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Molecular events and signalling pathways involved in skeletal muscle disuse-induced atrophy and the impact of countermeasures

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Abstract

Disuse-induced skeletal muscle atrophy occurs following chronic periods of inactivity such as those involving prolonged bed rest, trauma and microgravity environments. Deconditioning of skeletal muscle is mainly characterized by a loss of muscle mass, decreased fibre cross-sectional area, reduced force, increased fatigability, increased insulin resistance and transitions in fibre types. A description of the role of specific transcriptional mechanisms contributing to muscle atrophy by altering gene expression during muscle disuse has recently emerged and focused primarily on short period of inactivity. A better understanding of the transduction pathways involved in activation of proteolytic and apoptotic pathways continues to represent a major objective, together with the study of potential cross-talks in these cellular events. In parallel, evaluation of the impact of countermeasures at the cellular and molecular levels in short- and long-term disuse experimentations or microgravity environments should undoubtedly and synergistically increase our basic knowledge in attempts to identify new physical, pharmacological and nutritional targets to counteract muscle atrophy. These investigations are important as skeletal muscle atrophy remains an important neuromuscular challenge with impact in clinical and social settings affecting a variety of conditions such as those seen in aging, cancer cachexia, muscle pathologies and long-term space exploration.

Keywords: proteolysis • muscle wasting • ubiquitin ligase • NF-κB • proteasome • apoptosis • microgravity • exercise • growth factor • nutrition

Introduction

Skeletal muscle is one of the most affected tissues during chronic disuse situations resulting from prolonged bed rest, trauma to the neuromuscular apparatus and microgravity environment. These situations involve both hypokinesia and hypodynamia, thereby reducing neuromuscular activity and directly causing muscle atrophy. The loss of muscle can occur rapidly and significantly within in fact 2 days of disuse onset [1], and an abundant literature already describes the multitude of muscle adaptations

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affecting the expression of metabolic, structural and contractile proteins [2–6].

To date, our understanding of the effect of hypokinesia and hypodynamia on skeletal muscle is mainly derived from studies that focused on elucidating the specific contributions of transcriptional mechanisms, intracellular signalling molecules and the extent of cross-talks between these various pathways during muscle wasting. The patterns of mRNA changes have been defined in several well-characterized pathological or physiological states that cause muscle wasting, such as disuse-induced, age-related (sarcopenia) and disease-related (cachexia) atrophy [7]. Molecular targets affected in sarcopenia seem to be less related to cachexia and disused-induced atrophy. However, contrary to disuse-induced atrophy, tumour cachexia and sepsis are often associated with increased activation of signalling pathways such as tumour necrosis factor (TNF)-α. Thus differences in signalling pathways likely exist between these two latter models.

In parallel to defining the cellular and molecular events controlling the atrophic process, it appears crucial to also identify the best protocols to counteract muscle atrophy. The ultimate goal in this case, is to develop effective and therapeutic exercise paradigms, nutritional supplements, pharmacological agents and/or genetic countermeasures to prevent, limit or even reverse skeletal muscle atrophy.

In its first section, this review provides an up-to-date account of atrophy signalling pathways related mainly to chronic disuse, microgravity and unloading. Although phenotypic adaptations during muscle disuse affect a broad spectrum of genes and proteins [6, 8], we focus mostly on events causing the actual loss of muscle mass, and on the main transcription factors currently known to be involved in muscle atrophy, namely nuclear factor κB (NF-κB) and the Forkhead family of transcription factors (FOXOs). For this, special attention is given to studies using simulated atrophy models in both animals, such as immobilization, hindlimb suspension and denervation, and in human beings, such as immobilization and bed rest, as well as to the contributions of cellular and molecular experimentations. The second section presents several approaches to counteract muscle atrophy and discusses how the different types of countermeasures affect atrophic signalling pathways.

Overview of signalling pathways involved in disuse-induced muscle atrophy

Changes in protein synthesis and associated signalling pathways

A high rate of protein turnover is observed in adult human skeletal muscle. In normal conditions, this turnover is efficient as it maintains and stabilizes muscle mass while ensuring replacement of proteins incorrectly translated and others requiring degradation. Considering a classical diet involving consumption of 1 g of protein per kg of body weight per day, the estimated synthesized and degraded protein rate is approximately 3.5 to 4.5 g per kg per day, which translates into an average of 280 g of protein per day for an adult of 70 kg – an amount of protein equivalent to that contained in 1.0 to 1.5 kg of muscle [9]. In rats, the majority of muscle proteins is replaced every 1 or 2 weeks and myofibrillar proteins have relatively long half-lives, *e.g.* 7 to 10 days for myosin heavy chain [10]. Thus, small chronic changes in the rate of protein synthesis or degradation can markedly affect the equilibrium of protein turnover resulting in either gains or losses of muscle mass.

Changes in protein synthesis result in part from modulations of the mechanisms involved in mRNA translation which in turn involves the initiation, elongation and termination steps [11]. The initiation process appears to be a major regulatory event. Briefly, translation of most mRNAs is initiated by the binding of the 40S ribosomal subunit to the m^7GTP cap. In this case, translation initiation involves more than a dozen proteins named eukaryotic initiation factors (eIF) and is characterized by two main steps: The first one involving eIF2 and binding to the 40S ribosomal subunit, and the second one involving the eIF3–40S ribosomal subunit complex and the eIF4F complex (which comprises eIF4A, eIF4E and eIF4G). The binding of 4E-BP1 (a family of translational repressors) to eIF4E precludes the interaction of eIF4E mRNA complex to 40S and thus inhibits protein synthesis. By contrast, phosphorylation of 4E-BP1 suppresses the link with eIF4E and thus allows the interactions with 40S rRNA, which increases protein synthesis [12].

Recently, several studies have described signalling pathways involved in the translation initiation step. Most of this recent knowledge comes from the opposite field of muscle disuse atrophy, *i.e.* from studies focusing on muscle hypertrophy [11]. In this context, it has been shown that the overloading of muscle using maximal resistance exercise, compensatory hypertrophy and stimulation of muscle growth with anabolic agents, such as insulin-like growth factor 1 (IGF-1), leads to the induction of IGF-1 in muscle and the activation of the PI3K/Akt pathway [13–15]. This activation leads to the phosphorylation and activation of mammalian target of rapamycin (mTOR), which causes phosphorylation and activation of p70S6 kinase and 4E-BP1, and inhibition of glycogen synthase kinase 3-β, which is a repressor of eIF-2B. Thus, activation of this pathway leads to increases in muscle protein synthesis. However, although several studies have demonstrated that IGF-I is a critical factor involved in skeletal muscle hypertrophy, no investigation has convincingly demonstrated that the IGF-I receptor is the primary upstream signal necessary for load-induced activation of the Akt/mTOR signalling pathway. In fact, a recent study from the Bodine group, using MKR mice (transgenic mice expressing a mutated IGF-I receptor specifically in skeletal muscle to prevent activation of the IGF-1R in response to binding of endogenous or exogenous IGF-I), has demonstrated that the IGF-I receptor is not necessary for the activation of the Akt/mTOR signalling pathway and induction of skeletal muscle growth in response to mechanical loading [16].

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Conversely, recent studies have focused on trying to determine whether disuse muscle atrophy indeed causes deactivation of PI3K/Akt pathway thereby explaining a decrease in protein synthesis (Fig. 1A). Importantly, inhibition of the PI3K/Akt/mTOR pathway was sufficient to block the vast majority of genes normally regulated by IGF-1, demonstrating that Akt/mTOR is additionally required for the IGF-1-mediated transcriptional changes in skeletal muscle cells [17]. Furthermore, using *in vitro* experimentations of atrophy (treatment of C2C12 myotubes with dexamethasone [DEX]) and hypertrophy (treatment of C2C12 with IGF-1), Latres *et al.* showed that metallothioneins (MT-1, MT-2), MAFbx, proliferins and parvalbumin are significantly and inversely regulated after IGF-1 *versus* DEX treatment [18]. These last results indicated that indeed some of the same mechanisms are triggered but with opposite effects.

Muscle hypokinesia and hypodynamia have been shown to be associated with a rapid decrease in the rate of protein synthesis as seen in rat hindlimb suspended muscles [19] and in human muscles exposed to bed rest [20] or spaceflight [21]. Dephosphorylation and hence, deactivation of the PI3K/Akt pathway, leading to the decrease in protein synthesis rate, has been observed using experimentation with hindlimb unloading and denervation models [22, 23]. Two groups have generated mice lacking either Akt1, or Akt1 and 2, and have demonstrated that these mice exhibit severe growth deficiency with significant skeletal muscle atrophy [17, 24]. The phenotype of the Akt1-Akt2 double knockout mice appears similar to the IGF-1-receptor-deficient mice with a body weight decreased by ~50% [25]. Bodine *et al.* also observed that the amount of 4E-BP1 bound to eIF-4E was increased after 14 days of unloading in rat gastrocnemius muscle [22], and an increase in 4E-BP1 mRNA, as early as 1 day to 14 days of unloading, has been reported in a large-scale study of rat unloading [26]. These previous studies highlight the central role that Akt/mTOR signalling pathways have in the regulation of cell growth, but a number of questions remain. For example, the regulation of genes involved in ribosome assembly has been addressed but little is known about which genes are downstream by the Akt/mTOR pathway [27]. Additionally, because most studies have focused on short-term atrophy, it seems important to carry out experiments with long duration disuse atrophy to determine the involvement of this signalling pathway in prolonged atrophic events.

Several pathways contribute to increased protein degradation but to different extents

Different proteolytic systems have been described in skeletal muscle and they have been well reviewed [28]. In this context, chronic disuse leads to activation of the different proteolytic systems, mainly the calpain (Fig. 1B1), lysosomal (Fig. 1B2) and ubiquitin-proteasome systems (Fig. 1B3), in series or in parallel, ultimately resulting in enhanced protein breakdown and muscle wasting. However, it seems rather clear that although each of these systems is involved and operates to degrade cellular proteins, their relative involvement differs significantly.

The cytosolic non-lysosomal calcium-dependent protease system is involved in multiple functions in cells including proteolysis of proteins, cell cycle, apoptosis, cytoskeleton organization and signal transduction [29]. Skeletal muscle mainly expresses three ubiquitous calpains; calpain 1, 2 and 3 which are also called mu-calpain, m-calpain and p94, respectively [30]. Although the involvement of the calpain system in muscle atrophy is now confirmed, the relative contribution of each isoform is unclear. Increases in calpain 2 activity and its mRNA were reported following 9 and 14 days of hindlimb suspension [31, 32] and following 16 days of spaceflight in rats [32]. Moreover, an increase in calpain 3 mRNA following 60 days of bed rest in women was also observed [33]. The net activity of calpains depends largely on the calpain/calpastatin ratio – the latter being the endogenous inhibitor of calpains. The existence of this inhibitor has made it difficult to precisely ascertain the role and degree of involvement of the calpain system in disuse atrophy. However, studies using transgenic mouse models with muscle-specific overexpression of calpastatin brought clear insights on the involvement of Ca^{2+}-dependent protease system by showing a 30% decrease of muscle atrophy and prevention of the slow to fast fibre type switch following 10 days of unloading [34] (Table 1). Even if it remains unclear, several experiments strongly indicated that the calpain proteases and the proteasome could work in a coordinated manner to degrade muscle proteins and further suggested that the calpains act upstream of the ubiquitin-proteasome pathway [35–38]. As oxidative stress has been linked to skeletal muscle disuse-induced atrophy [39], a specific role for activated cysteine proteases (calpains and caspase 3), has been hypothesized as candidates in the disassembly of myofibrils and myofilaments release during the initial stages of muscle atrophy [39–42]. Actually, the specific role of these cysteine proteases in oxidant-induced muscle atrophy remains unclear. However, the recent study from Powers's group, using siRNA mediated gene silencing, clearly showed that the knockdown of calpain 1 attenuated H_2O_2-induced C2C12 myotube atrophy, whereas knockout of calpains 2 and 3 did not [43].

In mammalian cells, lysosomes, containing high concentrations of acid hydrolases, act as intracellular compartments for degradation of diverse macromolecules [44]. Among several types of hydrolases and proteases, the ubiquitous cathepsins L, B, D and H have a major role in proteolytic capacity of lysosomes, particularly in tissues with a high rate of protein turnover such as liver or kidney [45]. By contrast, low levels of cathepsins are present in skeletal muscle, but a higher expression has been reported in slow-twitch muscles [44]. Moreover, an increase in the expression and activity of cathepsins, and specifically cathepsin L, has been reported in atrophic muscle following short-term unloading [31] and 2 weeks of spaceflight [32]. However, inhibition in atrophying muscles of lysosomal acidification with leupeptin E-64 [42], or with agents inhibiting cathepsins [46], causes only a slight reduction in muscle proteolysis, indicating that the lysosomal system is not a major contributing pathway to protein degradation in muscle disuse atrophy, particularly for cytosolic degradation of myofibrillar proteins. Although autophagy, essential for cell

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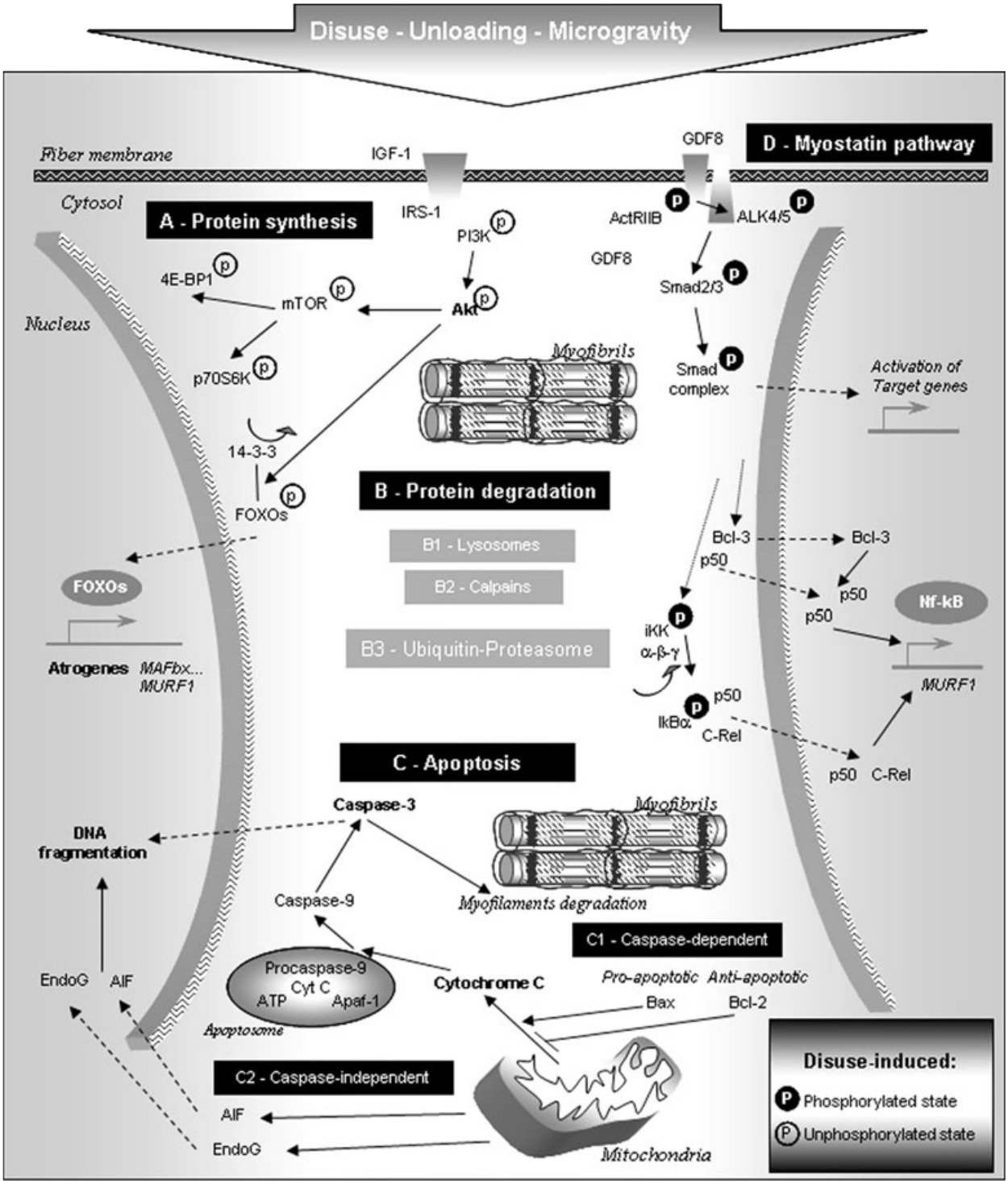

Fig. 1 Schematic diagram of signalling pathways involved in skeletal muscle remodelling and atrophy following disuse. (**A**) Skeletal muscle disuse is associated with a decrease in protein synthesis involving the deactivation of the PI3K/Akt/mTOR pathway. (**B**) Different proteolytic systems are also involved and operate to degrade cellular proteins, but their relative contribution and importance differ significantly. (**B**1) Lysosomal system. (**B**2) Cytosolic non-lysosomal calpain system. (**B**3) Ubiquitin-proteasome system. The latter of which appears to catalyse the majority of protein breakdown.

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survival in all eukaryotic organisms, has been shown to be activated in different conditions leading to muscle atrophy [44], recent work characterized the mechanisms that control the autophagy/lysosomal pathway during muscle atrophy and demonstrated that the lysosomal and proteasomal pathways for protein degradation are co-ordinately regulated and simultaneously activated by FOXO3 (*see later in Akt/FOXO section*) [47, 48].

The ubiquitin-proteasome complex has been largely studied and appears to catalyse the majority of protein breakdown in mammalian cells. It is therefore not surprising that this system significantly contributes to the control of muscle mass [49]. In this system, degradation of proteins requires hydrolysis of adenosine triphosphate (ATP), the protein co-factor ubiquitin and the 26S proteasome [50]. Polyubiquitination involves the sequential action of the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin-protein ligases (E3). Ubiquitin is first 'activated' by its ATP-dependent attachment to E1. The activated ubiquitin is then transferred to E2. E3 bound to the protein substrate then binds to the E2 carrying the activated ubiquitin and transfers the ubiquitin from E2 to the targeted protein. This process is repeated until a chain of four or more ubiquitin molecules has been formed. Ubiquitin-labelled proteins are marked for degradation by the proteasome through the ATP-dependent conjugation of ubiquitin to an internal lysine residue. Ubiquitin-conjugated proteins are bound to the 19S 'cap' that feeds the protein into small peptides of 6–12 amino acids. Thus, among the different step of polyubiquitination, E3 ubiquitin ligases play a critical role in determining which proteins are degraded and the rate of proteolysis thereby conferring specificity to the system. A major goal is to determine which ubiquitin-conjugating enzymes and especially ubiquitin-protein ligases (E3s), are responsible for proteolysis during muscle atrophy [51].

A number of studies have now shown that the ubiquitin-proteasome system is necessary for most of the increase in proteolysis during inactivity [52], cachexia [53] and diabetic atrophy [49]. More specifically, several mRNAs encoding proteins involved in the ubiquitin-proteasome pathway are increased in muscle atrophy highlighting the key role of this system in controlling protein degradation following either hindlimb unloading [54, 55], spaceflight [32] and denervation [56].

In skeletal muscle undergoing disuse atrophy, two specific E3 ligases were identified some time ago. Indeed, two groups simultaneously described MAFbx/atrogin1 [57, 58], and a ring finger-containing ligase called MURF1 [58, 59]. Additionally, two others E3s have been described, namely Nedd4 [60], whose expression is increased after 14 days of unloading [61] and after 1 and 3 months of denervation [62], and E3α, also known as Ubr1 [63] which acts together with the ubiquitin carrier protein E214k. The role of these ligases has been highlighted in atrophying muscles from tumour-bearing or septic rats in which E3α/Ubr1 inhibitors reduce the accelerated rate of ubiquitin conjugation [64]. However, the identification of the upstream signals leading to ubiquitination, and the nature of the substrates for these E3 ligases in atrophic muscle is relatively unclear.

MURF1 belongs to the RBCC (for RING, B-box, coiled-coil domain) or TRIM (for tripartite motif) protein family [65] and has been shown to have ubiquitin-ligase activity that depends on the presence of the RING domain [58]. MURF1$^{-/-}$ mice, either following 14 days of denervation [58] (Table 1), or when a constitutively active form of the NF-κB activator IkB kinases (IKK) is coexpressed [66], showed significantly less muscle wasting compared to wild-type animals. Although it is currently clear that MURF1 is directly responsible of protein breakdown during atrophy, questions remain about atrophy-relevant MURF1 substrates. Several MURF1 binding partners have been identified. For example, the giant proteins titin and nebulin, along with troponin I, telethonin and myotilin are known substrates [59]. However, no differences in the level of these proteins were reported between MURF$^{-/-}$ and control mice [67, 68]. Only troponin I [68] and myosin heavy chain [69] have been shown to be a substrate of MURF1, respectively, in cardiac myocytes and in the *in vitro* DEX model of atrophy. This latter study, reporting MURF1 as an E3 ubiquitin ligase for myosin heavy chain, suggested an important mechanism for how sarcomeric protein breakdown proceeds.

Although the major function of MURF1 may be to control the ubiquitination-dependent degradation of several myofibrillar proteins, a recent study led to the idea that it could act also as a connector in the regulation of energy metabolism and protein synthesis in muscle [70]. More precisely, this study demonstrated that under fasting conditions, muscle-type creatine kinase, a critical enzyme for energy metabolism that reversibly produces phosphocreatine and ATP, could be degraded following its ubiquitination by MURF1. In this way, MURF1 allows down-regulation of energy consumption in atrophying muscle, and may act as an energy homeostasis regulator for muscle. The authors also showed that 3-hydroxyisobutyrate dehydrogenase (HIBADH), a key enzyme in the valine catabolic pathway, is also ubiquinated by MURF1. In this way, degradation of HIBADH results in the suppression of free amino acid consumption as a source for energy catabolism, and maintenance of serum level of free amino acids, thus allowing a role for MURF1 in amino acid homeostasis and in suppression of protein synthesis [70].

MAFbx/atrogin1 contains a F-box domain allowing targeting of protein substrates for ubiquination and degradation. MyoD [71]

On the left, the Akt/FOXO transcriptional control pathway is shown leading to the induction of atrogenes (ubiquitin ligases). On the right, a specific NF-κB pathway is activated during disuse atrophy. (**C**) Apoptotic pathways are also shown as they represent additional events controlling muscle atrophy. (**C**1) and (**C**2) highlight apoptosis induced in a caspase-dependent *versus* caspase-independent manner, respectively. (**D**) Myostatin pathway. Myostatin, growth differentiation factor-8 (GDF8), a member of the TGF-β, is a negative regulator of muscle mass acting *via* Smad transcription factors. *See text in first section for details.*

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Table 1 Genetic manipulations shown to counteract disuse muscle atrophy

Genetic model	Gene target	Model of atrophy	Fibre size atrophy (%)			Others main effects	References
			WT	TM	Rescue		
Bcl-3$^{-/-}$	B cell lymphomia 3 gene, IkB family member	Hindlimb suspension 10 days	Soleus			The seven fold increase of NF-κB reporter activity is abolished in SOL. Inhibition of slow-to-fast shift in myosin isoforms. Lack of atrophy of fast fibres in Nfkb1$^{-/-}$, and not the Bcl-3 $^{-/-}$ mice.	[87]
			–29	0	100		
Nfkb1$^{-/-}$	p105/p50 gene		Plantaris				
			–28	–10	65		
MISR	Ikb· gene superrepressor	Denervation 14 days	Gastrocnemius			NF-κB reporter activity decrease from 9 in WT to 1.5 in TM.	[66]
			–53	–28	47		
Murf1$^{-/-}$	Muscle RING Finger 1	Denervation 14 days	Gastrocnemius			36% of muscle weight sparing.	[58]
			No data				
Mafbx$^{-/-}$	SCF family of E3 ubiquitin ligases /(Atrogin1)		–50	–26.5	47	56% of muscle weight sparing.	
PGC1-α	Transgenic expression of PPARγ coactivator 1	Denervation 12 days	Tibialis anterior			Minor shift in fibre size distribution Counteract increases in mRNA for Mafbx/atrogin-1, MuRF-1 and cathepsin-L by 40%. Suppresses FOXO3 action.	[215]
			–40	–10	75		
Myostatin $^{-/-}$	GDF-8 gene TGF-β family	Hindlimb suspension 7 days				Myostatin$^{-/-}$ lost more body and muscle mass → more susceptible than WT to HS-induced atrophy.	[121, 139]
			No data				
Calpastatin	Overexpression of calpastatin: endogenous inhibitor of calpains	Hindlimb suspension 10 days	Soleus			Prevention of the shift in myofibrillar myosin content from slow to fast isoforms.	[34]
			–29	–20	31		

Knockout mice, or overexpression transgenic models, in which the extent of skeletal muscle disuse atrophy was attenuated or abolished. The duration and type of disuse experimentation are indicated, as well as the percentage of reduction in fibre size atrophy compared to wild-type animals (WT: Wild-type, TM: Transgenic Model, Reduc.: Percentage of reduction in atrophy compared to WT).

as well as calcineurin [72] have been shown to be targeted by MAFbx. Mice deficient in either MAFbx or MURF1 display a ~35–55% reduction in the extent of atrophy following 14 days of muscle denervation (Table 1). Although this study shows the direct involvement of these E3 ligases in the atrophic process, it also indicates that additional events are regulating atrophy because the atrophy was not completely prevented [58]. Up-regulation of both MAFbx and MURF1 is now well demonstrated in early and short-term disuse atrophy (1 to 14 days). In fact, these two proteins are often selected as markers of muscle atrophy. Yet, the induction of these ligases may not be such a general phenomenon. During sarcopenia atrophy [73] and in human beings following 60 days of bed rest [33], no change in the expression of MAFbx and MURF1 was reported. Thus, additional mechanisms are likely involved in the atrophic process of muscle. In this context, the localization of ubiquitin ligases in nuclei of atrophic muscle suggests that these proteins may also be implicated in the control of transcription [74] and/or that some proteasome degradation occurs in nuclei as showed previously for MyoD degradation [75].

Akt/FOXO transcriptional control and induction of the ubiquitin ligase pathway

Akt has multiple functions. Although Akt2 is mainly involved in glucose metabolism [76], Akt1, acts as a nodal point (see Fig. 1), integrating anabolic signals leading to increases in muscle mass and catabolic signals causing reductions in muscle mass [22, 77]. Anabolic signals cause activation of Akt which translocates into the nucleus where it directly phosphorylates members of the FOXOs. More specifically, phosphorylated FOXOs bind to 14–3–3 proteins and are exported from the nucleus into the cytoplasm where they remain sequestered by 14–3–3 proteins and are transcriptionally inactive [78]. By contrast, catabolic signals resulting from disuse for example, inactivate Akt thereby preventing phosphorylation of FOXOs [22, 78]. Unphosphorylated FOXOs bind target

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promoters *via* the consensus site TTGTTAC [78] and can thus exert their transcriptional activity on target genes such as MAFbx/atrogin-1 (Fig. 1).

Among the four members of FOXO family in mammalian cells, FOXO1 and FOXO3 have been shown to be up-regulated during various models of atrophy [7, 79, 80]. Furthermore, muscle-specific overexpression of FOXO1 and FOXO3a is sufficient to cause skeletal muscle atrophy [81, 82]. In addition, FOXO1 activity has been shown to be required for MAFbx/atrogin1 [81] and indirectly for MURF1 [83] induction whereas FOXO3a activity has been shown to induce transcriptional regulation of MAFbx/atrogin1 [81, 84] and MURF1 [84]. Interestingly, the study of Mammucari *et al.* demonstrated that FOXO3a also controls the activation of the autophagic/lysosomal pathway in fasting and denervation models of atrophy [48]. Together, these data strongly support the notion that FOXO transcription factors play a direct role in the progression of muscle disuse atrophy and highlight that common transcription factors, like FOXO3, could regulate muscle atrophy *via* the proteasome and lysosomal system in a coordinated manner.

NF-κB Pathways activated during disuse atrophy

The NF-κB represents a family (Rel) of five transcription factors (p65, Rel B, c-Rel, p52 and p50) ubiquitously expressed and involved in a multitude of processes such as immune and inflammatory responses, cell survival and cell adhesion [85]. NF-κB is a mediator of the effects of cytokine TNF-α during inflammation. In cachexia, characterized by an accelerated loss of muscle mass due to a chronic inflammatory response, p65/p50 heterodimers are activated and are considered as the canonic mode of activation. Briefly, the major upstream activators in this case are circulating cytokines, especially TNF-α (first identified as 'cachectin'), which induce phosphorylation of NF-κB inhibitor protein (I-kBα) by activating the IKK kinase. Upon phosphorylation, I-kBα becomes polyubiquitinated and degraded by the proteasome thereby enabling NF-κB nuclear translocation and transcriptional activation.

Disuse atrophy has also been shown to activate NF-κB [86, 87]. Because in disuse atrophy, there is no evidence of enhanced TNF-α production or other inflammatory cytokines [88], it appears that the trigger for NF-κB activation is independent of these cytokines leading to the idea that a specific NF-κB pathway is activated during unloading atrophy instead of the more classical pathway acting *via* TNF-α [88, 89]. This alternative pathway of NF-κB activation has been partly elucidated and shown to require p50 and Bcl-3 but not p65 (Fig. 1B, right). Bcl-3 (B cell lymphoma 3) is known to activate p50 homodimer-mediated gene expression *via* several possible mechanisms [90]. In this context, the group of Hunter and Kandarian has clearly shown that muscles from NF-κB $1^{-/-}$-p105/p50 knockout as well as Bcl-$3^{-/-}$ mice did not atrophy following disuse and also failed to display the typical slow-to-fast fibre transformation, indicating that both proteins, p50 and Bcl-3, are required for atrophy (Table 1) [87]. More recently, this group confirmed that whereas p50 and Bcl-3 are required for atrophy, c-Rel is not [91]. Studies have also been conducted to address the role of the upstream proteins that regulate NF-κB-dependent transactivation during atrophy [66, 91–93]. It has been shown that IkBα is a component of NF-κB activation in disuse atrophy [66, 88] and, furthermore, that IkBα degradation is required for normal atrophy [91]. The Kandarian group recently reported that the use of a dominant negative IkBα, a super repressor of NF-κB (IkBαΔN), could down-regulate NF-κB signalling during muscle unloading, attenuate disuse atrophy and regulate the increased protein ubiquitination associated with atrophy [91]. In addition, the role of IKK, which phosphorylate IkBα leading to its proteasomal degradation, has also been recently described [66, 92, 93], indicating that both kinases of the IKK complex, IKKα and IKKβ, are necessary and sufficient for muscle atrophy [93].

Although the role of a specific NF-κB pathway in muscle atrophy seems firmly established, the identity of its targets remains almost unknown. Studies on cachexia-induced atrophy revealed interestingly, that NF-κB could inhibit muscle differentiation by suppressing MyoD expression by preventing accumulation of its mRNA *via* post-transcriptional mechanisms [89]. Cai *et al.* demonstrated an *in vivo* role for NF-κB independent of its activation by cytokines and causing an increase in MURF1 expression [66]. In this last study, the NF-κB pathway was activated through a constitutively active IKKβ (MIKK mice) or inversely inhibited through a dominant inhibitor form of IkBα (MISR mice, see Table 1) expressed selectively in skeletal muscle of transgenic mice. In muscles from MIKK mice, the degree of activation of NF-κB matched that seen in the study of Hunter *et al.* with 10 days of hindlimb suspension, and a severe (32% of normal) atrophy was noted directly implicating this pathway in the regulation of muscle size [88]. Supplemental experimentation with microarrays revealed that the super-activation of NF-κB in MIKK mice was accompanied by increases in the levels of mRNAs encoding MURF1, and the C2 and C9 subunits of the proteasome. Ablation of MURF1 expression in MIKK mice (by crossing MIKK mice with MURF$1^{-/-}$ mice) partially ameliorated the atrophic phenotype further highlighting an important role for the IKKβ/NF-κB/MURF1 pathway in muscle atrophy [66]. Finally, work by the Kandarian group brought new insights into NF-κB target genes by determining the effect of dominant negative IkBα on expression of several atrogenes during unloading, strongly suggesting that MAFbx/atrogin1, Nedd4, IEX, 4E-BP1, FOXO3a and cathepsins L are potential targets of NF-κB signalling [91].

Additional events controlling muscle atrophy

Apoptotic pathways are involved depending on the atrophy models

Muscle fibres, along with a few other selected animal cell types, such as osteoclasts and cytotrophoblasts, are exceptional in that they are multinucleated [94]. The multinucleated nature of muscle fibres has lead to the concept of 'myonuclear domains' which defines the theoretical amount of cytoplasm supported by a single muscle fibre nucleus (myonucleus) [95]. Several studies dealing with muscle hypertrophy using overload as an experimental model in animals [96, 97] or biopsies from power-lifters [98], have

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demonstrated a proportionate increase in muscle fibre size with myonuclear number. As a result, the ratio of nuclei to cytoplasmic volume, *i.e.* myonuclear domain, remains constant. On the other hand, disuse muscle atrophy following spaceflight in animals [99] or human beings [100], and following hindlimb suspension [101] or chronic denervation in rats [102] was shown to be accompanied by a decrease in the number of myonuclei. This decrease has been suggested to be partially responsible for the reduction in muscle fibre size. However, it has also been observed that fibre size decreased to a greater extent than myonuclear number following hindlimb suspension [101, 103], and space flight [104], resulting in a significant decrease in the size of myonuclear domains. Because it is also known that slow-twitch fibres have smaller myonuclear domains as compared to their fast-twitch counterparts [105], this reduction in the size of myonuclear domains cannot be ascribed to a shift in fibre types because disuse causes a slow to fast transition [106].

Under these conditions, a main objective is to understand the molecular basis for the reduction in myonuclei in disuse atrophy. In this context, it has been suggested that apoptosis (programmed cell death) may be a potential contributor to the atrophic response by reducing the number of muscle nuclei [107, 108]. Apoptosis is an evolutionary conserved cell-suicide program essential for embryogenesis, development and maintenance of tissue homeostasis. In skeletal muscle, apoptosis has also been shown to contribute to sarcopenia and cachexia [109]. In disuse-induced atrophy, chromatin condensation and DNA fragmentation has been observed [103, 110] but the precise cellular and molecular events leading to apoptosis as a potential mediator of muscle atrophy are still unknown. Thus, it is not clear whether loss of nuclei initiates or is a consequence of the reduction in muscle mass during atrophy.

Apoptotic events in muscle fibre seem to be distinct from those occurring in mononucleated cells where nuclear as well as cytoplasmic contents are cleared [111]. Mitochondria play a major role in regulating apoptosis *via* their ability to release cytochrome *c* into the cytosol (Fig. 1C). The Bcl-2 family of proteins was the first one described to affect the release of cytochrome *c*. This family consists of several proteins which are either anti-apoptotic by protecting against cytochrome *c* release (like Bcl-2, Bcl-X_L) or pro-apoptotic favouring cytochrome *c* release (Bax, Bak, Bad, Bid) [112]. Thus, the Bcl-2/Bax ratio is often used as an indicator of apoptotic potential where a high ratio protects against apoptosis and a low ratio favours apoptosis [113]. Bcl-2 family, together with heat shock proteins and inhibitors of apoptosis proteins are considered as anti-apoptotic proteins by directly participating in the control of the balance between pro- and anti-apoptotic proteins.

Among different mechanisms characterizing apoptosis (Fig. 1C), caspases have been shown to be central enzymatic determinants involved in the different steps of apoptosis, *i.e.* from its initiation (caspase 8, 9, 12) to execution of apoptosis (caspase 3, 6, 7) [111] (Fig. 1C1). These cysteine-dependent aspartate-specific proteases are normally found in the cytoplasm in an inactive procaspase state. However, these procaspases can be readily activated upon proteolytic cleavage [114]. Briefly, cytochrome *c* released into the cytosol is able to form an apoptosome (Apaf-1, ATP and procaspase 9) that cleaves procaspase 9 thereby activating itself into caspase 9. Once activated, caspase 9 in turn cleaves and activates procaspase 3 causing apoptosis. The functional relevance of this pathway is further illustrated by the notion that caspase 3 may also be responsible for the initial breakdown of myofibrillar proteins, even before their degradation by the proteasome [115]. In fact, caspase 3 could cleave myofibrillar proteins such as actinomyosin, troponin and tropomyosin, generating monomeric actin fragments (including a characteristic 14 kD fragment), which are subsequently degraded by the 26S proteasome [115]. However, recent experiments reported that the absence of caspase 3 protects against denervation-induced atrophy by suppressing apoptotic signalling rather than regulating actin substrate availability in ubiquitin-proteasome mediated proteolysis and up-regulating MAFbx/atrogin-1 or MURF1 [116]. The authors suggest that other proteases are required to disassemble the myofibril for the 26S proteasome and that caspase 3 regulates denervation induced signalling *via* the mitochondrial associated apoptotic pathway [116].

Apoptosis has been observed following denervation [108] and hindlimb suspension [107, 117] but different results were reported related to changes in caspase activities. For example, Siu *et al.* demonstrated that in gastrocnemius there is an up-regulation of caspase 3 and 9 following 14 days of denervation, but not following 14 days of hindlimb suspension [107, 108]. Dupont-Versteegden *et al.* also observed no change in caspase 3, 7 and 12 activity following 12 hrs to 7 days of HS [118], but an increase in caspase 8 activity has been reported with the same model of atrophy [117]. Thus, these data clearly suggest that the involvement of caspases in apoptotic events in atrophying muscle may depend on the selected experimental models. In this regard, apoptosis induced in a caspase-independent manner has been investigated in disuse-induced muscle atrophy (Fig. 1C2). In this case, mitochondria can release other pro-apoptotic proteins such as apoptosis-inducing factor (AIF) [119] and endonuclease G (EndoG) which can translocate to nuclei where they induce DNA fragmentation leading to apoptosis [120]. Of particular relevance is the observations of Dupont-Versteegden *et al.* who showed a lack of activation of caspase 3, 7 and 12, and a marked accumulation of EndoG to myonuclei following hindlimb suspension [118].

In general, the current available data indicate that apoptosis is indeed an important event in disuse-induced muscle atrophy but the extent of apoptotic events, as well as their nature, remains unclear. Additionally, it is also possible that the extent of apoptosis and nature of the signalling events differ according to the specific time-point of disuse atrophy. In this context, we can report the absence of studies with long-term disuse model (more than 14 days, as seen above). In fact, our recent microarray study showed, for example, a significant decrease of caspase 3 mRNA following 60 days of bed rest in soleus muscle of women, together with an increase in mRNAs encoding the anti-apoptotic proteins BAG1 (Bcl-2 family), as well as HSP90 and HSP27 [33]. Together, these results could lead to a model whereby muscle fibres display pronounced apoptosis in early phases of atrophy, followed by an important anti-apoptotic response which could represent an attempt to resist atrophy and maintain some mass.

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Preferential role of myostatin in inhibiting hypertrophy rather than inducing atrophy

Myostatin (growth differentiation factor-8, GDF8), a member of the transforming growth factor-β (TGF-β), is a negative regulator of muscle mass acting *via* Smad transcription factors [121, 122] (Fig. 1D). Thus, a number of investigations have been conducted in the last 10 years in order to understand the potential involvement of myostatin in muscle growth and regeneration. In human beings, the first natural myostatin mutation was recently identified in a child who showed a very similar phenotype to the myostatin-deficient mouse [123]. Knockout of the myostatin gene in mice induces a dramatic increase in muscle mass resulting from a combination of hyperplasia, hypertrophy [121]. In adult mice, inhibition of myostatin activity using injections of an anti-myostatin antibody for 4 days leads to an increase phosphorylation of p70S6 kinase and ribosomal protein S6 and demonstrates that myostatin exerts a tonic inhibitory influence on the rate of myofibrillar protein synthesis [124, 125]. In cattle, mutation of this gene results in a 'doubled-muscled' phenotype associated almost exclusively with fibre hyperplasia [126, 127], underlining different patterns of myostatin expression and distinct temporal roles for this factor during embryogenesis in various species.

By contrast, over-expression of myostatin has been shown to cause significant loss in muscle mass in mice, an effect reversible by follistatin administration [128]. During skeletal muscle development, it has been shown that myostatin overexpression inhibits myoblast proliferation, differentiation, and apoptosis [129, 130]. The group of Freyssenet reported recently that myostatin may also contribute to the regulation of skeletal muscle mass in adulthood by showing that myostatin overexpression induced a marked down-regulation of Akt/mTOR signalling pathway but failed to increase expression of atrogenes (MURF1, MAFbx, Nedd4 and ZNF216) and regulate the catalytic activities of the proteasome [131, 132].

Follistatin, an extracellular factor that binds and modulates activin, interacts with and inhibits the activity of several TGF-β family members [133]. Follistatin interacts directly with myostatin to inhibit its activity resulting in an increase in muscle mass [134, 135]. Thus, it has been reported that mice overexpressing follistatin display a very similar muscle phenotype of that seen in the absence of myostatin [135]. In disuse atrophy, follistatin mRNA levels have been shown to be highly induced in animal models [26], following long term bed rest in human beings [33], as well as during sarcopenia [136].

The role of myostatin in atrophy remains nebulous and conflicting results have been reported. Several studies have shown an increase in myostatin expression in fast type muscles in rodents following 17 days of microgravity [137] and 10 days of hindlimb suspension [138], indicating a preferential role for myostatin in fast fibre type. However, others failed to detect any change in its expression following 7 days of hindlimb suspension in different muscle types [139] and following four to 14 days of immobilization in fast type human muscles [140, 141]. Given that myostatin expression decreases with exercise [140, 142] and, as seen above, that its expression is associated with specific fibre type populations with greater levels in type IIB fibres [143], it seems reasonable to argue that myostatin may have a preferential role in inhibiting hypertrophy rather than in inducing atrophy.

However, several studies recently revealed the existence of a potential link between myostatin and FOXOs transcription factors. In models of cachexia for example, an increase in myostatin expression was linked to protein degradation through an increase in FOXO1 expression [144]. Furthermore, the expected increase in FOXO3a mRNA following dexamethasone-induced atrophy was abolished in myostatin-deficient mice [145]. These new findings are important as they seem to link myostatin to the atrophic pathways in specific cases of atrophy.

Different approaches to counteract muscle disuse-induced atrophy

A practical perspective when dealing with disuse atrophy is to identify adequate prescription in order to counteract the negative impact on human health and performance of such conditions as chronic inactivity, long-term spaceflight or aging. Preventive medicine, therapeutic intervention, clinical care and rehabilitation programs have together used a variety of approaches in attempts to prevent and reverse atrophy. These varied approaches are referred to as 'countermeasures'. Together with on-going efforts aimed at deciphering the cellular and molecular basis of muscle disuse-atrophy, it seems critical to concomitantly identify appropriate countermeasures and define their impact not only on the extent of muscle mass reduction but also, on the various signalling pathways and biomarkers triggered during the atrophic process. The ultimate goal is to develop effective countermeasures to prevent, limit or even reverse skeletal muscle atrophy and functional loss in response to reduced activity on earth, exposure to microgravity environment or following disease/injury. Although many varied approaches are being investigated, these can basically be grouped into three distinct classes: exercise and physical training, nutritional aids and manipulations of growth factors.

Exercise and physical training

Among the different types of available countermeasures, physical exercise with high resistance loading appears efficient to counteract the deficits in muscle structure and function during human bed rest [146, 147] and hindlimb suspension in animal models [148, 149]. Together with previous studies dealing with mechanical and physiological parameters, this body of work has led to the notion that the most efficient exercise program should include isometric and isotonic (concentric and eccentric) exercises as well as high-intensity exercises. A more marked positive effect of exercise has been described during the early phases of atrophy (until 7 days) which may be related to the prevention of decreased protein synthesis during the early phase compared to reducing degradation which becomes more prevalent at later time-points [2].

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Although the beneficial effects of exercise to counteract disuse atrophy is well known, there are few studies that have defined the impact of exercise countermeasures on signalling pathways and molecular events. Dynamic and maximal resistance exercise has been shown to allow for maintenance of muscle mass largely due to rates of muscle protein synthesis that remain elevated during hindlimb suspension in rat [150] or 14 days of bed rest in human beings [151]. Maximal resistance exercise, during limb unloading, also counteracts the deficits normally seen in total RNA, total mRNA and actin and myosin heavy chain mRNA levels following disuse atrophy [152]. At the protein level, it has been demonstrated that resistance exercise could prevent to a large extent the decrease in cytoskeletal and membrane-associated proteins such as for members of the dystrophin complex, in soleus and vastus lateralis muscles during prolonged bed rest in human beings [5, 153]. Similarly, daily resistance exercise was associated with a ~50% decrease in the induction of MAFbx and MURF1 and a clear attenuation of atrophy during 14 days of hindlimb suspension in rats [61].

Despite these dramatic results showing the benefits of exercise on atrophy-related signalling pathways, several other studies have had more moderate results. For example, resistance exercise had no effect on the typical increase in C2 and C9 proteasome subunit expression linked to hindlimb suspension [61]. Similarly, resistance exercise alone failed to prevent apoptosis or the decrease in satellite cell proliferation which occurs with hindlimb suspension [61]. Moreover, limited periods of muscle loading during unloading (30 min. daily during 10 days of hindlimb suspension) is not sufficient to counteract the increase in myostatin mRNA levels [138]. Along those lines, a study from Haddad *et al.* using electrical stimulation to induce maximal isometric contraction during initial stages of unloading (5 days of hindlimb suspension in rat) failed to maintain the content of myofibrillar proteins. Although in this same study, isomeric resistance exercise counteracted the large increase of catabolic markers including MURF-1, MAFbx/atrogin and myostatin, the anabolic pathways remained defective particularly at the level of IRS-1 which plays a pivotal role in transmitting and amplifying signalling events mediated *via* IGF-1 receptors [154]. Thus, it would appear that although exercise is beneficial, additional studies are needed to better define the impact of various regimens on signalling pathways involved in the atrophic response of muscle. This would then lead to better exercise prescription and optimization of various constraints including time and duration of exercise training programs.

The findings presented above also highlight the preferential benefits of combined exercise and physical training protocols in counteracting long-term disuse muscle atrophy. In this case, an ideal combination may be to perform exercise that involves both an aerobic training component together with maximal resistance exercise. Aerobic training is known to enhance the muscle respiratory capacity achieved mainly *via* mitochondrial biogenesis. These adaptations involve an increase in mitochondrial density and enzyme activity, and they arise along with coordinated changes in the contractile properties of myofibres that undergo fibre type conversions depending on the specific demands of the exercise protocol [155]. With the ultimate aim of also protecting several integrated systems, and particularly the cardiovascular apparatus and bone structure, it has been recently demonstrated that concurrent resistance and endurance exercise brings optimized results. Combined training, during long-term bed rest in human beings has been shown to almost completely counteract the decrease in the size of thigh and calf muscles [156] while preserving single muscle fibre function [157] and fibre size, and preventing the slow to fast phenotype switch [157, 158]. A transcriptomics approach showed that combined exercise was effective in preventing ~90–95% of the changes normally seen in mRNA expression during long-term bed rest. Importantly, combined exercise abolished the alterations observed in gene subclusters following bed rest particularly those relating to protein synthesis and degradation, as well as those involved in glycolysis and oxidative phosphorylation [33].

Nutritional aids

During bed rest, whole body protein turnover is mainly reflected by muscle protein turnover leading to a loss of lean body mass [20]. The reduction in protein synthesis is much greater (up to 45%) during long duration spaceflight [21]. This loss can be amplified by an increased level of circulating cortisol induced by the accompanying stress [159]. Under many different conditions, exercise cannot always be used as unique countermeasures. Therefore, other strategies have been studied in order to counteract or prevent catabolic processes during chronic disuse.

One of preferred approaches for nutritional aid consists of amino acid supplementation [160]. Amino acids are generally well recognized for their anabolic effects [161] and ability to stimulate protein synthesis in skeletal muscle [162, 163]. In this context, it is known that amino acids primarily exert their effects on protein synthesis by accelerating peptide chain initiation rather than elongation [164]. Among essential amino acids, leucine appears to mediate most of the effects of protein/amino acid intake on protein metabolism and is well recognized as an anabolic factor [165]. The specific mechanisms by which leucine exerts its potent effects on protein synthesis and degradation remain, however, unclear. Work by Anthony *et al.* showed that leucine had a stimulatory effect on the assembly of the eIF4F complex, a key component in the mRNA binding step in translation initiation [166]. Similarly, Carroll *et al.* also showed that amino acid administration can elevate fractional synthesis rate in the soleus muscle leading to the idea that slow muscle may be more dependent on nutritional status than on exercise [167]. Although several studies have reported a major role for leucine in protein synthesis through PI3K and mTOR signalling pathways *in vivo* [168] and *in vitro* [169, 170], further studies should evaluate the effects of essential amino acids on signalling pathways involved in disuse muscle atrophy.

Although amino acid supplementation can promote protein synthesis during the atrophy process, there are data indicating that it can also be beneficial by reducing proteolytic events. In this context, a study of Nakashima *et al.* is relevant because these

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authors showed that leucine supplementation inhibits ubiquitin and proteasome C2 subunit mRNA expression in chick myotubes and skeletal muscles, while also inhibiting expression of the m-calpain large subunit [171]. These findings indicate that leucine has the ability to suppress the ubiquitin-proteasome proteolytic pathway and degradation of myofibrillar proteins.

In contrast to the findings described above, recent study on women muscle showed that dietary protein supplementation during long-term bed rest with enrichment of leucine intake, failed to counteract the decrease in isometric and dynamic muscle strength [156], changes in single muscle fibre function [157], alterations in fibre size as well as the slow to fast phenotype transition [157, 158]. The modest impact of protein supplementation on the atrophic process was also described systematically by using a transcriptomics approach. Through this approach, it was shown that only 25% to 40% of the number of differentially expressed mRNAs induced by 60 days of bed rest was normalized by protein supplementation, including several mRNAS coding for proteins regulating degradation pathways [33].

Manipulations of growth factors

Signalling transduction pathways initiated by growth factors such as IGF-1, fibroblast growth factor (FGF) and TGF-β, exert a profound influence over muscle gene expression. In particular, a number of growth factors contribute to the regulation of satellite cell proliferation, differentiation and motility [172]. All members of the IGF-1, FGF and TGF-β family promote proliferation of satellite cells whereas myogenic differentiation is mainly driven by IGFs [173]. Several growth factors have been tested in attempts to attenuate or reverse muscle atrophy but the degree of success appears to largely depend on the specific actions of the growth factor studied, method of delivery and model of atrophy.

IGF-I, originally named somatomedin, was described as the factor by which the growth hormone (GH) exerted its anabolic actions in most tissues [174]. The complex regulatory system of IGFs comprises two IGFs (IGF-I and IGF-II), cell surface receptors (types I and II), six specific high-affinity binding proteins (IGFBP1–6), IGFBP proteases and others IGFBP-interacting molecules. The importance of IGFs in growth and development is well established. As described in first section, the IGF-1/PI3K/Akt signalling is able to suppress the activation of atrophy pathways by suppressing catabolic events associated with MAFbx and MURF1 [81, 83]. Thus, IGF-1/PI3K/Akt signalling is sufficient to induce hypertrophy *via* activation of protein synthesis pathways while concurrently suppressing activation of denervation- or dexamethasone-induced atrophy pathways [22]. The anabolic activity of IGF-I as an endocrine, circulating hormone produced by the liver, is clearly distinct from its activity as an autocrine/paracrine growth factor locally secreted [175]. Hence, it appears rather clear that IGF-I administration, resulting in higher levels of serum IGF-I, plays a role in the development and maintenance of muscle mass only in cases of GH deficiency, and not in healthy persons [176]. By contrast, locally produced, autocrine/paracrine IGF-I, has been shown to play a significant role in pre- and post-natal muscle growth. The hypertrophic effects of GH are mediated in this case by locally produced IGF-I [177]. In this context, it has been shown that muscle specific expression of IGF-1, *via* local injection of IGF-I expression construct followed by electroporation 3 days before unloading, is an effective inhibitor of muscle atrophy [178].

FGFs are stored in the extracellular matrix where they bind proteoheparan sulphates until used for local actions. Their biological cellular effects are initiated *via* high affinity tyrosine kinase (FGFRs) and low affinity heparin sulphate proteoglycan cell surface receptors [179]. The FGFs are involved in foetal skeletal muscle growth and development as well as in the development of cultured myogenic cells *in vitro* where they act as strong regulators of myoblast proliferation [179, 180]. Thus, treatment of cultured myoblasts with these factors stimulates proliferation and represses terminal differentiation. FGFR1, a receptor for several members of the FGF family highly expressed in adult skeletal muscle, has been used to counteract disuse atrophy. By electroporation of plasmid DNA into mice hindlimb muscles 2 days before 7 or 14 days of hindlimb suspension, it was shown that ectopic expression of FGFR1 stimulates protein synthesis and inhibits muscle atrophy [180]. However, although the role of FGFs in regulation of myoblast proliferation and differentiation has been clearly demonstrated, a definitive role for this family of growth factors in mature fibre maintenance, in regulating hypertrophy and/or in counteracting atrophy awaits further experimentation.

TGF-β is the prototypical family member of the cytokine family, and has a broad spectrum of effects ranging from proliferation, differentiation, adhesion, migration, apoptosis and regulation of cell growth [181]. In skeletal muscle, TGF-β has been shown to be involved in muscle development and maintenance of post-natal muscle mass. Generally, members of the TGF-β family inhibit muscle proliferation and differentiation by silencing the transcriptional activation of myogenic regulatory factors such as MyoD [182]. Among the large number of TGF-β superfamily members, myostatin has been shown to be expressed in skeletal muscle, heart and adipose tissue [183]. As mentioned previously, myostatin is known to act as an inhibitor of muscle mass growth. Blockade of myostatin, by a pharmacological approach consisting of the administration of an anti-myostatin monoclonal antibody to inhibit the binding of myostatin to its receptor ActRIIB, has been shown to result in growth of dystrophic muscles [184]. Moreover, another study showed that blockade of myostatin using myostatin knockout mice, harbouring a deletion of the third myostatin exon, could prevent glucocorticoid-induced muscle atrophy [145]. However, McMahon *et al.* have reported that contrary to their working model, genetic ablation of myostatin in mice does not attenuate the loss of body and muscle mass following hindlimb suspension [139]. In fact, the extent of atrophy for some muscles was even greater in mice deficient in myostatin (Table 1) [139]. Although the loss of myostatin activity improves muscle mass and regeneration in dystrophic muscles, it seems that its effects in atrophying muscles are controversial. Thus, the use of myostatin as a target for counteracting skeletal muscle disuse atrophy remains to be further explored [184].

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Additional nutritional, ergogenic supplements and drugs

In addition to the three main countermeasures discussed above, several other approaches have been used over the years for their potential in preventing, attenuating or reversing muscle atrophy. Here we present a few of the most cited in the literature.

The effects of dietary administration of antioxidants have been evaluated. Muscle unloading involves oxidative stress which contributes to the developing weakness [39, 185, 186]. The peak of oxidant activity has been observed at day 3 of hindlimb suspension in mice and persists for up to 12 days [187]. Whereas allopurinol administration (50 mg/kg/day), a xanthine oxidase inhibitor with antioxidant properties, was not able to counteract muscle atrophy of hindlimb suspended mice [188], diet supplementation with 1% Bowman–Birk inhibitor concentrate (BBI/C) during 12 days hindlimb unloading was shown to have beneficial effects [187]. BBI, a soy protein, widely recognized as a serine protease inhibitor, is able to decrease matrix metalloproteinase activity but not that of the proteasome [187]. As an antioxidant, BBIC was able to depress cytosolic oxidant activity, without quenching nitric oxide release in unloaded soleus muscle, thereby offering some protection [187]. Along these lines, the impact of the lipid-soluble antioxidant vitamin E on muscle atrophy has been examined in various studies. For example, long-term supplementation with vitamin E prior and during the phase of unloading, was recently shown to partially counteract unloading-induced soleus muscle atrophy, to abolish the increased mRNA expression of caspase 9 and 12 and MURF1 in unloaded muscles, and attenuate the increased mRNA expression of mu-calpain, caspase 3 and MAFbx/atrogin1 mRNA [41]. The latter study indicated that the beneficial effect of vitamin E may be related to modulation in the expression of muscle proteolysis-related genes rather than to its antioxidant function [41].

Countermeasures targeting NF-κB pathways have also been studied by using for example, curcumin (diferuloylmethane) and N-acetylcysteine (NAC, naturally found in several vegetables), two NF-κB inhibitors [189, 190]. Dietary curcumin (1% supplementation) was not able to inhibit the activation of NF-κB during unloading-induced atrophy and the accompanying weakness, whereas dietary NAC (1% supplementation in water) could prevent the activation of NF-κB but not muscle atrophy and weakness [191]. Importantly, these findings show that NF-κB inhibition alone is not sufficient to prevent skeletal muscle atrophy. β-2-adrenergic agonists have also been examined for their ability to prevent muscle atrophy. Even though the mechanisms of action remain unclear, clenbuterol (CB), a β-2-adrenergic agonist with growth-promoting properties, can stimulate increases in muscle mass [192] while reducing the extent of muscle atrophy following hindlimb suspension [193] or denervation *via* activation of the β-2-adrenergic receptor [194]. Additional work has revealed that CB treatment, administrated in the drinking water during unloading, failed to attenuate the increase in protein degradation by the proteasome normally seen in soleus muscles following 14 days of hindlimb suspension. However, CB treatment succeeded in preventing the reduction in total and myofibrillar proteins, and significantly reduced ubiquitin conjugates in fast muscles underlying a preferential reduction of proteolysis through inhibition of the ubiquitin-proteasome pathway in fast-twitch muscles [195].

Although several studies aimed at testing diverse countermeasures have been conducted with limited results, these have nonetheless contributed to increasing our understanding of the signal transduction pathways involved in muscle atrophy. Further experimentation thus appears warranted particularly to determine the combined effects of known countermeasures and for identifying novel physical, nutritional or pharmacological targets as to enlarge the spectrum of disuse-induced atrophy countermeasures.

Combined countermeasures for optimized effects

Knowledge on the effects of countermeasures is most often gained *via* experimentations evaluating isolated countermeasure. However, there are several studies that have used combined approaches to enhance skeletal muscle hypertrophy. By contrast, there are relatively few data available concerning the effects of combined countermeasures on disuse-induce muscle atrophy.

Although the exact nature of the signalling pathways involved remain unclear, it is well known that combination of dietary protein and resistance exercise, synergistically work together to ultimately promote muscle hypertrophy and increase whole body fat-free mass [196]. These previous results generally come from global training experiments in young and older athletes [197, 198] or in elderly persons to counteract sarcopenia [196]. Whey/milk protein supplementation, containing high levels of leucine, has been described to be advantageous for gaining muscle size and improving muscle protein balance after a resistance exercise bout [199]. Although these studies did not examine conditions of muscle atrophy, they nonetheless bring important information on the synergistic effects of resistance exercise and amino acid supplementation upon muscle contraction-induced augmentation of protein synthesis, when supplementation is provided either intravenously [162] or orally [200]. Interestingly, timing of the nutrient intake appears as a crucial parameter. Indeed, it has been well demonstrated that nutrient intake before and/or immediately after a resistance exercise session is more beneficial in terms of muscle protein anabolism [201]. Recently, an increase in the cell cycle kinase cdk2 mRNA expression was reported following 21 days of resistance exercise with protein supplementation, ingested immediately before and after each bout of RE, thereby suggesting a higher proliferating cell activation response [202]. These authors hypothesized, taking into account that leucine activates myogenic satellite cells through the mTOR pathway [203], that an increase in satellite cell proliferation had occurred following resistance exercise training when protein supplementation is ingested closely to the exercise session [202].

A number of studies have attempted to increase muscle adaptations to resistance training by increasing levels of IGF-I. In this context, IGF-I locally produced has been shown to play a much

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more important role in muscle growth and bring better results than IGF-1 alone [150]. Experiments using combined and locally applied GH/IGF-1 treatment, immediately before each maximal resistance exercise showed a more positive effect on muscle atrophy and apoptosis associated with hindlimb unloading in animals [101, 204]. More recently, a combination of resistance training and injection into muscle of an adenoviral vector encoding IGF-I led to increases in muscle mass and force production compared to exercise alone [205]. On the other hand, increasing levels of circulating IGF-I, together with exercise training, has failed to induce further benefits in elderly men or young persons performing resistance exercise training [206, 207].

In human bed rest studies, exercise countermeasures have also been combined to additional physical or mechanical stimulation, like penguin suit [208], artificial gravity using a human-powered short-arm centrifuge [209] or vibration [210, 211]. Unfortunately, there are few data available on the degree of positive or negative effects on the molecular events and signalling pathways associated with atrophy. Electrical stimulation has been used for decades as a way to preserve muscle integrity following disuse and denervation but very few studies have actually dealt with this type of countermeasure. This countermeasure has been proposed also as an alternative method to complement physical training, for avoiding or reducing the changes that occur during spaceflight [212]. More recently, electrical stimulation was shown to preserve muscle mass and maximal force, and *via* a transcriptomics analysis, to also substantially prevent the changes in mRNA expression following denervation [213]. Finally, a plantar vibration device has been used in atrophy experimentation which causes reflexive plus 'resistive-like' vibration muscle exercise. In parallel to maintaining calf muscle structure and force, this device caused an increase in the expression of dystrophin-associated nNOS in soleus muscle, which allowed for further increases in force and actomyosine ATPase activity [211], and adequate RyR1 expression important for maintaining activity-dependent Ca^{2+} homeostasis [214].

Although many physical protocols, nutritional and therapeutic trials have been investigated for many years, contributing to our knowledge and aiding the development of countermeasures, few data are available to explore the effects of these different countermeasures, isolated or combined, in relation with research on the molecular processes of signalling pathways involved in skeletal muscle disuse atrophy.

Conclusion and perspectives

As highlighted above, a substantial body or work shows the involvement of several distinct pathways in disuse-induced muscle atrophy. Among these various pathways, it seems that the events underlying the massive protein degradation that occurs during muscle atrophy are keys. Yet, a number of important questions remain unanswered particularly those dealing with specific upstream activators and downstream targets. In addition, many studies have shown the existence of cross-talks between the different atrophy pathways but the detailed interactions and cross-signalling events between, for example, myostatin and FOXOs, NF-κB and MURF1, and mTOR signalling in myogenic satellite cells, are largely unexplored.

Most studies conducted to date have used various animal models to examine disuse-induced atrophy pathways with only a few focusing on human beings. Thus, considering long-term clinical situations and space exploration programs, it seems that there is a clear need for human experimentation using both short and long periods of inactivity. The current lack of human experimentation is understandable and may be best explained by the ethical aspects involved in such studies and the rare opportunities for human spaceflight. However, such human studies together with continued cell culture and animal experimentation, are essential if the ultimate objective is to comprehensively define the molecular events and pathways regulating muscle loss in healthy persons and to identify specific cellular targets which could be used to counteract or minimize muscle wasting. These investigations are especially important as skeletal muscle atrophy remains a scientific challenge with clear clinical, social and economical ramifications.

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