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SYMPOSIUM REVIEW

Redox homeostasis, oxidative stress and disuse muscle atrophy

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Abstract A pivotal role has been ascribed to oxidative stress in determining the imbalance between protein synthesis and degradation leading to muscle atrophy in many pathological conditions and in disuse. However, a large variability in disuse-induced alteration of redox homeostasis through muscles, models and species emerges from the literature. Whereas the causal role of oxidative stress appears well established in the mechanical ventilation model, findings are less compelling in the hindlimb unloaded mice and very limited in humans. The mere coexistence of muscle atrophy, indirect indexes of increased reactive oxygen species (ROS) production and impairment of antioxidant defence systems, in fact, does not unequivocally support a causal role of oxidative stress in the phenomenon. We hypothesise that in some muscles, models and species only, due to a large redox imbalance, the leading phenomena are activation of proteolysis and massive oxidation of proteins, which would become more susceptible to degradation. In other conditions, due to a lower extent and variable time course of ROS production, different ROS-dependent, but also -independent intracellular pathways might dominate determining the variable extent of atrophy and even dispensable protein oxidation. The ROS production and removal are complex and finely tuned phenomena. They are indeed important intracellular signals and redox balance maintains normal muscle homeostasis and can underlie either positive or negative adaptations to exercise. A precise approach to determine the levels of ROS in living cells in various conditions appears to be of paramount importance to define and support such hypotheses.

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Abbreviations eIF3-f, eukaryotic initiation factor 3 subunit 5; Grp75, Hsp-70 isoform; Hsp, heat shock protein; HU, hindlimb unloading; HO-1, haem oxygenase-1; MV, mechanical ventilation; SOD, superoxide dismutase; ROS, reactive oxygen species.

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Introduction

Skeletal muscle is well known to undergo atrophy and a slower to faster shift in contractile and metabolic properties in several animal and human models of disuse (Thomason & Booth, 1990; Baldwin, 1996; Desplanches, 1997; Baldwin & Haddad, 2001).

Muscle atrophy, which is the major determinant of disuse muscle weakness, is due to an imbalance between protein synthesis and degradation. At least in the most studied animal models of disuse, it is believed that an initial decrease in protein synthesis is followed by a sustained and likely predominant increase in protein breakdown (Thomason & Booth, 1990; Powers *et al.* 2005). Most studies have focused on protein degradation and have shown a complex and still developing picture in which the ubiquitin–proteasome pathway and the activation of caspase-3 and calpains are likely to play a major role (Powers *et al.* 2005, 2010). Importantly, myofibrillar proteins have been shown to be lost at a higher rate that other muscle fibre proteins (Thomason & Booth, 1990; Jackman & Kandarian, 2004).

Oxidative stress has been shown to occur in disuse and in many pathological conditions, and is now widely considered a major trigger of the imbalance between protein synthesis and degradation leading to muscle atrophy (Powers *et al.* 2005, 2010; Moylan & Reid, 2007). However, the causal role of oxidative stress in determining disuse atrophy has not been definitely established yet. Some contradictory results have been reported, suggesting that care should be taken over generalizing conclusions through different species, disuse models and muscles. We will focus on the most widely studied models of disuse in animals (mechanical ventilation, limb immobilization and hindlimb unloading), and in humans (bed rest, unilateral lower limb suspension, limb immobilization), and we will consider how conclusive the evidence for a causal role of oxidative stress is.

Oxidant antioxidant balance and oxidative stress

It has been known for a long time that reactive oxygen species (ROS) are present in skeletal muscle (Commoner *et al.* 1954) and can be generated during exercise (Dillard *et al.* 1978; Davies *et al.* 1982). As ROS can damage cell proteins, DNA and lipids through oxidation, they have been considered to just be damaging agents, and antioxidants are used to scavenge them (Dillard *et al.* 1978; Davies *et al.* 1982). The term oxidative stress has been consequently defined as the 'imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage' (Sies, 1997). Importantly, more recently it has been understood that ROS are major signals involved in muscle homeostasis, i.e. in maintaining normal skeletal muscle structure and function (Droge, 2002; Smith & Reid, 2006; Brigelius-Flohe, 2009; Musaro *et al.* 2010). ROS production due to heavy exercise training (Sastre *et al.* 1992; Vina *et al.* 2000; Palazzetti *et al.* 2003; Aguilo *et al.* 2005; Silva *et al.* 2010) has been shown to determine muscle damage, documented by increased lipid peroxidation, protein carbonylation, increase in serum creatine kinase and altered glutathione redox status. On the contrary, ROS production during moderate exercise caused positive adaptations among which are increases in insulin sensitivity, mitochondria biogenesis and antioxidant defence systems (Powers & Jackson, 2008; Jackson, 2009; Ristow *et al.* 2009; Silva *et al.* 2009; Strobel *et al.* 2011). Consequently, antioxidant administration may counter muscle damage following heavy exercise (Sastre *et al.* 1992; Vina *et al.* 2000; Palazzetti *et al.* 2004; Silva *et al.* 2010), but also positive adaptations following moderate exercise (Ristow *et al.* 2009).

The mechanisms underlying the opposite effects of ROS on muscle homeostasis in different conditions are still unclear. It could be that small, compartmentalized, or transient (minutes) increases in ROS mostly modulate intracellular signals by reversible oxidation of specific protein residues (Ghezzi, 2005; Janssen-Heininger *et al.* 2008) and consequently affect gene expression (Jackson *et al.* 2002; Ji *et al.* 2004; Powers *et al.* 2005, 2010). The latter phenomenon could occur in response to moderate exercise. In heavy exercise, disuse and pathological conditions, sustained (hours, days), large increases in ROS could (i) have a direct, non-specific, large scale oxidative effect on proteins, which would become more susceptible to proteolysis; (ii) damage plasma membrane and sarcoplasmic reticulum altering calcium homeostasis and activating proteases (e.g. calpains), enhancing proteolysis, (iii) damage lysosome and cause a leakage of catabolic enzymes in the cytosol. Oxidized proteins could be more susceptible to proteolysis because they are more easily targeted by the ubiquitin–proteasome system, which is up-regulated by ROS (Davies, 1987; Shang *et al.* 1997), because their recognition by calpain and caspase is enhanced (Smuder *et al.* 2010), or because they could be directly degraded by proteasome without being ubiquitinated (Grune *et al.* 2003), or for all the above causes.

As ROS are short lived and the direct determination of their concentration is complex and exposed to error (Smith & Reid, 2006; Palomero *et al.* 2008), in most studies on disuse atrophy, ROS activity has been studied 'indirectly', namely by measuring protein oxidation and lipid peroxidation (Lawler *et al.* 2003; Urso & Clarkson, 2003). The latter approaches have been mostly combined with another indirect index of ROS activity, namely the activity or content of antioxidant defence systems among them superoxide dismutase, catalase and glutathione peroxidase. The correlation between muscle atrophy, increase in protein and lipid peroxidation and impairment

of antioxidant defence systems has been taken as a strong indication that oxidative stress occurred and was involved in muscle wasting (Lawler *et al.* 2003; Powers *et al.* 2005). The administration of antioxidants has been used to counteract oxidative stress with the goal of confirming the role of ROS through amelioration of muscle atrophy (Kondo *et al.* 1992; Appell *et al.* 1997; Arbogast *et al.* 2007).

Animal models of disuse

Disuse atrophy due to mechanical ventilation. Studies on the mechanical ventilation model can be considered a paradigm of the relevance of studying a disuse model for clinical practice and of how an analysis can successfully develop providing strong evidence of the role of oxidative stress. The topic has been recently reviewed in detail (Powers *et al.* 2009). Table 1, presented as online Supplemental Material, summarizes findings of the major references cited regarding human and animal models of disuse in order of appearance.

Mechanical ventilation (MV) is extremely important in clinical practice, as it save lives of patients with critical respiratory problems due to a variety of conditions, e.g. respiratory diseases, spinal cord injuries, neuromuscular diseases, coma, general anaesthesia, drug overdoses.

In the MV model, as animals are tracheostomized and a ventilator delivers all breaths, the diaphragm is completely inactive (McClung *et al.* 2007). As early as 2002 available data suggested that MV induces an extremely rapid (12–18 h) force loss and diaphragm atrophy, and that oxidative stress plays a major role in the phenomenon (Shanely *et al.* 2002). Lately, the picture of the role of oxidative stress in MV was further refined showing that MV cause a very early depression of protein synthesis (Shanely *et al.* 2004), besides increasing protein degradation; that insoluble proteins, likely myosin and actin, are oxidized as early as 6 h into MV (Zergeroglu *et al.* 2003); and that the increase in ROS depends on both an increase in production and a decrease in removal due to down-regulation of antioxidant defence systems (e.g. glutathione, glutathione peroxidase, superoxide dismutase) (Falk *et al.* 2006).

Finally, a very recent work has brilliantly confirmed in humans undergoing MV some conclusions obtained in the rat model of MV: (i) muscle fibre atrophy, (ii) decrease in an antioxidant defence system (glutathione), and (iii) increase in caspase-3 and increase in expression of two key ubiquitin ligases (MurF-1 and atrogin-1) of the ubiquitin–proteasome pathways (Levine *et al.* 2008).

Importantly, administration of an antioxidant, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), a vitamin E analogue with strong and specific antioxidant properties, blunted diaphragm atrophy in mechanically ventilated rats (Betters *et al.* 2004; McClung *et al.* 2007; Whidden *et al.* 2010) (Fig. 1). Even more importantly, Trolox prevented activation of calpain and caspase-3 indicating that oxidative stress is a requirement for the activation of a major proteolytic pathway underlying MV muscle atrophy (Whidden *et al.* 2010). Such an example illustrates how a direct link between oxidative stress and proteolysis can be established, whereas most evidence of the role of oxidative stress in other models

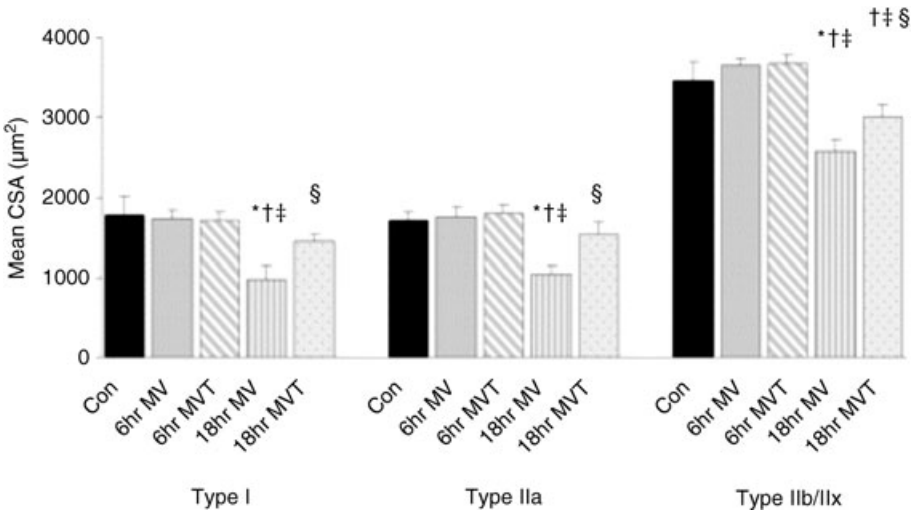

Figure 1. The impact of mechanical ventilation and antioxidant administration (Trolox) on cross-sectional areas (CSA) of different muscle fibres (type I, IIa and IIb/x)
Five groups of rats were studied: controls (Con), mechanically ventilated for 6 and 18 h without and with Trolox treatment (6 h MV, 6 h MVT, 18 h MV, 18 h MVT). (Reprinted from McClung *et al.* 2007 with permission from Wiley-Blackwell.) *Significantly ($P < 0.05$) different from control values. †Significantly ($P < 0.05$) different from 6 h MV values. ‡Significantly ($P < 0.05$) different from 6 h MVT values. §Significantly ($P < 0.05$) different from 18 h MV values.

relies just on correlations, e.g. on the presence at the same time of atrophy and signs of oxidative stress.

Disuse atrophy due to hindlimb unloading and limb immobilization. Several models of disuse have been used in small mammals, among which the most used have been hindlimb unloading (HU) and limb immobilization.

The potential role of oxidative stress in determining disuse atrophy was studied by Kondo *et al.* (1992, 1993) in pioneering works using the limb immobilization model. No change in two antioxidant enzymes, glutathione peroxidase and catalase, but an increase in the Cu,Zn cytoplasmic isoform of superoxide dismutase and in several indicators of oxidative stress (free iron, xanthine oxidase activity, lipid peroxidation and oxidized glutathione/reduced glutathione ratio) suggested, for the first time, a major role of oxidative stress in disuse atrophy.

The issue was reconsidered in detail 10 years later by Lawler *et al.* (2003, 2006) using the HU model. The data confirmed Kondo's hypothesis showing increase in ROS, alterations in antioxidant defence systems (increase in Cu,Zn superoxide dismutase (SOD), decrease in catalase and glutathione peroxidase activities), decrease in non-enzymatic antioxidant scavenging capacity (Lawler *et al.* 2003) and decrease in heat shock proteins, Hsp25 and Hsp70 (Lawler *et al.* 2006), which can play relevant roles in protecting cells against oxidative damage (Senf *et al.* 2008).

As the presence in the same muscle of atrophy, of indirect signs of ROS increase, and of an impairment of antioxidant defence systems cannot be considered a direct demonstration of the causal role of oxidative stress, antioxidants have been used in an attempt to prevent muscle atrophy, thereby proving the role of ROS. Whereas early experiments in the limb immobilization model suggested that antioxidant administration (vitamin E) can blunt soleus atrophy (Kondo *et al.* 1992; Appell *et al.* 1997), contradictory results were obtained in HU. No effect either in soleus or in gastrocnemius was observed in the first study on HU using vitamin E (Koesterer *et al.* 2002), and using allopurinol, an xanthine oxidase inhibitor (Matuszczak *et al.* 2004). The Bowman–Birk inhibitor, a soy protein extract which directly buffers ROS, ameliorated soleus atrophy (Arbogast *et al.* 2007), but as the drug also inhibits serine protease activity (Arbogast *et al.* 2007), its effect could be independent from its antioxidant activity. In another study, cysteine administration was shown to partially prevent unweighting-induced ubiquitination and degradation of proteins in parallel with redox system normalization, but the same group further showed no benefit of vitamin E (Ikemoto *et al.* 2002*a*,*b*). Interestingly, in a more recent study, a moderate effect of vitamin E on soleus atrophy was observed, but was accounted for by vitamin E inhibition of proteolytic enzymes, rather than its antioxidant capacity (Servais *et al.* 2007).

We very recently applied the proteomic approach (Brocca *et al.* 2010) and electrophysiological measurements (Desaphy *et al.* 2010) to mice following HU in the presence and absence of Trolox administration. Soleus data recapitulated what was previously observed (Lawler *et al.* 2003): muscle fibre atrophy (Fig. 2*A*), increase in lipid peroxidation and protein oxidation (Fig. 2*B*), and impairment of several antioxidant defence systems (Fig. 2*C*). In an electrophysiological study on the same animals, a slow to fast shift in muscle phenotype and an increase in chloride conductance and ClC-1 chloride channel expression were found, which are early markers of disuse atrophy (Pierno *et al.* 2002; Desaphy *et al.* 2005). Therefore, soleus data seemingly supported the idea that oxidative stress could be a major trigger of disuse atrophy. Interestingly, the level of lipid peroxidation was studied in soleus, gastrocnemius, tibialis anterior and EDL muscle and was found to be linearly related to percentage muscle atrophy (Fig. 2*D*) (Desaphy *et al.* 2010).

The analysis of HU gastrocnemius and of Trolox treated HU soleus and gastrocnemius casted doubts about the role of oxidative stress in determining muscle atrophy. HU gastrocnemius, a fast muscle rarely analysed in disuse models as it is considered to be less susceptible to atrophy, did show atrophy (11%), but surprisingly no increase in lipid peroxidation and protein oxidation, and an increase, rather than a decrease, of antioxidant defence systems (Brocca *et al.* 2010). No change in antioxidant defence systems was observed in the only previous work on HU gastrocnemius, although, based on increased lipid peroxidation, a pathogenetic role of oxidative stress was suggested (Siu *et al.* 2008). Moreover, Trolox administration, which increased antioxidant defence systems in both soleus and gastrocnemius muscles, fully prevented lipid peroxidation and protein oxidation, counteracted the increase in chloride conductance and partially counteracted the slow to fast shift in muscle phenotype, but did not have any impact on muscle and muscle fibre atrophy (Brocca *et al.* 2010; Desaphy *et al.* 2010).

Interestingly, these data suggest that the existence of a correlation between an indirect index of ROS production and muscle atrophy (Fig. 2*D*) does not necessarily imply a causal role for ROS. Indeed, protein oxidation might follow muscle atrophy or occur in parallel and the relationship still hold. In neurons, it has been shown that ROS production by mitochondria depends on Bax, a member of the Bcl-2 family of apoptotic regulators, and that caspase 3, a major enzyme involved in disuse induced proteolysis, mediates part of Bax induced ROS production (Kirkland *et al.* 2010). In principle, the latter finding opens the possibility that protein oxidation could, at least to some extent, follow proteolysis.

We reasoned that, notwithstanding Trolox administration, oxidative stress could have occurred at early times during HU, still determining muscle atrophy but dying away by the time of our analysis, i.e. 14 days of unloading. Therefore, we analysed four animals of the same strain and age as those used by Brocca *et al.* (2010) following 3 days of HU. Although muscle fibres (type 1 and 2A in soleus and type 2B in gastrocnemius) were significantly atrophic (Fig. 3*A*) (16% on average), no protein oxidation was detectable (Fig. 3*B*) and no impairment was observed regarding three components of the antioxidant defence systems previously found to be differentially expressed after 14 days of HU, namely superoxide dismutase, Hsp70 and α,β-crystallin. The latter results, consistent with the lack of benefits from antioxidant administration in mice, support the hypothesis of a marginal role of oxidative stress in muscle atrophy induced by HU, which is clearly in contrast with the causal role played in diaphragm atrophy following mechanical ventilation.

Human models of disuse

Notwithstanding the large amount of work on structure and function of skeletal muscle in several disuse models

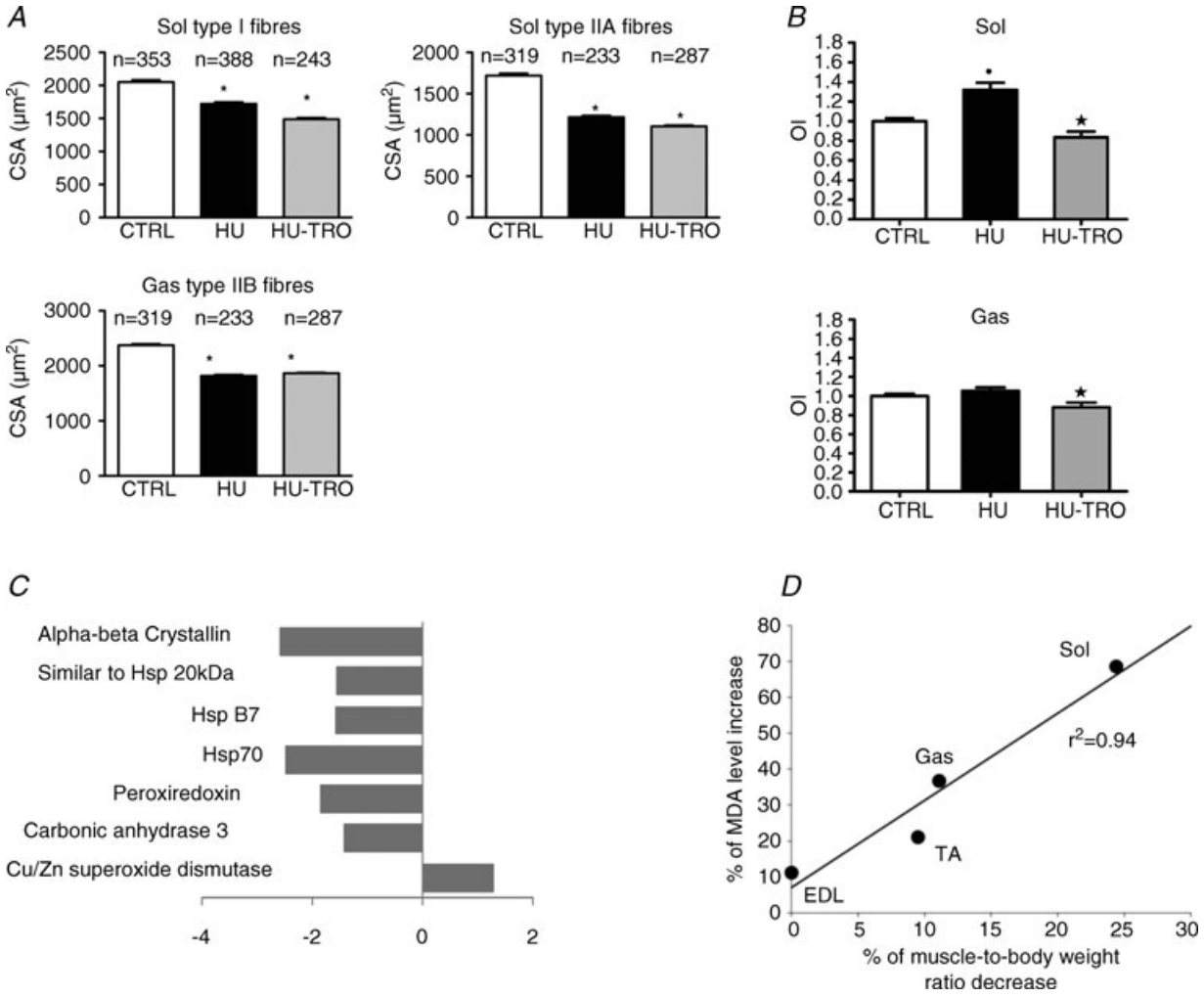

Figure 2. The impact of hindlimb unloading (HU) and antioxidant administration (Trolox) in a slow, soleus (Sol), and a fast, gastrocnemius (Gas), muscle of the mouse
A, cross-sectional area of type I and IIA fibres from Sol and of type IIB fibres from Gas in control mice (CTRL), hindlimb unloaded mice for 14 days (HU-14) and hindlimb unloaded mice for 14 days treated by Trolox (HU-TRO). *Significantly different from CTRL ($P < 0.05$). (Redrawn from Brocca *et al.* (2010).) *B*, protein oxidation index (OI). The height of each vertical bar represents the mean ± SEM. *Significantly different from all groups ($P < 0.05$). (Reprinted from Brocca *et al.* (2010) with permission from Wiley-Blackwell.) *C*, differentially expressed proteins belonging to antioxidant defence systems in soleus muscles of HU mice, identified by proteomic analysis. (Redrawn from Brocca *et al.* 2010.) *D*, malondialdeyde (MDA) levels following 14 days HU plotted against the mean percentage of muscle-to-body weight ratio decrease for EDL, tibialis anterior (TA), Gas and Sol muscles of the mice. The points were linearly correlated ($r^2 = 0.94$). (Reprinted from Desaphy *et al.* 2010 with permission from Elsevier.)

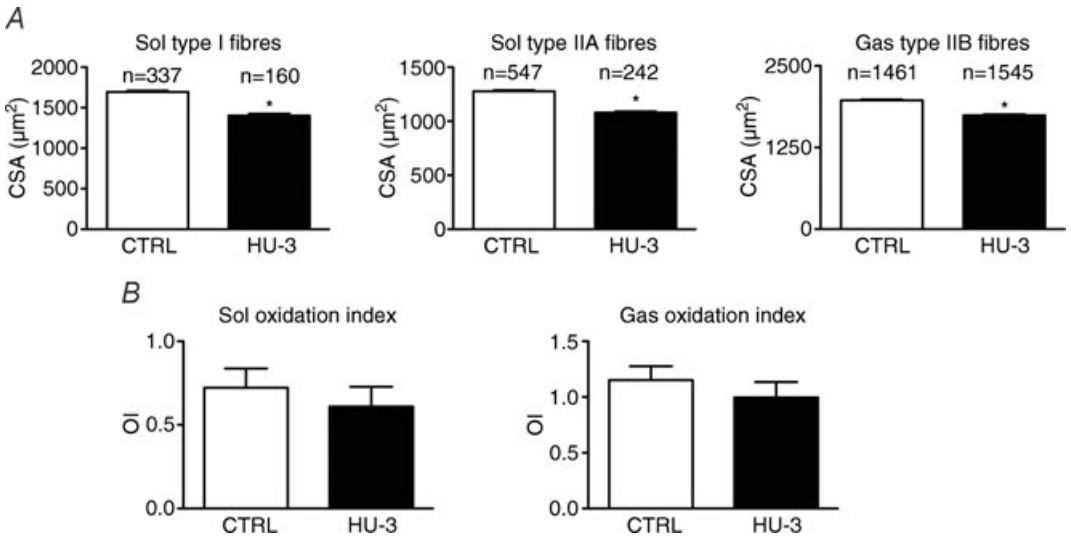

Figure 3. The impact of 3 days hindlimb unloading (HU-3) on cross-sectional area (CSA) of identified types of muscle fibres (*A*) and on protein oxidation index (OI) (*B*) of soleus (Sol) and gastrocnemius (Gas) muscles of the mouse
The number (*n*) of fibres measured is indicated above each bar. Both CSA and OI were determined exactly as by Brocca *et al.* (2010). *Significantly different from CTRL ($P < 0.05$). Four mice were used.

in humans (Fitts *et al.* 2000; Trappe *et al.* 2001; Narici *et al.* 2003; Hortobagyi & Devita, 2006; de Boer *et al.* 2007*a*; Pavy-Le Traon *et al.* 2007; Rittweger *et al.* 2009), information on oxidative stress and on the potential mechanisms involved in increased proteolysis is scanty. The first of such studies on bed rest suggested a potential role of oxidative stress in human disuse in line with what was previously observed in small mammals (Dalla Libera *et al.* 2009). Following 35 days (T35), but not 8 days (T8), of bed rest, vastus lateralis muscle samples showed

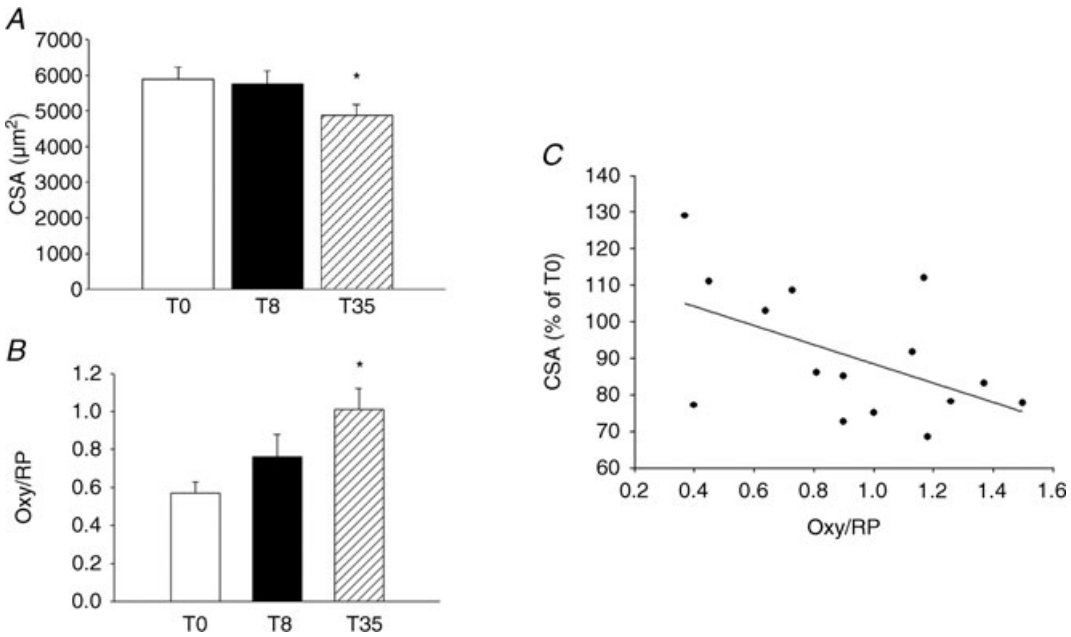

Figure 4. The impact of 8 (T8) and 35 (T35) days of bed rest on cross-sectional area (CSA) of muscle fibres and on protein oxidation (Oxy/RP) of muscle samples from the vastus lateralis muscle of humans
A, mean values of CSA of muscle fibres before bed rest (T0) and at T8 and T35. *B*, protein oxidation index (Oxy/RP). *Significantly different from T0 ($P < 0.05$). *C*, regression analysis of normalized values of muscle protein oxidation (Oxy/RP) plotted against the percentage change of fibre CSA of the same muscles, determined at T8 and T35; the slope of the line was significantly different from zero ($P < 0.05$), reprinted from Dalla Libera *et al* 2009 used with permission from The American Physiological Society.

muscle fibre atrophy (18%) (Fig. 4*A*) and increased protein carbonylation (Fig. 4*B*). Interestingly, an inverse linear relationship was found between normalised levels of protein oxidation and CSA of muscle fibres of biopsy samples (Fig. 4*C*), consistent with what was reported for mice muscles in Fig. 3*D* (Desaphy *et al.* 2010). At T8 the transient increase in two heat shock proteins known to be up-regulated in response to ROS production (Motterlini, 2005) (Ryter *et al.* 2006), haem oxygenase-1 (HO-1) and mitochondrial Hsp-70 isoform (Grp75), suggested the occurrence of oxidative stress. A very recent global analysis of gene expression (Reich *et al.* 2010) has shown an up-regulation of the pathways involved in oxidative stress response following 48 h of unilateral lower leg suspension (ULLS), consistent with the increase in HO-1 observed by Dalla Libera *et al.* (2009). However, some inconsistencies emerged, as discussed by the authors. For instance the content of both heat shock proteins was normal at T35, suggesting that some mechanism had blunted the stress response after T8, although increased carbonylation was actually observed at T35.

More recently, in a limb immobilisation human model, muscle (5.7%) and muscle fibre (5.6–11.8%) atrophy were observed at the end of 14 days of immobilisation (T14) in the absence of lipid peroxidation (Fig. 5*A*) and protein oxidation (Fig. 5*B*) in vastus lateralis muscle. Ubiquitin conjugates were higher at day 2, but normal at T14 (Fig. 5*C*), and caspase 3/7 activity was normal at both 2 and 14 days (Fig. 5*D*) (Glover *et al.* 2010). Based on their own data and on previously published findings, which did not show increased protein breakdown in human disuse (Paddon-Jones *et al.* 2004; Symons *et al.* 2009), the authors suggested that muscle atrophy was likely to be independent of oxidative stress and increased proteolysis, although the rate of protein breakdown was not determined (Glover *et al.* 2010).

The lack of increased proteolysis in muscle atrophy following immobilisation is consistent with a number of studies strongly suggesting that, contrary to what observed in small mammals, the major phenomenon involved in humans is a decrease in protein synthesis exacerbated by anabolic resistance, namely by a decreased stimulation of protein synthesis by exogenous amino acids (Rennie *et al.* 2010). In muscle atrophy following ULLS, rates of myofibrillar protein synthesis fell and no clear signs of increased expression of MuRF-1 and atrogin-1, used as markers of proteolysis, were observed (de Boer *et al.* 2007*b*). The increase in whole-body protein synthesis following amino acid feeding was blunted in bed rest (Biolo *et al.* 2004) and the infusion of amino acids increased proteins synthesis less in the immobilised leg than in the non-immobilised leg (Glover *et al.* 2008) suggesting anabolic resistance.

Although most evidence points to a decreased protein synthesis as the dominant phenomenon in disuse atrophy in humans, some findings suggested an early and transient increase in protein breakdown (Tesch *et al.* 2008; Glover *et al.* 2010; Gustafsson *et al.* 2010). A very recent global gene expression analysis showed up-regulation of pathways involved in protein ubiquitination and oxidative

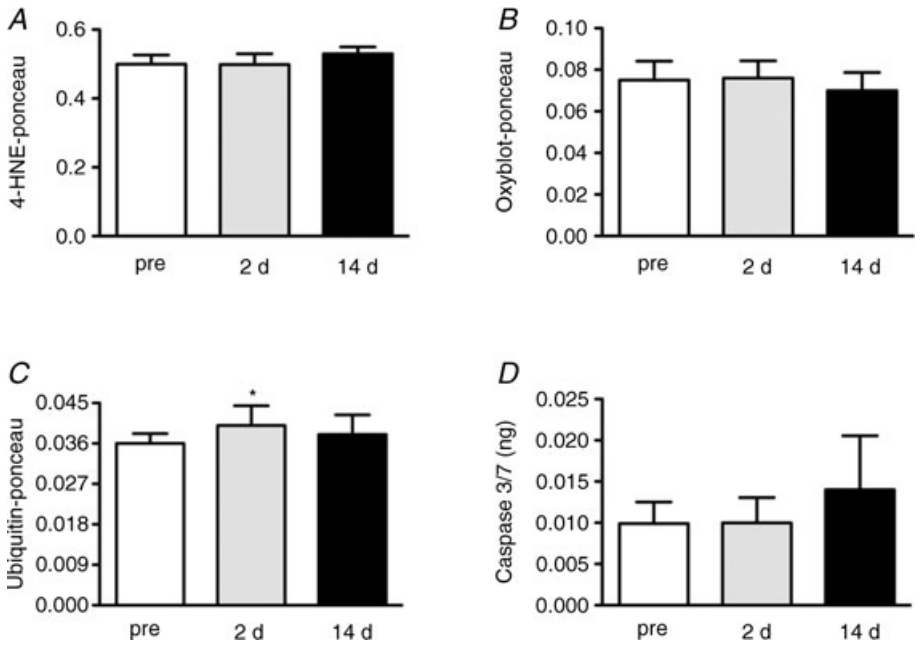

Figure 5. The impact of 2 days (2 d) and 14 days (14 d) leg immobilization on lipid peroxidation (4-HNE-ponceau) (*A*), protein carbonylation (oxyblot-ponceau) (*B*), ubiquitin protein conjugates content (ubiquitin-ponceau) (*C*) and caspase 3/7 activity (*D*)
Redrawn from Glover *et al.* 2010.

stress response following 48 h ULLS (Reich *et al.* 2010). Moreover, a pivotal role of oxidative stress and increased proteolysis in human chronic disease (e.g. respiratory, kidney and cardiac disease, and muscular dystrophy) has been suggested by a number of studies (Moylan & Reid, 2007) in agreement with what was previously observed in many disuse conditions in small mammals (Powers *et al.* 2007). In humans following 2–5 days of spinal cord injury, muscle atrophy was associated with an increased expression, although not with an increased content of key components of the ubiquitin proteasome pathway (Urso *et al.* 2007). However, it has been strongly argued that, in the absence of inflammation or other phenomena occurring in chronic diseases, disuse muscle atrophy can be fully explained by a decreased protein synthesis with little evidence of a possible role of increased proteolysis and of oxidative damage or protein carbonylation (Murton *et al.* 2008; Glover *et al.* 2010; Rennie *et al.* 2010). Consequently, caution has been suggested in transferring the results obtained on small mammals to humans (Rennie *et al.* 2010).

To the best of our knowledge, there are no more studies which assessed the presence of oxidative stress in human limb muscles in disuse. The lack of a major role for proteolysis, the key phenomenon supposedly triggered by oxidative stress, suggests that the latter may not be a major phenomenon in disuse atrophy of limb muscles in humans. However, studies on oxidative stress and disuse atrophy in humans are still limited. Moreover, ROS might still play a role, but not through an increase in proteolysis. Indeed, it has been recently suggested that a ROS induced increase in atrogin-1 following 20 days of bed rest could cause atrophy by impairing protein synthesis (Ogawa *et al.* 2006), reconciling the strong evidence of a predominating decrease in protein synthesis in humans with the observation of a transient increase in atrogin-1, a well established index of proteolysis. The latter hypothesis, which is still to be confirmed, finds some support in several observations. Atrogin-1 is up-regulated in disuse atrophy through a potentially ROS-dependent mechanism (Li *et al.* 2003; Powers *et al.* 2010). It is a key component of the ubiquitin–proteasome proteolytic pathway, but has been also shown to have a modulatory role on eukaryotic initiation factor 3 subunit 5 (eIF3-f), a component of the AKT/mTor pathway (Lagirand-Cantaloube *et al.* 2008), which controls protein synthesis (Sandri, 2008).

Table 1 (online Supplemental Material) summarizes findings of the major references cited regarding human and animal models of disuse in order of appearance.

Why oxidative stress may not be equally relevant in all disuse conditions

The role of an alteration of redox homeostasis in disuse atrophy appears to widely vary through muscles, models and species. Oxidative stress very likely plays a causal role in diaphragm following MV. It is less clear whether it is a cause or consequence of disuse atrophy in soleus and even more so in gastrocnemius of HU mice, and very limited evidence exists that it could play a determinant role in humans. The discrepancy between the strong and rapid oxidative stress-dependent atrophy of human diaphragm following mechanical ventilation (Levine *et al.* 2008) and the smaller and slower oxidative stress-independent atrophy of human limb muscles (Glover *et al.* 2010) strengthens the relevance of variability among different muscles and experimental conditions.

ROS acutely affects muscle force and are necessary for normal muscle homeostasis (Reid *et al.* 1993; Smith & Reid, 2006; Brigelius-Flohe, 2009). They play a significant role in positive adaptations following moderate exercise, being intracellular signals modulating gene expression (Jackson *et al.* 2002; Ji *et al.* 2004; Brigelius-Flohe, 2009; Jackson, 2009; Ristow *et al.* 2009); they cause muscle damage in heavy exercise; they modulate intracellular signalling pathway involved in disuse atrophy (Powers *et al.* 2010); and they can directly oxidize proteins on a large scale enhancing their degradation rate (Smuder *et al.* 2010). Consequently, it is very likely that differences in the time course of ROS production, in the levels of ROS, in the cellular locations of ROS production and possibly in the nature of the ROS can occur in different tissues and conditions supporting different responses. Interestingly, NADPH oxidases, a major source of ROS, are a family of enzymes whose members have different tissue localizations (Geiszt & Leto, 2004) and are present in different subcellular compartments (Ushio-Fukai, 2006).

Given the large spectrum of ROS potential effects it is not surprising that their role in disuse atrophy can vary according to muscles, species and models. The difficulties in differentiating the role of ROS in different disuse conditions might depend on the experimental approach used and on technical limitations. The analysis of correlations between atrophy and indirect indexes of ROS activity might be misleading. It can be argued that such correlations cannot definitely discriminate between oxidative stress being a cause or a consequence of muscle atrophy (Fig. 3*D*) (Desaphy *et al.* 2010). Moreover, determination of carbonyls and lipid peroxidation is likely to be sensitive to large scale oxidative phenomena missing more subtle, but still potentially modulating, levels of ROS.

The precise determination of ROS levels in living cells is, therefore, of paramount importance and its lack is a major drawback in all attempts to differentiate ROS effects in different conditions. Recent advances in this respect may open important opportunities (Palomero *et al.* 2008). Notwithstanding the latter problems, some phenomena potentially responsible for the variability in the response

to disuse and in the ascertained roles of ROS can be put forward.

The major muscles studied have different fibre type composition. Soleus has the highest percentage of slow fibres, gastrocnemius the lowest and diaphragm is intermediate (Pellegrino *et al.* 2004; Desaphy *et al.* 2010). Interestingly, diaphragm muscle has the higher capillary and mitochondrial density than any other skeletal muscle (Hoppeler *et al.* 1981) and soleus is well known to be much more oxidative than gastrocnemius. Considering that mitochondria are among the major potential sources of ROS and that a proportion of electron flow (0.15%, which is small in relative terms, but relevant in absolute values) gives rise to hydrogen peroxide (Chance *et al.* 1979; St-Pierre *et al.* 2002), soleus and especially diaphragm could be more exposed to ROS production.

The decrease in load could have different effects on diaphragm, which is chronically active, and on soleus, which is a postural muscle, than on gastrocnemius, which is a fast, phasic muscle. MV, limb immobilisation, and HU could decrease neuromuscular activity to different extents. In MV diaphragmatic fibres are totally inactive (Powers *et al.* 2002) and go through passive shortening during mechanical expansion of the lungs (Froese & Bryan, 1974). Inactivity in the shortened position is known to favour atrophy (Loughna *et al.* 1987). Immobilisation (Fischbach & Robbins, 1969) might decrease neuromuscular activity more than HU, in which some authors have shown no significant decrease in integrated EMG (Alford *et al.* 1987), and others have shown an initial decrease in the first 6 days followed by recovery towards normal levels (De-Doncker *et al.* 2005).

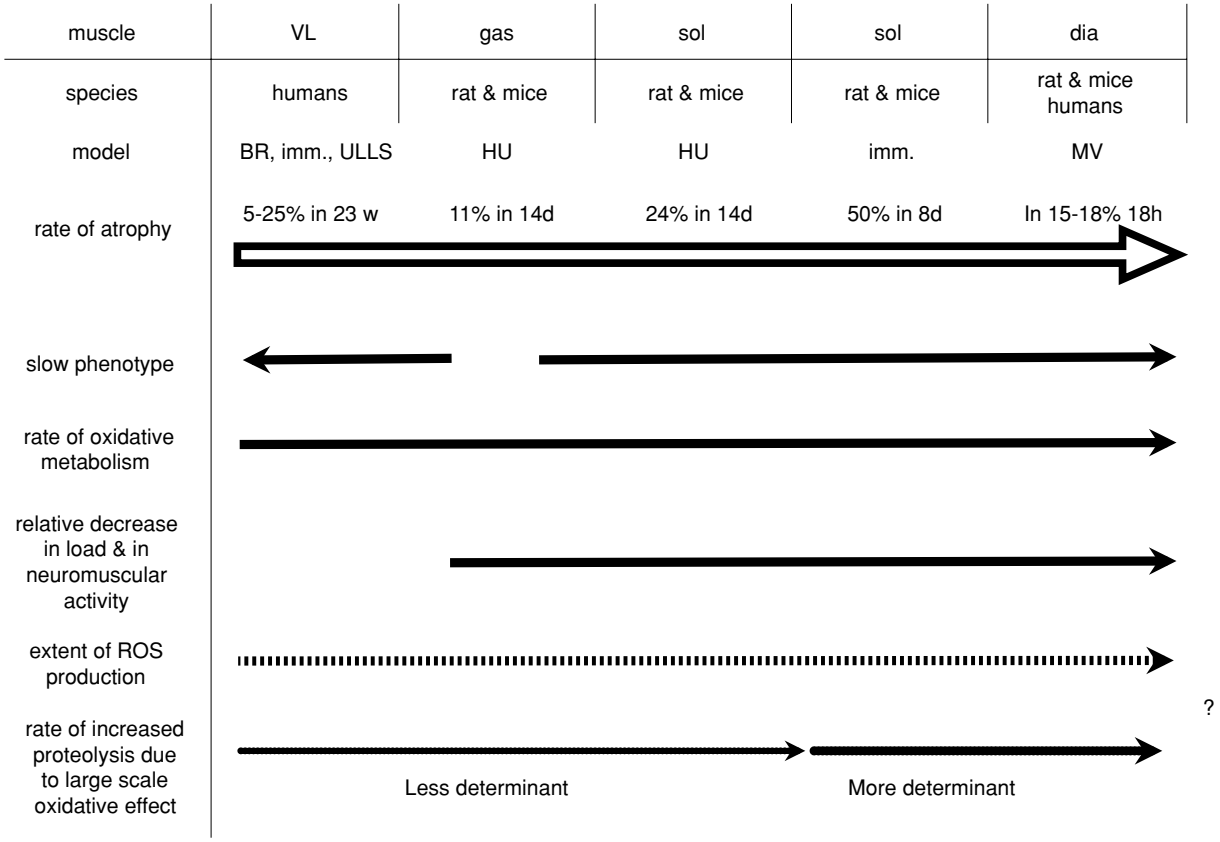

Figure 6. A scheme of the factors potentially involved in determining variable alterations of redox homeostasis through muscles, species and models reported in the first three rows
Arrows point to the direction of an increase in the parameter. The large open arrow refers to the rate of atrophy, which increases from left to right. The dashed arrow hypothesizes an increase in the extent of ROS production, from left to right, depending (i) on the increase in the rate of oxidative metabolism due to small species having higher metabolic rate and to the progressively slower phenotype (i.e. relative content of slow, type 1 fibres) of muscles and (ii) on the increase in the relative extent of unloading at least from HU gastrocnemius towards diaphragm subjected to MV. Consequently, the dotted arrow hypothesizes an increase in the rate of proteolysis, from left to right, which is less determinant (or minor) in humans, and in HU gastrocnemius and soleus of rat and mice, but more determinant (or major) in immobilized soleus and in diaphragm following MV due to a progressively more evident large scale oxidation of proteins. Abbreviations: BR, bed rest; imm., immobilization; HU, hindlimb unloading; MV, mechanical ventilation; d, days; w, weeks; h, hours.

Therefore, diaphragm might go through higher ROS production then soleus and soleus than gastrocnemius. In diaphragm all ROS inducible pathways could be activated, whereas in soleus and especially in gastrocnemius protein oxidation could not be an earlier (Fig. 3*B*) and major (Fig. 2*B*) phenomenon and other mechanisms independent from ROS or induced by a more subtle increase in ROS could play a role.

Interestingly, the possibility that ROS production occurs at different extents and rates and that different mechanisms prevail in different models and muscles is consistent with the very variable rate of muscle atrophy. The rate of muscle atrophy is, in fact, extremely fast in rat diaphragm following MV reaching 15–30% in 18 h, very fast in rat soleus following limb immobilisation (∼50% in 8 days) and much slower in soleus (24%) and in gastrocnemius (11%) of mice following 14 days HU (Brocca *et al.* 2010; Desaphy *et al.* 2010).

The discrepancies between animal and human models appear even larger than within the different animal models. It has been known for a long time that oxidative metabolism and heat production per unit body weight are inversely related to body weight (Kleiber, 1947), and that small animals are more exposed to ROS production and, possibly for this reason, have shorter lifespan (Demetrius, 2005). Interestingly, it has been observed that the higher the metabolic rate the higher muscle atrophy and that small mammals, having a smaller percentage of muscle mass, have less metabolic resilience than large mammals (Demetrius, 2005). Moreover, disuse models in small mammals could determine some stress and this might make muscle more prone to an increase in protein breakdown (Paddon-Jones, 2006; Paddon-Jones *et al.* 2006). Finally, xanthine oxidase, a major source of ROS, might be less expressed in human than in rat muscle (Linder *et al.* 1999). Indeed, the rate of disuse atrophy is much higher in small mammals (∼3% a day) (Thomason & Booth, 1990) than in humans (5–25% in 23 weeks) (de Boer *et al.* 2007*b*).

Figure 6 reports a scheme summarizing the major factors potentially involved in determining variable alterations of redox homeostasis through muscles, species and models.

Conclusions

The observation that ROS play a major role in muscle homeostasis, but can cause muscle wasting as well, indicated that ROS production can be finely tuned and controlled. It is thus very likely that the extent and time course of ROS production, the ROS-dependent intracellular pathways activated and, therefore, roles of ROS widely vary through different muscles, species and disuse models.

It could be wise to apply the term 'oxidative stress' only to conditions in which oxidative damage is documented to avoid misunderstanding by pooling phenomena in which ROS modulate intracellular pathways and gene expression contributing to muscle homeostasis and plasticity and possibly to muscle atrophy, and those in which ROS enhance proteolysis acting directly and non-specifically on proteins.

In the absence of readily available approaches to determine ROS levels in living cells, the evaluation of the time course of changes in muscle mass, antioxidant defence systems and signals involved in protein synthesis and breakdown in both the absence and presence of antioxidants should be the more correct approach to clearly define the cause and effect relationship between such phenomena. The simple correlation between muscle atrophy and indirect indexes of enhanced ROS production at any given time during the process does not seem to be able to provide definitive conclusions (Fig. 2*D* and Fig. 4*C*).

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