. The chloride-free solution was prepared by equimolar substitution of methylsulfate salts for NaCl and KCl and nitrate salts for $CaCl_2$ and $MgCl_2$. The cable parameters of myofiber sarcolemma were determined from the electrotonic potentials elicited by square-wave hyperpolarizing current pulse of 100-ms duration, using two intracellular microelectrodes in current-clamp mode, as previously described [14]. The membrane conductance is calculated from the values of input resistance, space constants and time constant and assuming a myoplasmic resistivity of 125 Ω cm. The mean chloride conductance (gCl) is calculated as the mean total membrane conductance (gm) measured in NP solution minus the mean potassium conductance gK measured in chloride-free solution.

Sarcolemma excitability parameters were determined by applying 100 ms-long depolarizing current pulses of increasing amplitude to elicit first a single action potential (AP) then a train with the maximal number of APs. The membrane potential was held at −80 mV between test pulses. The excitability parameters, determined off-line on digital AP recordings, were the current threshold to elicit the first AP (or Rheobase current, I_{th}), the latency of the AP (Lat), the AP amplitude (APA), the maximal number of elicitable AP (N spikes), and the ratio between I_{th} and the current threshold needed to elicit more than one AP (I_{th}/I_2). The Lat is inversely correlated to the gCl, whereas the other parameters depend on various ion channels/carriers.

The mechanical threshold (MT) for contraction was determined using a two-microelectrode point voltage clamp method in the presence of 3 μM tetrodotoxin, as described previously [4]. The holding potential was set to −90 mV. Depolarizing current pulses of various durations (5–500 ms) were applied at a frequency of 0.3 Hz, while the impaled fiber was continuously inspected with a stereomicroscope. The command voltage was increased until contraction was visible and the threshold membrane voltage was read at this time from a digital sample-and-hold voltmeter. The mean threshold voltage V (mV) ± S.E.M. (n fibers) was plotted as a function of the pulse duration t (ms), and the relationship was fit using a nonlinear least squares algorithm with the equation,

$$V(t) = \frac{[-90 - R \cdot \exp(-t/t_R)]}{[1 - \exp(-t/t_R)]}, \quad (1)$$

where R (mV) is the Rheobase voltage and t_R (ms) the time constant to reach R. The MT values were expressed as the calculated R value along with the standard error of the fit.