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PGC1-α over-expression prevents metabolic alterations and soleus muscle atrophy in hindlimb unloaded mice

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Key points

- Oxidative stress is widely considered a major cause of muscle loss not only in disuse but also in most chronic diseases, triggering carbonylation of proteins and activation of catabolic pathways involved in their degradation.
- Here we show that administration of an antioxidant prevents redox imbalance, but does not prevent activation of catabolic pathways and muscle atrophy.
- We indicate that alterations of oxidative metabolism, occurring in slow soleus muscle, are not just a consequence of disuse, but a major cause of activation of catabolic pathways and loss of mass.
- This conclusion is confirmed by the observation that muscle-specific overexpression of PGC-1α, a master regulator of mitochondrial biogenesis, prevents activation of catabolic systems and disuse muscle atrophy.
- These findings contribute to a better mechanistic understanding of disuse muscle loss.

Abstract Prolonged skeletal muscle inactivity causes muscle fibre atrophy. Redox imbalance has been considered one of the major triggers of skeletal muscle disuse atrophy, but whether redox imbalance is actually the major cause or simply a consequence of muscle disuse remains of debate. Here we hypothesized that a metabolic stress mediated by PGC-1α down-regulation plays a major role in disuse atrophy. First we studied the adaptations of soleus to mice hindlimb unloading (HU) in the early phase of disuse (3 and 7 days of HU) with and without antioxidant treatment (trolox). HU caused a reduction in cross-sectional area, redox status alteration (NRF2, SOD1 and catalase up-regulation), and induction of the ubiquitin proteasome system (MuRF-1 and atrogin-1 mRNA up-regulation) and autophagy (Beclin1 and p62 mRNA up-regulation). Trolox completely prevented the induction of NRF2, SOD1 and catalase mRNAs, but not atrophy or induction of catabolic systems in unloaded muscles, suggesting that oxidative stress is not a major cause of disuse atrophy. HU mice showed a marked alteration of oxidative metabolism. PGC-1α and mitochondrial complexes were down-regulated and DRP1 was up-regulated. To define the link between mitochondrial dysfunction and disuse muscle atrophy we unloaded mice overexpressing PGC-1α. Transgenic PGC-1α animals did not show metabolic alteration during unloading, preserving muscle size through the reduction of autophagy and proteasome degradation. Our results indicate that mitochondrial dysfunction plays a major role in disuse

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atrophy and that compounds inducing PGC-1α expression could be useful to treat/prevent muscle atrophy.

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Abbreviations CSA, cross-sectional area; CuZnSOD1, superoxide dismutase1; DHE, dihydroethidium; DNP, dinitrophenylhydrazone; DRPI, dynamin-related protein 1; 4EBP1, eukaryotic translation initiation factor 4E-binding protein 1; HU, hindlimb unloading; MHC, myosin heavy chain; MuRF-1, muscle-specific ring finger protein-1; NRF2, nuclear factor erythroid derived 2 like 2 (Nfe2l2); OI, oxidative index; PGC-1α, peroxisome proliferative activated receptor-γ coactivator 1α; ROS, reactive oxygen species.

Introduction

The maintenance of muscle mass is critical for health and quality of life. It is widely recognized that loss of muscle mass can impair the ability to perform daily tasks, increase the incidence of injuries, prolong the period of rehabilitation and be a major risk factor of chronic diseases. Prolonged skeletal muscle inactivity results in the loss of muscle protein, fibre atrophy and impaired muscle function. Understanding of the signalling pathways responsible for the imbalance between protein synthesis and degradation leading to disuse muscle atrophy is an important step toward the development of therapeutic strategies to delay or prevent skeletal muscle atrophy.

Prolonged periods of contractile inactivity lead to increased production of reactive oxygen species (ROS) in muscle fibres, suggesting that oxidative stress could be a major trigger of disuse muscle atrophy (Powers *et al.* 2005, 2010; Moylan & Reid, 2007). However, whether ROS play a causal role in disuse atrophy of limb muscles is still debated (Brocca *et al.* 2010; Pellegrino *et al.* 2011; Powers *et al.* 2012). Recent findings challenge the role of oxidative stress in disuse atrophy (Desaphy *et al.* 2010; Glover *et al.* 2010; Kuwahara *et al.* 2010). The ineffectiveness of anti-oxidant treatments to prevent muscle atrophy (Ikemoto *et al.* 2002; Koesterer *et al.* 2002; Servais *et al.* 2007), including our recent observation that antioxidant trolox prevents alterations in antioxidant defence systems and protein carbonylation, but fails to prevent soleus atrophy (Brocca *et al.* 2010), casts further doubts on the causative role of oxidative stress.

Interestingly, alterations in mitochondrial function characterize different conditions of muscle wasting such as ageing (Mishra & Misra, 2003), diabetes (Patti *et al.* 2003; Mootha *et al.* 2004), chronic obstructive pulmonary disease (Balasubramanian & Varkey, 2006) and muscular dystrophies (Bernardi & Bonaldo, 2008). Mitochondria are highly dynamic organelles that change in shape and function as a consequence of physical activity. The mitochondrial pro-fusion or fission shaping proteins and autophagy–lysosome system regulate mitochondrial size and network. Emerging evidence suggests that mitochondrial dynamics related to muscle atrophy play a critical role in the regulation of signalling pathways controlling muscle mass. Recently it has been shown that an imbalance of mitochondrial dynamics, i.e. an overexpression of fission factors, contributes to the development of muscle atrophy induced by denervation, fasting and FoxO3 overexpression (Romanello *et al.* 2010).

A key player controlling mitochondrial shape and content is peroxisome proliferative activated receptor-γ coactivator 1α (PGC-1α), a master regulator of mitochondrial biogenesis (Lin *et al.* 2002). PGC-1α is a transcriptional coactivator affected by muscle activity and energy stress (Brault *et al.* 2010). An early decrease of PGC-1α in muscle has been found in denervation-induced atrophy (Sandri *et al.* 2006) and in several catabolic states such as diabetes (Patti *et al.* 2003; Roberts-Wilson *et al.* 2010) and ageing (Anderson & Prolla, 2009). Forced expression of PGC-1α in cultured mammalian cells, in muscles of transgenic mice and in transfected adult muscle fibres inhibits muscle atrophy induced by denervation (Sandri *et al.* 2006; Brault *et al.* 2010), and inhibits amyotrophic lateral sclerosis (Da Cruz *et al.* 2012) by directly interfering with a FoxO3-dependent pathway.

We formulated the hypothesis that (i) metabolic stress mediated by PGC-1α down-regulation plays a major role in disuse atrophy and (ii) oxidative stress is not a major cause of muscle atrophy. To test this hypothesis we studied two different time points (3 and 7 days) in the early phases of disuse muscle atrophy in soleus (anti-gravitary muscle, preferentially affected by unloading) hindlimb unloaded mice with and without antioxidant treatment. In an attempt to better define the link between mitochondrial dysfunction and muscle atrophy we unloaded muscle-specific PGC-1α transgenic mice. Our data show that mitochondrial dysfunction induced by hindlimb unloading (HU) plays a critical role in the onset of muscle atrophy activating catabolic systems. Elevated PGC-1α levels in muscle significantly mitigate soleus atrophy induced by HU, prompting an attractive therapeutic strategy for maintaining muscle mass during disuse.

Methods

Ethical approval

Experiments were approved by the Italian Health Department and complied with the Italian guidelines for the use of laboratory animals, and conform to the principles of UK regulations, as described by Drummond (2009).

Animal care and hindlimb unloading

Six-month-old male C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA) and transgenic mice overexpressing PGC-1α (TgPGC-1α) in skeletal muscle, previously described by Lin *et al.* (2002), were used. Mice were unloaded for 3 and 7 days as previously described (Brocca *et al.* 2010). Briefly, animals were suspended individually in special cages by thin string tied at one end to the tail and at the other end to the top of the cage; the length of the string was adjusted to allow the animals to move freely on their forelimbs, while the body was inclined at 30–40 deg from the horizontal plane. All mice had access to water and food ad libitum. The ground C57BL/6 and TgPGC-1α mice were maintained free in single cages and killed after 3 or 7 days. A hindlimb-suspended group was treated with an antioxidant, trolox (HU-3-Tr). These mice received daily an intraperitoneal injection of 0.25 ml of a 1 M $NaHCO_3$ solution containing 5 g l^{-1} trolox, corresponding to ∼45 mg kg^{-1} day^{-1} for 10 days (Brocca *et al.* 2010), commencing 1 week before HU and continuing for the 3 days of HU. Furthermore, a placebo treated mice group (HU-3-Pl) received an intraperitoneal injection of 1 M $NaHCO_3$ solution with a timeline identical to HU-3-Tr mice. For each experimental group six animals were used.

The animals of all experimental groups were killed at 10.00 h after 2 h without food with cervical dislocation to allow removal of soleus muscle. Muscles were immediately frozen in liquid nitrogen and stored at −80°C. Six muscle samples were used for each analysis.

Cross-sectional area (CSA) analysis

CSA of individual muscle fibres was determined in the mid-belly region of soleus muscle as previously described (Brocca *et al.* 2010). Briefly, muscle serial transverse sections (10 μm thick) were immunostained (see list of antibodies below). Images of the stained sections were captured from a light microscope (Leica DMLS) and transferred to a personal computer using a video camera (Leica DFC 280). Fibre CSAs were measured with Image J analysis software (NIH, Bethesda, MD, USA) and expressed in μm^2.

Detection of superoxide by dihydroethidium (DHE) fluorescence staining

DHE is a commonly used indicator of ROS production both *in vitro* (Benov *et al.* 1998) and *in vivo* (Robinson *et al.* 2006). It is oxidized by superoxide, forming ethidium bromide, which fluoresces red when intercalated with DNA (Benov *et al.* 1998). Muscle transverse sections (10 μm thick) were collected and incubated with DHE (5 μM) in a light-protected place at 37°C for 30 min. After rinsing with PBS, fluorescence was assessed using fluorescence microscopy.

Hydrogen peroxide quantification

Frozen muscle samples were pulverized in a steel mortar with liquid nitrogen to obtain a powder that was immediately re-suspended in the assay buffer provided with the kit (Abcam, Cambridge, MA, USA). After protein quantification, samples were cleared of all proteins. A standard curve with known H_2O_2 concentration was generated following the supplied protocol. Samples and H_2O_2 standards were incubated at room temperature for 10 min in HRP and OxiRed Probe that reacted with the H_2O_2 to produce red fluorescence (Ex/Em = 535/587 nm), which was measured with a micro-plate reader (Tecan Infinite 200 Pro) (Li *et al.* 2012).

Analysis of myosin heavy chain (MHC) isoform content

The MHC isoform content was determined using an electrophoretic approach previously described in detail (Brocca *et al.* 2010). Briefly, about 6 μg of each muscle sample were dissolved in lysis buffer (20 mM Tris-HCl, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 5 mM EDTA, 100 mM NaF, 2 mM NaPPi, 1× inhibitor protease phosphatase (Protease Inhibitor Cocktail, Sigma-Aldrich, St Louis, MO, USA) and 1 mM phenylmethylsulfonyl fluoride (PMSF)). The lysates were loaded onto 8% polyacrylamide SDS-PAGE gels. Electrophoresis was run for 2 h at 200 V and then for 24 h at 250 V; the gels were stained with Coomassie Blue. Four bands could be separated in the region of MHC isoforms. Densitometric analysis of MHC bands was performed to assess the relative proportion of the four MHC isoforms, MHC-I, MHC-IIA, MHC-IIX and MHC-IIB, in each sample (Pellegrino *et al.* 2003)

Western blot analysis

Frozen muscle samples were pulverized and immediately re-suspended in a lysis buffer (20 mM Tris-HCl, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 5 mM EDTA, 100 mM NaF and 2 mM NaPPi supplemented with 1× protease,

phosphatase inhibitors (Sigma-Aldrich) and 1 mM PMSF). The lysate was left for 20 min in ice and the homogenate obtained was centrifuged at 18000 *g* for 20 min at 4°C. Protein concentration was determined on the supernatant using the RC DCTM protein assay kit (BioRad, Hercules, CA, USA). The supernatant was stored at –80°C until ready to use.

Equal amounts of muscle samples were loaded on gradient precast gels purchase from BioRad (AnyKd). Proteins were electro-transferred to polyvinylidene fluoride (PVDF) membranes at 35 mA overnight. The membranes were probed with specific primary antibodies (see below). Thereafter, the membranes were incubated in HRP-conjugated secondary antibody. The protein bands were visualized by an enhanced chemiluminescence method. The content of each protein investigated was assessed by determining the brightness–area product of the protein band as previously described (Gondin *et al.* 2011).

Oxyblot analysis

Muscle samples previously stored at −80°C were pulverized and homogenized at 4°C in an antioxidant buffer containing protease inhibitors, 25 mM imidazole and 5 mM EDTA, pH 7.2 adjusted with NaOH as previously described in detail (Brocca *et al.* 2010). The lysate was left for 20 min in ice and the homogenate obtained was centrifuged at 18000 *g* for 20 min at 4°C. Protein concentration was determined on the supernatant using the RC DCTM protein assay kit (BioRad). The supernatant was stored at −80°C until ready to use.

The protein carbonylation level was detected using the OxyBlot Kit (AbNova, Taipei City, Taiwan), which provides reagents for sensitive immunodetection of these carbonyl groups. The carbonyl groups in the protein side chains are derivatized to 2,4-dinitrophenylhydrazone (DNP hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH); 6 μg of the DNP-derivatized protein samples were separated by PAGE (15% SDS-polyacrylamide gels) and then blotted for 2 h at 100 V to a nitrocellulose membrane. Membranes obtained were stained with Ponceau Red and then scanned. Membranes were incubated with primary antibody, specific to the DNP moiety of the proteins and subsequently with an HRP–antibody conjugate directed against the primary antibody (secondary antibody: goat anti-rabbit IgG). Blots were developed by using an enhanced chemiluminescence method. Positive bands emitting light were detected by short exposure to photographic films. Protein oxidation was quantified by defining the oxidative index (OI), i.e. the ratio between densitometric values of the oxyblot bands and those stained with Ponceau Red.

Gene expression analysis

Total RNA, from skeletal samples, was extracted using the Promega SV Total RNA isolation kit; the concentration of RNA were evaluated by using a NanoDrop instrument (Thermo Scientific, Waltham, MA, USA) and 300 ng was reverse-transcribed with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) to obtain cDNA. The cDNA was analysed by real time-PCR (AB 7500) with the SYBR Green PCR kit (Applied Biosystems, Foster City, CA, USA) and the data were normalized to HPRT rRNA (hypoxanthine-guanine phosphoribosyl transferase).

Primers

Forward (FP) and reverse (RP) primers used for RT-PCR were: *MuRF1* FP: ACCTGCTGGTGGAAAACATC, RP: CTTCGTGTTCCTTGCACATC; *Atrogin-1* FP: GCAA ACACTGCCACATTCTCTC, RP: CTTGAGGGGAAAG TGAGACG, *p62* FP: CCCAGTGTCTTGGCATTCTT, RP: AGGGAAAGCAGAGGAAGCTC; *Beclin1* FP: GCTCCT GAGGCATGGAGGGGTCT, RP: GGTTTCGCCTGGGCT GTGGTAA; *NRF2* FP: TTCTTTCAGCAGCATCCTCTC CAC, RP: ACAGCCTTCAATAGTCCCGTCCA; *SOD1* FP: GAGACCTGGGCAATGTGACT, RP: GTTTACTGC GCAATCCCAAT; *Catalase* FP: CACTGACGAGATGG CACACTTTG, RP: TGGAGAACCGAACGGCAATAGG; *PGC-1α* FP: ACCCCAGAGTCACCAAATGA, RP: CGAAGCCTTGAAAGGGTTATC; *HPRT*: Quanti Tect Primer Assay (Qiagen, Valencia, CA, USA)

Antibodies

Antibodies used were: anti-rabbit superoxide dismutase 1 (Abcam); anti-rabbit catalase (Abcam); anti-rabbit α tubulin (Sigma-Aldrich); anti-mouse OXPHOS complexes (Abcam); anti-rabbit PGC-1α (Abcam); anti-rabbit DRP1 (Cell Signaling Technology, Inc., Danvers, MA, USA); anti-rabbit p-AKT$^{(ser473)}$ (Cell Signaling); anti-rabbit AKT (Cell Signaling); anti-rabbit p-S6Rp$^{(ser235/236)}$ (Cell Signaling); anti-rabbit S6Rp (Cell Signaling); anti-rabbit p-4EBP1$^{(thr37/46)}$ (Cell Signaling); anti-rabbit 4EBP1 (Cell Signaling); anti-mouse myosin heavy chain 1 isoform (BA-F8); anti-mouse myosin heavy chain 2A isoform (SC-71); anti-mouse IgG (Dako North America Inc., Carpinteria, CA, USA); and anti-rabbit IgG (Cell Signaling).

Statistical analysis

Data were expressed as mean ± SEM. Statistical significance of the differences between means was assessed by one-way ANOVA followed by Student–Newman–Keuls

test. A probability of less than 5% was considered significant ($P < 0.05$).

Results

To define the mechanism responsible for the loss of muscle mass, all experiments were performed in the early phases of disuse (3 and 7 days following HU) on soleus muscle, which is well known to go through disuse atrophy in the early phases of HU.

CSA is reduced and MHC isoform distribution does not change in the early phases of HU

To assess the impact of disuse on muscle size, CSA of skeletal muscle fibres was analysed in the mid-belly region of muscles in control and HU mice on cryosections stained with monoclonal antibodies specific for MHC-1 and MHC-2A isoforms (Fig. 1). At both 3 and 7 days of HU, myofibre CSA was significantly lower in slow fibres (17.2% at 3 days and 20% at 7 days) as well as in type 2A fast fibres (15.4% at 3 days and 14.9% at 7 days). MHC isoform composition was unchanged (Table 1).

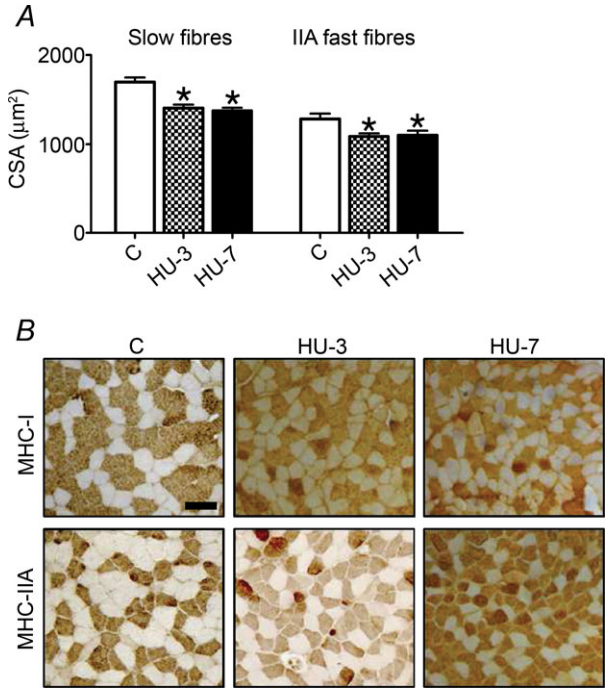

Figure 1. CSA is reduced in soleus in the early phases of HU
A, mean (±SEM) CSA of slow and fast fibres of soleus in control (C) and following 3 (HU-3) and 7 (HU-7) days of unloading; *significantly different from control ($P < 0.05$). *B*, representative cross-cryosections of soleus muscles using anti-MHC-I (BA-F8) and anti-MHC-IIA (SC-71) monoclonal antibodies. Scale bar: 100 μm.

Table 1. Myosin heavy chain (MHC) isoform composition does not change in the early phases of HU

MHC isoform	C	HU-3	HU-7
MHC-I	28.27 ± 6.31	40.06 ± 13.34	32.29 ± 12.5
MHC-IIA	50.82 ± 7.12	46.75 ± 13.42	36.72 ± 14.47
MHC-IIX	7.78 ± 3.49	9.17 ± 7.25	17.82 ± 9.65
MHC-IIB	13.13 ± 7.67	4.02 ± 2.23	13.17 ± 11.12

MHC isoform composition of soleus muscle of control mice (C), following 3 days (HU-3) and 7 days (HU-7) of HU. MHC composition was determined by SDS-PAGE and densitometric analysis of MHC bands. Data are expressed as mean ± SD.

Redox imbalance occurs following HU

Based on evidence from the literature and from our previous findings (Brocca *et al.* 2010, 2012), we hypothesized that redox imbalance occurs in the HU mice model, but it does not trigger the early activation of catabolic systems. We first assessed whether oxidative stress occurred by studying: (i) NRF2 (nuclear factor, erythroid derived 2, like 2, Nfe2l2) transcription factor and antioxidant defence system adaptations, (ii) superoxide and H_2O_2 content and (iii) protein carbonylation.

Figure 2*A* shows the mRNA expression levels of NRF2, the major sensor of cell redox balance, SOD1 (superoxide dismutase1) and catalase, the major enzymes of ROS scavenging. Soleus had an increased expression of NRF2 mRNA at 3 days of HU only, SOD1 mRNA expression was significantly higher at both 3 and 7 days of HU compared to control, while catalase mRNA level was significantly up-regulated at 3 days of HU only and underwent a significant drastic reduction at 7 days of HU. The protein levels (Fig. 2*B*) of SOD1 were significantly higher at both 3 and 7 days of HU, reflecting the gene expression levels, while no change of catalase content at both 3 and 7 days of HU in comparison to control was found.

Figure 2*C* shows representative muscle cryosections stained with fluorescence probe DHE to visualize accumulation of superoxides. A higher superoxide content was observed at 7 days of HU, but not at 3 days, compared to control muscle. H_2O_2 concentration was determined by a fluorimetric method (Fig. 2*D*). A significantly higher H_2O_2 concentration was found following 7 days of HU than in controls. Protein carbonylation was determined by Oxyblot analysis by calculating the protein OI. As shown in Fig. 2*E* the protein OI was not different at 3 and 7 days of HU compared to controls.

Disuse induces catabolic pathways and reduces protein synthesis

The adaptations to disuse of the two major catabolic systems, the ubiquitin proteasome and the autophagy

systems, were studied. Figure 3*A* shows the mRNA level of MuRF1 (muscle-specific ring finger protein-1) and atrogin-1 ubiquitin ligases (ubiquitin proteasome) and of Beclin1 and p62 (autophagy system). A significant MuRF1 and atrogin-1 mRNA up-regulation was observed at 3 days of HU. At 7 days of HU, there was a lower but still significant MuRF1 up-regulation compared to control muscle, whereas atrogin-1 expression was not different from the control level. As regards autophagy, p62 mRNA relative expression was significantly higher at 3 days of HU compared to control. Beclin1 did not change at both times considered.

In addition to the increase of protein degradation, muscle atrophy can result from a decrease in protein synthesis. To study the effect of disuse on the protein synthesis pathway, phosphorylation levels of key components of the PI3K-AKT pathway were analysed. Figure 3*B* shows representative Western blots and relative contents of phosphorylated/whole expression level of AKT kinase, 4EBP1 (eukaryotic translation initiation factor 4E-binding protein 1) and S6 ribosomal protein. A significantly lower phosphorylated AKT was found at 3 and 7 days of HU compared to control. The phosphorylated S6Rp was significantly lower at 3 days of HU in comparison to control and further decreased at 7 days of HU. The phosphorylation level of 4EBP1 did not change at any of the experimental times analysed.

Antioxidant treatment does not prevent induction of catabolic systems following HU

To clarify whether the early redox imbalance was responsible for the early activation of catabolic systems,

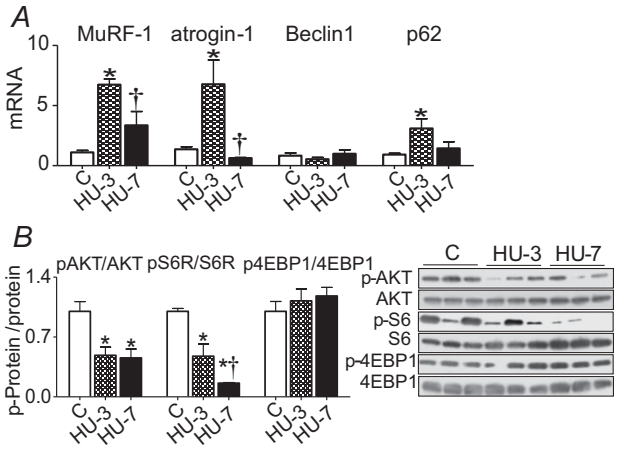

Figure 3. Catabolic pathways are induced and protein synthesis is reduced in soleus muscle in the early phases of HU
A, quantification of mRNA levels of MuRF-1 and atrogin-1 (ubiquitin proteasome system) and of Beclin1 and p62 (autophagy system) by real-time PCR. *B*, activity levels of AKT, S6R and 4EBP1 by Western blot analysis of the ratio between the content in the phosphorylated (p) and total forms. C, control; HU-3, 3 days of hindlimb unloading; HU-7, 7 days of hindlimb unloading. *Significantly different from C ($P < 0.05$); †significantly different from HU-3 ($P < 0.05$). Data are presented as means ± SEM.

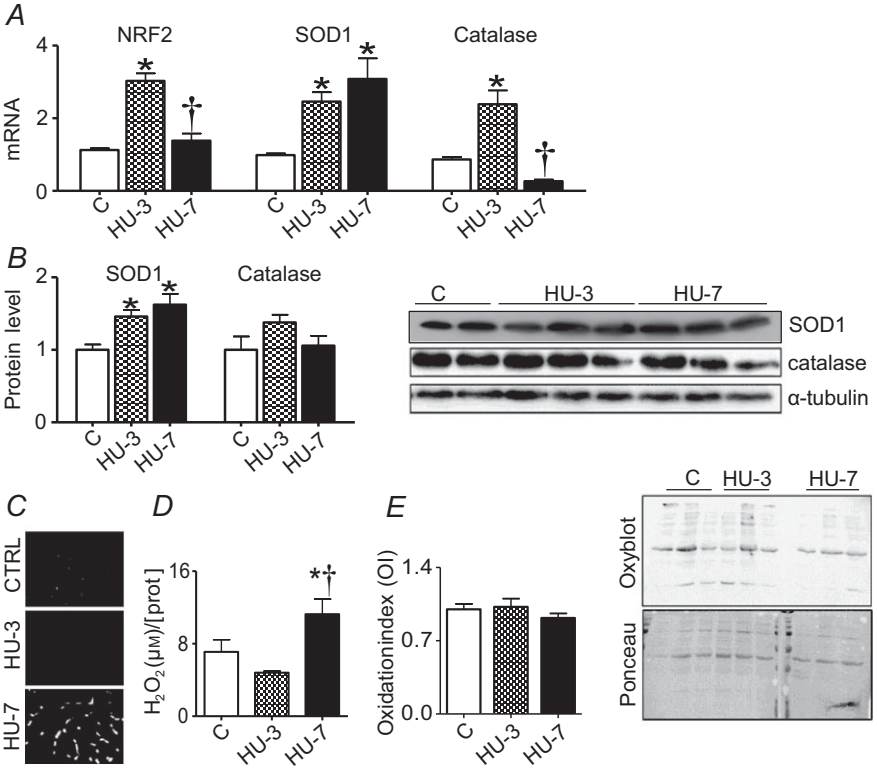

Figure 2. Redox imbalance occurs in soleus muscle following HU
A, quantification of mRNA levels of NRF2, SOD1 and catalase by real-time PCR. *B*, quantification of protein levels of SOD1 and catalase by Western blot. *C*, superoxides accumulation on muscle cryosections stained with fluorescent dye DHE. *D*, H_2O_2 concentration. *E*, level of protein carbonylation in muscle by oxidation index and relative oxyblot. C, control; HU-3, 3 days of hindlimb unloading; HU-7, 7 days of hindlimb unloading. In *A*, *B*, *D* and *E*: *significantly different from C ($P < 0.05$); †significantly different from HU-3 ($P < 0.05$). Data are presented as means ± SEM.

we unloaded mice treated with the antioxidant trolox, a vitamin E analogue, for 3 days, i.e. the time point at which we found both atrophy (Fig. 1) and activation of catabolic systems (Fig. 3).

First we checked the effectiveness of the antioxidant treatment through NRF2, SOD1 and catalase expression. The data in Fig. 4*A* show significantly higher NRF2, SOD1 and catalase expression in HU-3 placebo mice in comparison to control placebo mice. Trolox completely prevented the induction of NRF2, SOD1 and catalase mRNAs in unloaded mice, indicating that the treatment was able to blunt oxidative stress.

CSA of individual muscle fibres was determined in soleus of control placebo, HU-3 placebo and HU-3 trolox-treated mice. Trolox did not prevent muscle fibre atrophy in slow or fast fibres (Fig. 4*B*). Type I and IIA fibres of unloaded trolox mice showed a degree of atrophy similar to that of placebo-treated animals (type I 19 and 16%, respectively; type IIA 14.5 and 15.5%, respectively).

To test the role of oxidative stress in triggering muscle protein breakdown in disuse atrophy, gene expression of MuRF1, atrogin-1, Beclin1 and p62 was analysed following trolox treatment. mRNA levels of MuRF1 and atrogin-1 were significantly higher both in HU-3 placebo and in HU-3 trolox in comparison to control placebo. As regards autophagy, p62 mRNA level was significantly higher in HU-3 placebo and HU-3 trolox groups than in control placebo. No difference of Beclin1 expression among the different groups was found (Fig. 4*C*).

Oxidative metabolism is impaired in the early phases of HU

A metabolic programme has been suggested to play a role in controlling muscle mass (Sandri *et al.* 2006). Metabolic adaptations occur in disuse. The latter observations prompted the hypothesis that metabolic adaptations might not just be a consequence of disuse, but could be involved in the pathogenesis of muscle atrophy (Brocca *et al.* 2010). Therefore, we analysed expression of (i) PGC-1α, a transcription factor involved in mitochondria homeostasis; (ii) mitochondrial complexes (OXPHOS); and (iii) DRP1 (dynamin-related protein 1), an important factor involved in mitochondrial fission machinery. PGC-1α mRNA (Fig. 5*A*) and protein levels (Fig. 5*B*) were significantly lower at 3 and 7 days of HU compared to control. As regards protein levels in mitochondrial complexes, a significantly reduced expression of Complex I, Complex III and Complex V was found at both 3 and 7 days of HU (Fig. 5*D*). DRP1 level was determined by

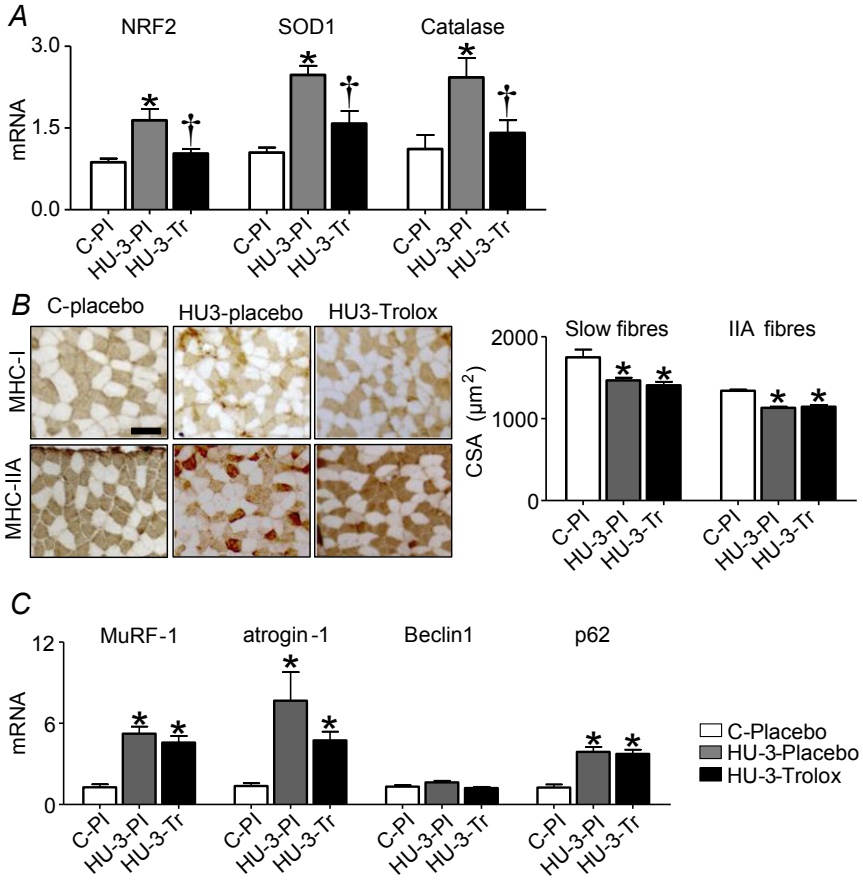

Figure 4. Antioxidant treatment does not prevent catabolic systems induction and atrophy in the early phases of HU
A, quantification of mRNA levels of NRF2, SOD1 and catalase by real-time PCR. *B*, Cross-cryosections of soleus muscles using anti-MHC-I (BA-F8) and anti-MHC-IIA (SC-71) monoclonal antibodies and relative CSA measurements. *C*, quantification of mRNA of MuRF-1, atrogin-1 (ubiquitin proteasome system), Beclin1 and p62 (autophagy system) by real-time PCR. C-Pl, placebo control; HU-3-Pl, placebo 3 days of hindlimb unloading; HU-3-Tr, Trolox 3 days of hindlimb unloading. *Significantly different from C-placebo ($P < 0.05$); †significantly different from HU-3-Pl ($P < 0.05$). Data are presented as means ± SEM.

Western blot and a significant up-regulation at 3 days of HU was observed (Fig. 5*C*).

Elevated PGC-1α levels preserve CSA in soleus unloaded muscle

We tested the hypothesis that a metabolic programme could trigger muscle atrophy by assessing whether its overexpression would lead to beneficial effects in HU. Hind-limb suspended muscle-specific PGC-1α transgenic (TgPGC-1α) and wild-type mice were studied at 3 days of HU.

As shown in Fig. 6, maintenance of PGC-1α expression in hind limb suspended soleus was sufficient to mitigate muscle atrophy. In fact, slow and fast fibres showed only 8 and 7% of atrophy, respectively.

Elevated PGC-1α levels prevent early catabolic pathways induction in soleus unloaded muscle without affecting protein synthesis

As the peak of expression of catabolic systems was reached at 3 days of HU, we monitored whether PGC-1α expression elicited any effect on atrogenes induction early in HU. As shown in Fig. 7*A*, high levels of PGC-1α in HU muscles significantly blunted the up-regulation of MuRF-1 and atrogin-1 genes and completely prevented p62 induction.

To test whether PGC-1α elicited an effect on the protein synthesis pathway we studied AKT, S6 and 4EBP1 mRNA expression. As shown in Fig. 7*B*, PGC-1α transgenic mice showed an attenuation of S6 dephosphorylation and no significant protection of AKT inhibition, which was significantly decreased as in unloaded wild-type mice. The phosphorylation level of 4EBP1 remained similar at both experimental time points. Therefore, PGC-1α had minor effects on pathways related to protein synthesis, as recently described (Bonaldo & Sandri, 2013).

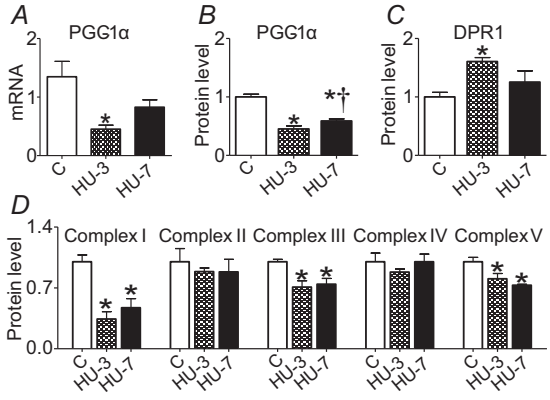

Figure 5. Mitochondrial dysfunction is established early during HU in soleus muscle
A, quantification of mRNA levels of PGC-1α by real-time PCR. *B*, quantification of protein levels of PGC-1α by Western blot. *C*, quantification of protein levels of DRP1 involved in fission machinery by Western blot. *D*, quantification of protein levels of mitochondrial complexes by Western blot. C, control; HU-3, 3 days of hindlimb unloading; HU-7, 7 days of hindlimb unloading. *Significantly different from control ($P < 0.05$); †significantly different from HU-3 ($P < 0.05$). Data are presented as means ± SEM.

Discussion

It is widely believed that disuse skeletal muscle atrophy is caused by an imbalance between muscle protein synthesis and muscle protein break-down. The general aim of this study was to clarify the adaptations of the intracellular signalling pathways involved in muscle protein synthesis and muscle protein break-down and to determine the underlying triggering phenomena. HU, in which the hind-limbs are suspended off the ground to remove the normal gravitational load on the muscles, is a commonly used model to study disuse muscle atrophy. The present work confirms the widely accepted view that HU is able to induce significant atrophy of the antigravitary soleus (Fitts *et al.* 2001; Hurst & Fitts, 2003) and extend it to early times, i.e. 3 days following HU (Fig. 1).

Redox imbalance has been considered one of the major triggers of skeletal muscle disuse atrophy (Powers *et al.* 2005, 2010; Moylan & Reid, 2007). The role of oxidative stress has been elegantly demonstrated in respiratory muscles following mechanical ventilation (Zergeroglu *et al.* 2003; Shanely *et al.* 2004; Falk *et al.* 2006). However, notwithstanding the large amount of work devoted to the issue, it is still unclear whether redox imbalance is actually the major cause of disuse atrophy of limb muscles in a model which mimics moderate decrease in neuro-muscular activity (Brocca *et al.* 2010; Pellegrino *et al.* 2011; Powers *et al.* 2012). Here we show that redox imbalance is not a major trigger of muscle atrophy in limb muscles following HU, whereas a major role is played by a metabolic programme controlling muscle mass.

Catabolic pathways are induced and protein synthesis is decreased in soleus in the early phases of HU

The ubiquitin proteasome and the autophagy lysosome systems play a critical role during myo-fibre shrinkage. The activation of these pathways requires a transcriptional-dependent up-regulation of a subset of genes named atrophy-related genes or atrogenes. The role of MuRF-1 and atrogin-1 in breaking down myofibrillar proteins in disuse in small mammals is well established (Ikemoto *et al.* 2001). The induction of these atrogenes precedes muscle atrophy (Sandri, 2008). Consistently we found an early increase of MuRF1 and atrogin-1 mRNA (Fig. 3*A*). The activation was transient, fading away at 7 days of HU, in agreement with other studies on HU

(Bodine *et al.* 2001; Haddad *et al.* 2006; Kline *et al.* 2007), cast immobilization (Krawiec *et al.* 2005; Caron *et al.* 2009), denervation (Bodine *et al.* 2001; Kline *et al.* 2007; Sacheck *et al.* 2007; Bertaggia *et al.* 2012) and several other atrophic conditions such as corticosteroid administration (Cho *et al.* 2010), nerve crash (Caron *et al.* 2009) and spinal cord isolation (Sacheck *et al.* 2007).

Autophagy is known to play a crucial role in the turnover of cell components both in constitutive conditions and in response to various stimuli, such as cellular stress, nutrient deprivation, amino acid starvation and cytokines (Mizushima *et al.* 2008). However, excessive stimulation of the autophagy machinery is documented to be deleterious and could lead to cell death (Levine & Yuan, 2005). It has been recently understood that an increase in activity of the autophagy system can play a relevant role in muscle atrophy (Sandri, 2010*a*,*b*). In other words, too much autophagy impairs myofibre homeostasis, causing excessive removal of cellular components and leading to muscle atrophy when excessive catabolic activity is sustained for long periods. We found a slight activation of autophagy in the early phase of disuse atrophy suggested by the increased expression of p62, the major protein involved in delivering ubiquitinated proteins to the autophagosome (Fig. 3*A*). To our knowledge there are few and conflicting data concerning autophagy activity in HU. They have all been obtained after chronic disuse, showing induction (Maki *et al.* 2012), suppression (Liu *et al.* 2012) and no modification of autophagy (Andrianjafiniony *et al.* 2010). Collectively, the results concerning the activation

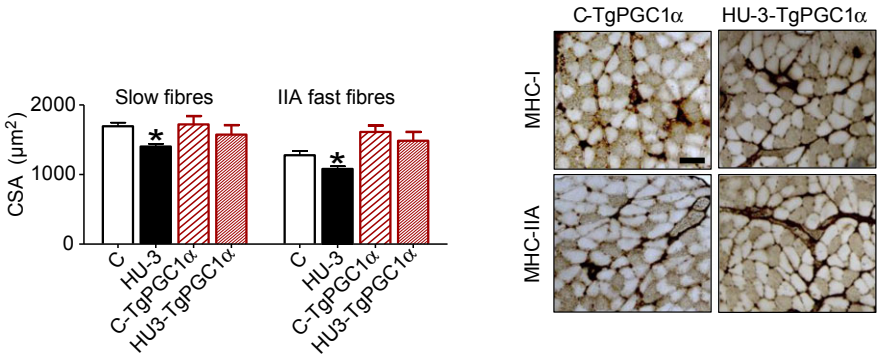

Figure 6. Increased PGC-1α expression in muscle prevents muscle atrophy during HU
CSA of slow and fast fibres of soleus stained anti-MHC-I (BA-F8) and anti-MHC-IIA (SC-71) monoclonal antibodies and relative measurements of CSA. Scale bar: 100 μm. C, control, wild type; HU-3, 3 days of hindlimb unloading, wild type; C-TgPGC-1α, control, transgenic PGC-1α; HU3-TgPGC-1α, 3 days of hindlimb unloading, transgenic PGC-1α. *Significantly different from control ($P < 0.05$). Data are presented as means ± SEM.

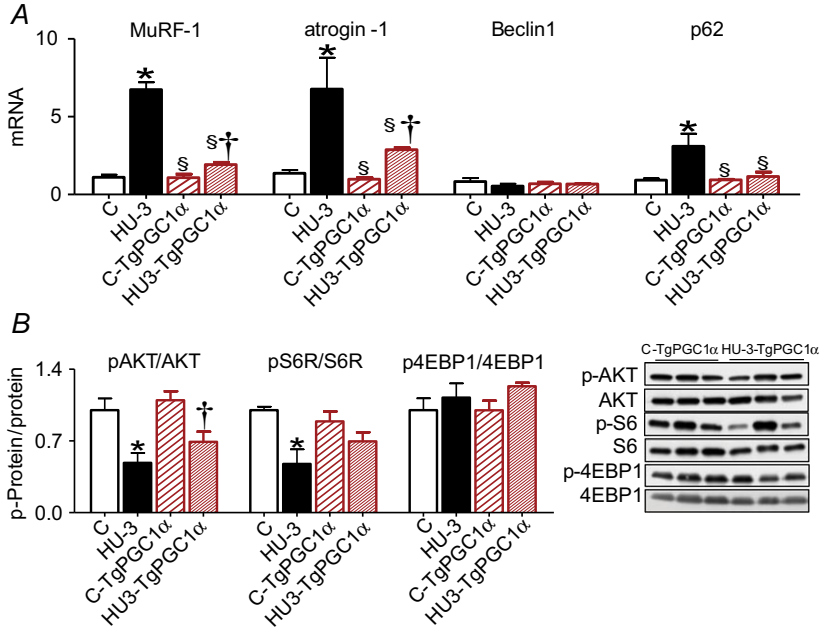

Figure 7. Increased PGC-1α expression in muscle prevents the activation of catabolic systems and mitigates decrease of protein synthesis
A, quantification of mRNA levels of MuRF-1 and atrogin-1 (ubiquitin proteasome system) and of Beclin1 and p62 (autophagy system) by real-time PCR. *B*, activity levels of AKT, S6R and 4EBP1 by Western blot analysis of the ratio between the content in the phosphorylated (p) and total forms. C, control, wild type; HU-3, 3 days of hindlimb unloading, wild type; C-TgPGC-1α, control, transgenic PGC-1α; HU3-TgPGC-1α, 3 days of hindlimb unloading, transgenic PGC-1α. *Significantly different from control ($P < 0.05$); §significantly different from HU-3 ($P < 0.05$); †significantly different from C-TgPGC-1α ($P < 0.05$). Data are presented as means ± SEM.

of catabolic systems show that, in the early stages of disuse, soleus atrophy is mainly supported by the ubiquitin proteasome system, whereas autophagy does not seem to be a major phenomenon.

The PI3K-AKT pathway is known to play a key role in activating muscle protein synthesis (Rommel *et al.* 2001; Sandri, 2008). We found a down-regulation of the anabolic PI3K-AKT pathway through the reduced phosphorylation of AKT and S6Rp (Fig. 3*B*) indicating that a decrease in muscle protein synthesis plays a role in both the early and the later phases of disuse. This is consistent with earlier studies showing a decrease in the PI3K-AKT pathway following acute (12 h) (Hornberger *et al.* 2001), short (7 days) (Hornberger *et al.* 2001; Dupont *et al.* 2011) and chronic disuse (Sugiura *et al.* 2005; Dupont *et al.* 2011) in soleus muscle. As found in other conditions (Magne *et al.* 2013) 4E-BP1 phosphorylation level following HU was unchanged. This was unexpected because a decrease in AKT phosphorylation is generally able to decrease phosphorylation of its downstream targets, including 4E-BP1. One possible explanation is that the very fast kinetics of phosphorylation and dephosphorylation of the different kinases of the PI3K-AKT pathway could make their concomitant activation an unlikely event.

Redox imbalance does not trigger disuse muscle atrophy

The adaptations in the expression of NRF2, SOD1 and catalase (Fig. 2) indicate that redox imbalance occurred early into HU. NRF2 is, in fact, a constitutively active transcription factor sensing redox balance and controlling genes of antioxidant defence systems (Baird & Dinkova-Kostova, 2011; Hur & Gray, 2011). The lack of accumulation of superoxides and H_2O_2 at 3 days of HU indicates that, in the early phase of disuse, the antioxidant defence system efficiently reacts to the initial ROS increase. A prompt response to redox imbalance in soleus is expected due to its high oxidative metabolism and therefore to its natural exposure to superoxides.

SOD1 catalyses the reduction of superoxide anions to hydrogen peroxide (H_2O_2) preventing superoxide accumulation and catalase converts H_2O_2 into H_2O and O_2 preventing H_2O_2 accumulation. The mismatch between SOD1 and catalase expression and H_2O_2 accumulation found at 7 days of HU indicates a clear alteration of the antioxidant defence system, providing a likely basis for protein carbonylation (Lawler *et al.* 2003). However, no signs of carbonylation were detected with Oxyblot (Fig. 2) probably because protein carbonylation is a massive and late phenomenon, which is unlikely to be a sensitive index of redox imbalance. Consistent with an early alteration of antioxidant defence systems and with protein carbonylation being its late consequence, protein carbonylation was found following 15 days of HU in our previous study (Brocca *et al.* 2010). Regardless of the lack of protein carbonylation, redox imbalance could still be responsible for the early activation of catabolic systems (Fig. 3). Therefore, the data discussed so far could still fit with the hypothesis that oxidative stress plays a major role in disuse atrophy.

However, antioxidant treatment successfully counteracted redox imbalance (Fig. 4*A*), but did not prevent activation of catabolic systems and muscle atrophy (Fig. 4*C*), indicating that activation of catabolic systems is not causally linked to redox imbalance. The observation of no impact of antioxidant treatment on muscle atrophy is in agreement with several studies (Ikemoto *et al.* 2002; Koesterer *et al.* 2002; Servais *et al.* 2007; Brocca *et al.* 2010; Desaphy *et al.* 2010; Glover *et al.* 2010). Recently, a partial prevention of muscle atrophy after inhibition of xanthine oxidase by allopurinol during hindlimb unloading was shown, suggesting a causal role of oxidative stress in determining muscle atrophy (Derbre *et al.* 2012). By contrast, Matuszczak *et al.* (2004) found no muscle atrophy mitigation after allopurinol treatment in the same disuse model.

The lack of a causal link with muscle atrophy in HU does not necessarily imply a minor role of redox imbalance in all models of disuse. The role of an alteration of redox homeostasis in disuse atrophy has been shown, in fact, to vary through muscles, models and species (Pellegrino *et al.* 2011). The causal role of oxidative stress has been clearly demonstrated in mechanical ventilation (Shanely *et al.* 2002, 2004; Zergeroglu *et al.* 2003; Falk *et al.* 2006) and immobilization in humans and rodents (Kondo *et al.* 1992, 1993) also by the use of a mitochondrially targeted antioxidant that prevented myofibre atrophy (Min *et al.* 2011). One of the possible reasons for a differential role of oxidative stress may be that mechanical ventilation, limb immobilization and HU could decrease neuromuscular activity and muscle size to different extents (Fischbach & Robbins, 1969; Froese & Bryan, 1974; Alford *et al.* 1987; De-Doncker *et al.* 2005). Moreover, redox imbalance could be responsible of other disuse-induced phenomena. To our knowledge, this is the first report in which superoxide and H_2O_2 have been measured at different experimental times in the early stages of disuse and correlated with the expression of the major endogenous antioxidant systems and catabolic systems.

Oxidative metabolism is impaired in soleus in the early phases of HU

Alternatively to redox imbalance, we considered metabolic alterations as potential triggers of disuse atrophy. We found an early impairment of oxidative metabolism and signs of mitochondrial dysfunction, suggesting that a metabolic programme could be responsible for disuse atrophy.

PGC-1α is one of the master regulators of mitochondrial biogenesis and oxidative metabolism (Puigserver & Spiegelman, 2003) and is known to inhibit FoxO3 (Sandri *et al.* 2006). The observed PGC-1α down-regulation (Fig. 5*A* and *B*) could therefore account for the reduced expression levels of the respiratory chain complexes I, III and V (Fig. 5*D*). The latter results are in agreement with the observation that a variety of genes related to glycolysis and oxidative phosphorylation are coordinately suppressed in atrophying muscles (Lecker *et al.* 2004). Furthermore, a down-regulation of metabolic enzymes (Brocca *et al.* 2010) and a decrease of mitochondrial oxidative capacity (Momken *et al.* 2011) have been shown in the later phases of muscle disuse. As the early phases of disuse did not change MHC isoform composition (Table 1), the observed adaptations in oxidative metabolism could not be dependent on a shift of muscle phenotype.

A decrease in PGC-1α mRNA expression has been found in the acute phase (Mazzatti *et al.* 2008) and chronic phase (Momken *et al.* 2011; Liu *et al.* 2012) of hindlimb unloading, in denervation atrophy (Sandri *et al.* 2006) and in several catabolic states such as diabetes (Patti *et al.* 2003) and ageing (Anderson & Prolla, 2009). PGC-1α down-regulation (Fig. 5) could be responsible for the activation of the catabolic systems (Fig. 3*A*) through FoxO3 disinhibition and the subsequent enhancement of ubiquitine ligase and autophagy gene expression, even in the absence of ROS accumulation (Fig. 2).

Moreover, it has been recently understood that expression of the mitochondrial fission machinery is sufficient to cause muscle wasting in mice, whereas inhibition of mitochondrial fission prevents muscle loss during denervation, indicating that disruption of the mitochondrial network is a crucial amplificatory loop of the muscle atrophy programme (Romanello *et al.* 2010; Romanello & Sandri, 2010). Therefore, the early DRP1 overexpression in soleus could play a role in disuse atrophy (Fig. 3).

Collectively, the results indicate that mitochondrial alterations could sustain muscle protein breakdown in soleus muscle disuse.

Elevated PGC-1α levels preserve CSA and prevent the early catabolic pathways induction in soleus unloaded muscle

To test the hypothesis of a causative role of a metabolic programme in disuse muscle atrophy, we subjected HU to Tg-mice overexpressing PGC-1α (TgPGC-1α). The maintained high levels of PGC-1α prevented MuRF-1 and atrogin-1 induction as well as autophagy activation, protecting muscle from proteolysis. Consistently, atrophy following 3 days of HU was mitigated in soleus (Fig. 6*C*). The latter findings are consistent with the observations that PGC-1α overexpression prevented muscle atrophy induced by denervation (Sandri *et al.* 2006) and amyotrophic lateral sclerosis (Da Cruz *et al.* 2012) through the inhibition of FoxO3 transcriptional activity and catabolic pathway activation.

CSA of muscle fibres of unloaded transgenic mice was lower than CSA of muscle fibres of control transgenic mice (7% *vs.* 16.3%), although the difference did not reach statistical significance. The lack of complete recovery of mass could depend on the protein levels of PGC-1α, which in transgenic mice were similar to and not higher than those observed in control wild-type mice, probably due to the promoter driving expression of the PGC-1α transgene, MCK, which is less expressed in slow than in fast muscles (Lin *et al.* 2002). Transgene PGC-1α expression may not be sufficient to prevent the complete activation of catabolic systems and consequently the reduction of protein synthesis (Bonaldo & Sandri, 2013). The latter hypothesis would explain why the administration of resveratrol in unloaded rats slightly blunts soleus mass loss, despite maintenance of PGC-1α expression at normal levels (Momken *et al.* 2011). It can be hypothesized that, to completely prevent disuse muscle atrophy, it is not sufficient to prevent the disuse-induced drop in PGC-1α, but it is essential to raise PGC-1α levels above basal levels. Moreover, the lack of complete recovery of atrophy in soleus of TgPGC-1α mice could also be accounted for by inhibition of the AKT/mTOR pathway and therefore of protein synthesis playing a role in disuse atrophy, consistent with several previous observations (Thomason & Booth, 1990). Interestingly, PGC-1α has been shown not to affect the AKT/mTOR pathway (Bonaldo & Sandri, 2013). Consistently, PGC-1α overexpression did not counteract the disuse-induced lower activation of such pathway (Fig. 7*B*) failing to counteract the loss of mass due to lower protein synthesis.

Note that mitochondrial dysfunction could not only trigger muscle atrophy but could also cause ROS production and redox imbalance. Disuse-induced redox imbalance (Fig. 2) could therefore be a secondary phenomenon to a decrease in PGC-1α. It could not play a major role at least in the early adaptations leading to muscle atrophy, but still cause relevant phenomena, i.e. protein carbonylation and altered protein function. In fact, it has been suggested that oxidative stress in the myoplasm plays a pivotal role in altering muscle function rather than in triggering muscle atrophy (Kuwahara *et al.* 2010).

The almost complete recovery of muscle mass in unloaded TgPGC-1α mice indicates that mitochondrial alteration plays a major role in disuse atrophy and that compounds inducing PGC-1α expression could be useful to treat or prevent muscle atrophy.

Conclusions

Oxidative stress is widely considered a major cause of muscle loss not only in disuse, but in most chronic diseases, triggering carbonylation of proteins and activation of catabolic pathways involved in their degradation. Here we show that administration of an antioxidant prevents redox imbalance, but does not prevent activation of catabolic pathways and muscle atrophy. We indicate that alterations of oxidative metabolism and mitochondrial dynamics, occurring in soleus muscle, are not just a consequence of disuse, but a major cause of activation of catabolic pathways and loss of mass. This conclusion is confirmed by the observation that muscle-specific overexpression of PGC-1α, a master regulator of mitochondrial biogenesis, prevents activation of catabolic systems and disuse muscle atrophy.

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Additional information

Competing interests

The authors declare no conflict of interest.

Author contributions

R.B. and M.A.P.: conception and design of the experiments; J.C. and L.B.: collection, analysis and interpretation of data; M.S., R.B. and M.A.P.: drafting the article or revising it critically for important intellectual content. All authors made comments on the manuscript and read and approved the final version. All experiments were performed at the Department of Molecular Medicine, University of Pavia, Italy.

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