

## Review Article

# Various Jobs of Proteolytic Enzymes in Skeletal Muscle during Unloading: Facts and Speculations

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Skeletal muscles, namely, postural muscles, as soleus, suffer from atrophy under disuse. Muscle atrophy development caused by unloading differs from that induced by denervation or other stimuli. Disuse atrophy is supposed to be the result of shift of protein synthesis/ proteolysis balance towards protein degradation increase. Maintaining of the balance involves many systems of synthesis and proteolysis, whose activation leads to muscle adaptation to disuse rather than muscle degeneration. Here, we review recent data on activity of signaling systems involved in muscle atrophy development under unloading and muscle adaptation to the lack of support.

## 1. Introduction

Functional unloading caused by prolonged weightlessness or bed rest (after trauma, etc.) leads to sufficient physiological alterations of skeletal muscles, mainly postural, as soleus. Studies on animals [1] and volunteers [2] showed that unloading leads to changes at the molecular level, which manifest in muscle mass loss and deterioration of its function.

In this paper, we paid attention mainly to the atrophy caused by hindlimb suspension, since this is the most correct experimental disuse mode, while muscle denervation, for instance, leads not only to disuse, but also to impaired trophic regulation of the muscle, which complicates atrophy data interpretation.

To study molecular processes during disuse, special models are used for animals (hindlimb unloading) [3] and volunteers (bed rest, dry immersion). Each of the models leads to development of a complex of structural and functional changes, as decrease of cross-sectional area and contractile activity of muscle itself and its single fibers [4–7]. There are also changes in myosin phenotype observed [8, 9] as well as enzyme activity [1]. Such hypogravitational syndrome [10] is supposed to be caused by changes in concentration and activity of factors circulating in blood (glucocorticoids and anabolic steroids) [11]. At the same time, cortisol was

shown to affect muscle under disuse indirectly [12]; while in muscle cytosol, quite high concentration of glucocorticoid receptors was observed [13]. Consequently, there are other mechanisms involved in muscle atrophy stimulation and development during unloading.

Muscle atrophy development under hypokinesia/hypogravity involves different signaling systems directed towards proteolysis activation and protein balance maintaining. Thus, an increase in content of components of the TGF- $\beta$ /Smads (transforming growth factor- $\beta$ ) signaling pathway [14] and decrease of activity of phosphatidylinositol-3-kinase (PI3K/Akt) cascade were shown. These changes reflect activation of both proteolytic and synthetic signaling pathways in skeletal muscle [15]. Therefore, we aimed at the analysis of the known pathways of proteolysis in postural muscles during functional unloading.

## 2. Main Systems of Proteolysis in Skeletal Muscles

Three catabolic pathways are known to be involved in the atrophy process during hypokinesia/hypogravity: Ca<sup>2+</sup>-dependent, lysosomal, and ATP-ubiquitin-dependent proteolytic pathways. However, these systems participate differ-

ently in muscle atrophy development caused by denervation and disuse. Thus, under disuse, activity of lysosomal proteases does not increase significantly [16].

Animals hindlimb suspension (HS) and human head-down bed-rest lead to an increase in mRNA of 14 kDa ubiquitin-binding enzyme, and 20S proteasome subunits [17, 18] and to an increment of ubiquitinated proteins [19], which proves significant contribution of ubiquitin proteasome system (UPS) in muscle atrophy under unloading.

Besides,  $\text{Ca}^{2+}$ -dependent proteases (calpains) also play an important role in skeletal muscle atrophy under disuse [17, 20, 21], being a system of primary protein degradation, since they do not degrade proteins to amino acids or small peptides [22].

Complex effect of activation of different proteolytic systems results in loss of muscle structural proteins and, thus, in decrease of muscle functional properties. A number of results obtained showed that atrophic changes during a space flight or under head-down bed-rest are accompanied by decrease of total muscle protein [23] and myofibril proteins degradation [24].

We suppose that first stage of muscle protein degradation can be  $\text{Ca}^{2+}$ -dependent proteases, since  $\text{Ca}^{2+}$  overloading is the first event observed in unloaded muscles, and it is calcium ions which stimulate primary myofibrils degradation. Stimuli inducing  $\text{Ca}^{2+}$  accumulation are not studied well. Thus, we start our review of proteolytic systems from calcium-dependent system.

Further, we will discuss proteolytic systems in details.

### 3. Calcium-Dependent Pathways of Proteolysis

Looking for a trigger of proteolysis researchers paid attention to  $\text{Ca}^{2+}$ -dependent proteases. Calcium redistribution between cytoplasm and sarcoplasmic reticulum (SR) during muscle atrophy caused by different diseases was first demonstrated about 30 years ago [25, 26]. Later, it was shown that 14 days of rat HS significantly increased induced by caffeine  $\text{Ca}^{2+}$  efflux from soleus SR [27]. However, myoplasm overloading with calcium was demonstrated only in 2001 by Ingalls et al. [28] who registered  $\text{Ca}^{2+}$  quantity increase at the 3rd day of rats functional unloading. Dihydropyridine-sensitive channels (DHPCs) and ryanodine receptors (RYRs) are main  $\text{Ca}^{2+}$  channels. DHPCs are the L-type  $\text{Ca}^{2+}$  channels, m.w. 165 kDa, specifically blocked by dihydropyridine [29]. They are localized predominantly in T-tubes of muscle fibers and are activated by depolarization of fiber membrane. At that,  $\text{Ca}^{2+}$  enters myoplasm without consumption of ATP hydrolysis energy.

We demonstrated that DHPCs specific inhibitor, nifedipin, caused significant decrease of  $\text{Ca}^{2+}$  in soleus fibers during unloading [30]. Since DHPCs are the voltage-dependent structures, they should be activated by changes of electrochemical potential at the myofiber membrane under disuse. Some authors showed membrane potential decrease in disused rat soleus [31, 32]. Six percent of decrease of membrane potential was observed at the 3rd day of animal hind limb suspension (HS) [32]. However, potential alteration was not

significant, and there were no direct evidences of membrane potential influence to  $\text{Ca}^{2+}$  accumulation, so the question of possible trigger of  $\text{Ca}^{2+}$  accumulation remains unclear.

RYR is another source of  $\text{Ca}^{2+}$ , localized in terminal cisternae membrane near DHPCs. These channels efficacy significantly exceeds that of DHPCs. RYR main function is fast  $\text{Ca}^{2+}$  influx to cytosol, where calcium ions interact with troponin C stimulating mechanism of fiber contraction. RYR and DHPC are known to be connected with each other structurally and functionally in fiber T-system. RYRs are activated when interact with  $\text{Ca}^{2+}$ -activated DHPC [29].

$\text{Ca}^{2+}$  accumulation in myoplasm stimulates  $\text{Ca}^{2+}$ -dependent proteolytic processes. Experiments with activators of  $\text{Ca}^{2+}$  transport demonstrated the increase in protein degradation [33], while dantrolene (specific blocker of  $\text{Ca}^{2+}$  exit from SR) decreased proteolysis rate [34].

However,  $\text{Ca}^{2+}$ -dependent proteolysis localization is not clear, since there are a number of data supporting [35] and contradicting [33] its lysosomal localization. Though, calcium can affect indirectly lysosomal proteases like cathepsin B [35], and nonlysosomal ones localized in cytosol of myofibers [33].

In myoplasm,  $\text{Ca}^{2+}$  activates cystein proteases, calpains, which are divided into two groups according to their sensitivity to  $\text{Ca}^{2+}$ :  $\mu$ - and m-calpains (calpain 1 and calpain 2); they are activated by micromolar and millimolar calcium concentrations, correspondingly. Skeletal muscles contain also calpain 3 (p94), which has common structure with calpains 1 and 2.  $\text{Ca}^{2+}$  dependence of p94 was proved only in 2006 [36]. At the early stages of functional unloading, calpains are activated and redistributed from cytoplasm fraction to the membrane in slow and fast muscles [37].

The fact that activity of soluble and membrane fractions significantly increased after 12 hours of animal hindlimb suspension and remains high till 9th day of disuse proves that calpains take part in muscle atrophy development under hypokinesia/hypogravity [37]. Moreover, we found twofold increase in soluble calpain fraction activity and fivefold increment of membrane fraction activity after 3 days of animal disuse [38] and were first to show directly that increase in  $[\text{Ca}^{2+}]$  stimulates  $\mu$ -calpain activity, which in its turn activates m-calpains [20, 36]. Local application of  $\text{Ca}^{2+}$  chelator 1,2-bis(2-aminophenoxy)ethan-N,N,N',N'-tetraacetate (BAPTA-AM) to soleus during unloading allowed us to demonstrate directly dependence of calpain activity on  $[\text{Ca}^{2+}]$  in vivo [38]. Importantly, techniques for determination of calpain activity used in the works [37] and [38] registered the enzymes activity at high millimolar concentration of calcium in the incubation medium, where higher activity of all calpain molecules was observed. Increase in calpains activity under disuse can be caused by their expression intensification or by other  $\text{Ca}^{2+}$ -independent regulators, as calpastatin. Thus, hindlimb suspension leads to pronounced decrement of calpastatin expression in soleus, while calpain 1 expression increases slightly [39]. Activity of calpains decreased in unloaded muscle under local application of calcium chelator [38] can be evidence of  $\text{Ca}^{2+}$ -dependence of regulation of calpastatin expression.

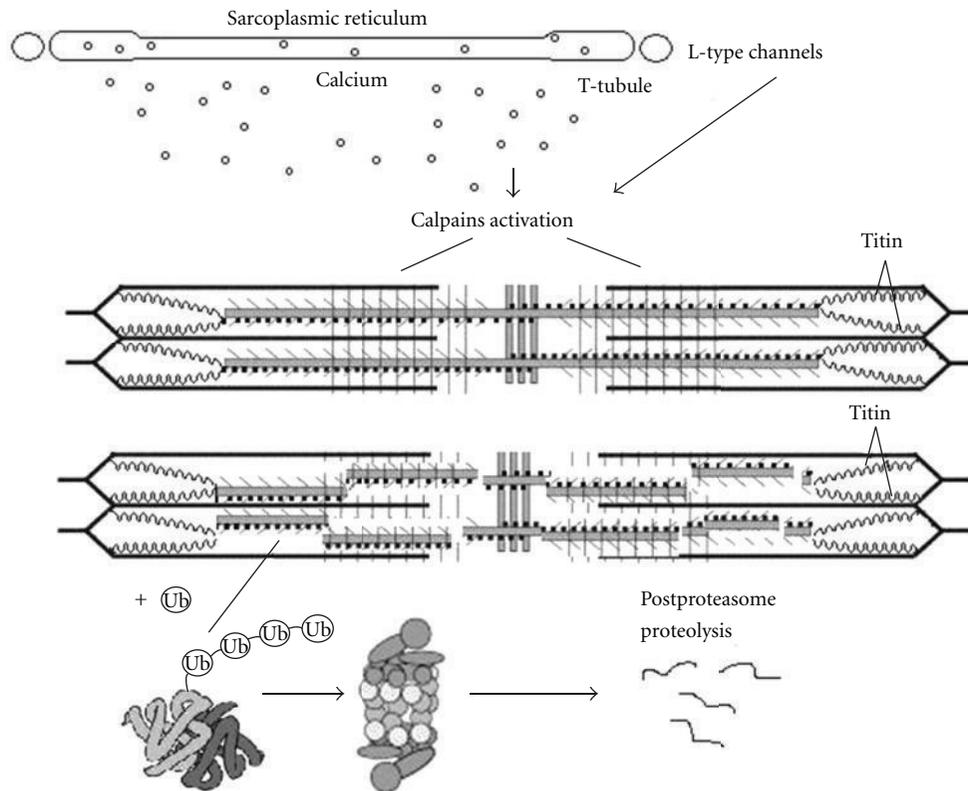


FIGURE 1: Interaction between proteolytic systems in mammals skeletal muscles under disuse atrophy. Preliminary disorganization of myofibrils by calpains helps to degrade myofibril proteins by UPS.  $\text{Ca}^{2+}$  accumulation can activate calpains localized near Z-disk. Main calpains target is titin, whose cleavage leads to E3-ubiquitin ligases activation.

Calpains contribute about 10% to disuse muscle atrophy. Their key role was demonstrated in HS experiments with mice overexpressing calpastatin, in which no reduction of muscle fiber size was observed [21]. These results are in agreement with recent data on calpastatin expression decrement under HS [39]. Interestingly, hypokinesia/hypogravity caused decrease of NO content leading to calpains inhibition [40]. At that, content of neuronal NO-synthase in soleus fibers under functional unloading decreased [41, 42].

Calpains cannot degrade proteins to small peptides or amino acids and cause only withdrawal of proteins from myofibrils, making them accessible to ubiquitination. Recently, titin was found to bind calpain-3 (p94) through a p94-specific region, suggesting that titin can regulate calpain-3 activity [43]. Calpain-3 was shown to activate expression of components of ubiquitin-proteasome system (UPS) [44–46]. P94 blocked binding of ankyrin repeats with titin molecule [47]. Ankyrin repeats normally are translocated to the nucleus, where they activate NF $\kappa$ B binding with DNA, hence stimulating ubiquitin ligase expression [47].

Thus, calpains and UPS work jointly (Figure 1) [23]. At that UPS, most probably, realizes final protein degradation. Close interrelation between  $\text{Ca}^{2+}$ -dependent proteases and UPS complicates studies of their individual contribution into atrophy development under disuse.

Intracellular localization of calpains in association with titin molecule makes possible the interrelation between UPS and calpains. Calcium accumulation under disuse activates calpains, which partly degrade titin molecule or change its conformation [41, 48]. Titin not only is involved in fiber contractility, but also realizes its signaling function through phosphorylation of ubiquitin ligases (MuRF-1 and MuRF-2) localized near titin kinase domain (in M-line), which can affect the titin molecule itself [49, 50]. 14-day HS is known to significantly decrease relative content of titin in rat soleus (Table 1) [51–53]. The same decrease of titin content was observed in human soleus after 7 days of dry immersion experiment [10], interestingly, that titin molecule remains intact at the 3rd day of animal HS [7, 51]. These results prove that, at the early stage of disuse, titin remains intact in spite of myoplasm overloading with calcium [7, 28] and increase in calpain activity. Consequently, phosphorylation of titin bound UPS components and their low activity is maintained. Meanwhile, calpastatin overexpression in mice under disuse was shown to prevent slow-to-fast shift in myosin heavy chains (MHCs) [21]. Probably, degradation of one of the calpain targets under disuse impairs regulation of slow MHC expression, which determines MHC phenotype transformation. Moreover, calpain 2 under HS translocates into the nucleus, where it initiates apoptosis [54]. Thus,

TABLE 1: Changes of titin and nebulin quantity in rat soleus under disuse [7].

Group	Titin 1/MHC	Titin 2/MHC	Titin 2/titin 1	Nebulin/MHC
Control	0.162 ± 0.022	0.033 ± 0.011	0.190 ± 0.048	0.040 ± 0.008
7 days of HS	0.090 ± 0.027	0.158 ± 0.037	1.890 ± 1.200	0.019 ± 0.005
14 days of HS	0.081 ± 0.014	0.084 ± 0.010	1.020 ± 0.035	0.020 ± 0.004

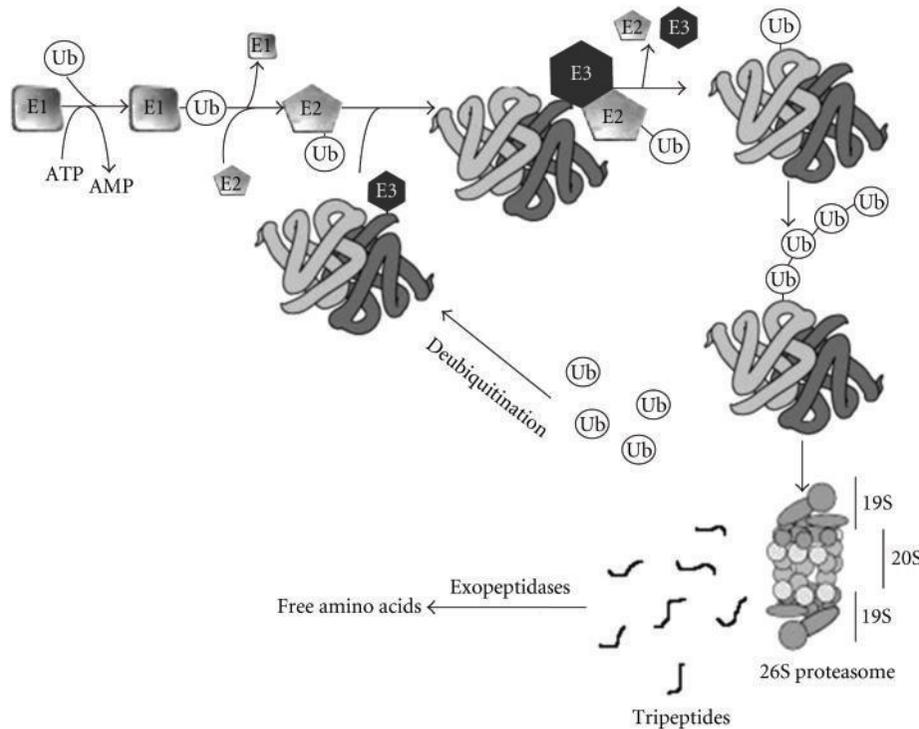


FIGURE 2: Scheme of UPS activity.

calpains in skeletal muscles besides their direct proteolytic activity possess also signaling properties, which are realized partly through the E3-ubiquitin ligases [50].

#### 4. Ubiquitin Proteasome System

Components of ubiquitin proteasome system (UPS) [19, 55, 56] were shown to be actively synthesized under disuse [15, 17, 19, 57] and under muscle atrophy of other nature [55, 58]. Intensification of proteolytic activity is caused by increase in quantity of mRNA encoding main UPS members (polyubiquitin, ubiquitin binding enzymes, ubiquitin ligases, 20S proteasome subunits) and following synthesis of corresponding proteins [59]. The scheme of UPS-dependent degradation of proteins is shown at the Figure 2. Ubiquitination process needs activation of three UPS enzymes: ubiquitin-activating enzyme (E1), ubiquitin-binding enzyme (E2), and ubiquitin ligases (E3). At first ubiquitin binds to E1 (ATP-dependent process) and then translocates to E2. E3 ligases covalently bind protein substrate and then interact with E2, which carries activated ubiquitin. Ubiquitin in its turn translocates from E2 to the target protein. The process repeats till target protein binds

a chain of 4-5 ubiquitin molecules. Then, the ubiquitinated protein degrades into peptides inside proteasome [60]. E3 ligases play an important role in recognition of proteins to be degraded. E2 enzyme and E3 ligase are tissue specific, individual E2 interacts with particular E3 ligase. Usually two main markers of UPS activity are studied, atrogin-1 or muscle atrophy F-box (MAFbx) and MuRF1 (muscle RING finger protein 1) [60]. MAFbx participates in formation of functional ligase complex. MuRF-1 binds conservative domain of titin molecule localized between titin kinase domain and titin C-terminal (in M-line) [48, 61]. This interaction is supposed to regulate metabolism of myofibrils, their trophic state, and maintains entirety of M-line region [62].

MAFbx is supposed to ubiquitinate and degrade MyoD [63] and eukaryotic factor of translation initiation 3 (eIF3) [64], thus playing role in muscle protein synthesis inhibition rather than in proteolysis activation in wasting muscle. MuRF-1 ubiquitinates and degrades troponin I [65] and myosin heavy chains [66].

*4.1. E3 Ubiquitin Ligases under Functional Unloading.* Expression rate of E3 ubiquitin ligases genes increased after denervation, immobilization, hindlimb suspension, and

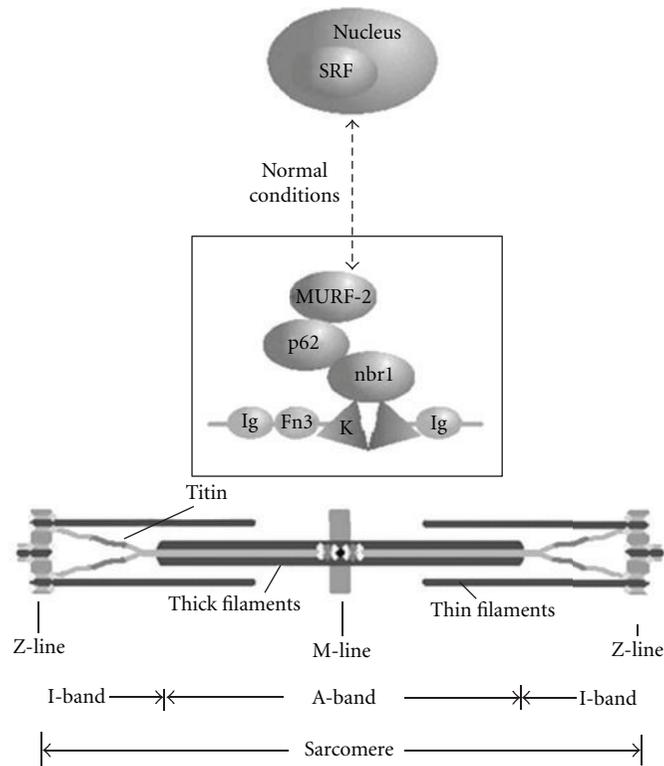


FIGURE 3: MuRF-2 localization on titin molecule [73].

after 11 days of animal space flight, which demonstrated MAFbx- and MuRF-1-dependent proteolysis under disuse [15, 58, 67]. Generally, MAFbx and MuRF-1 are universal proteases participating in skeletal muscle atrophy of different kinds [15].

Results of studies on volunteers using biopsy of vastus lateralis revealed difference between muscle atrophy development in animal and in human, because, in human biopsy significant decrement of protein synthesis and lack of proteolysis increase were observed [68]. At the same time, studies on volunteers during antiorthostatic head-down bed-rest showed increased MuRF-1 quantity in soleus (slow muscle), rather than in vastus lateralis (containing predominantly fast fibers) [69]. Later, expression intensification of MAFbx and ubiquitin ligase cbl-b was found in vastus lateralis after 20 days of disuse [70]. After 48 hours of unilateral lower limb suspension of volunteers, complete genomic analysis revealed increase in expression rate of mRNA of E3 ubiquitin ligases [71], also accumulation of 3-methylhistidine, product of degradation of myofibril proteins (actin and some myosin) was observed [72]. Ten days of immobilization caused threefold increase in MuRF-1 mRNA content in quadriceps femoris, which, however, was diminished to the control level to the 21st day of unloading [73]. We also observed changes in expression rate of ubiquitin ligases under HS of animals. Thus, we found that expression rate of MuRF-1 and MAFbx mRNA in rat soleus increased 3.3 and 2.1 times, correspondingly, at the 3rd day of disuse. To the 7th day of HS, this parameter decreased but was 1.27 and 1.52 times higher than in control (Figure 3) [74]. The 37% increase in

total level of protein ubiquitination at the 4th day of functional unloading [75] confirms our data.

**4.2. MuRF-2 and Signaling Role of E3 Ubiquitin Ligases.** Recently, information appeared about functions of another E3 ubiquitin ligase, MuRF-2, which is splice variant of MuRF-1. MuRF-2 is usually found in embryonic muscle, while, in adult animals, its quantity decreased. In spite of its predominant localization in cytoplasm, partly MuRF-2 is bound to nbr1 and p62 proteins, which localize at titin molecule near M-line (Figure 4) [49, 76]. Normally, one of the main functions of this protein is stabilization of microtubules population and several proteins of sarcomeric cytoskeleton (desmin, vimentin) during myofibrillogenesis [62]. Besides, along with MuRF-1, MuRF-2 participates in ubiquitination of myofibril proteins [77]. It should be noticed that MuRF-2 is regulated, at least partly, by titin-dependent mechanism [62]. Changes in spatial arrangement of titin kinase domain after denervation can cause loss of its main function. Consequently, dephosphorylated MuRF-2 dissociates from titin and translocates into the nucleus, where it forces out serum response factor protein (SRF), which is responded for c-fos-mediated stimulation of protein synthesis, cytoskeletal molecules expression, and expression of several growth factors. Our preliminary data showed MuRF-2 translocation to the nucleus and its increased expression under disuse. Thus, 7 days of HS caused increase in MuRF-2 concentration as in myoplasm (from 32.2+5.2 r.u. to 291.9 + 58.5 r.u., according to western blot densitometry

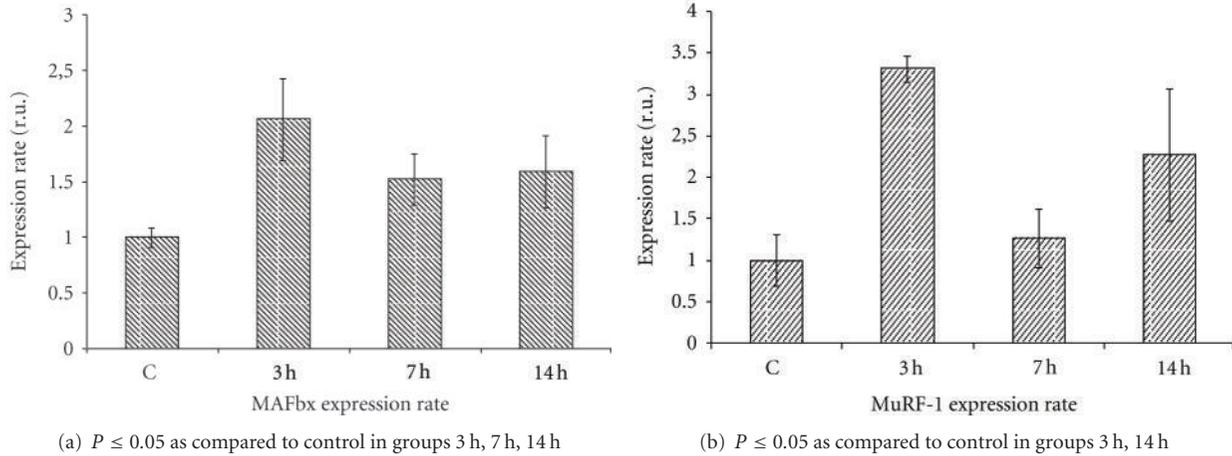


FIGURE 4: Changes in expression rate of MuRF-1 and MAFbx after different periods of unloading. HS3, HS7, HS14-hindlimb suspension for 3, 7, and 14 days, respectively. GAPDH and beta-actin were used as reference genes.

data), so in nucleus (from  $56.1 + 6.1$  to  $769.4 + 62.2$  r.u.) (unpublished data). Increment of MuRF-2 protein quantity increased further being almost twice as higher at the 14th day of disuse in myoplasm and nucleus. Thus, disuse induces synthesis and translocation of MuRF-2 into the nucleus. According to the data of Lange et al. [49], it seems that titin conformational changes under unloading should cause degradation of signaling complex, associated with titin kinase domain, and allow MuRF-2 migration to the nucleus. Such change in protein conformation was observed after 2 days of unloading [53], while data of other authors did not reveal any changes in titin content at the 3rd day of disuse [7, 53]. MuRF-2 concentration in nucleus fraction of rat soleus did not change, as compared to control, at the 3rd day of HS. However, data exist, which demonstrate titin stability at the 3rd day of HS; only after 7 days of HS, titin was degraded noticeably (Table 1). These data confirm that at the early stage of unloading myoplasm overloading with calcium [7, 28] and calpains activation cannot stimulate titin proteolysis and, thus, ubiquitin ligases dephosphorylation. Therefore, two triggers of MuRF-1 and MuRF-2 dephosphorylation can exist:  $\text{Ca}^{2+}$ -dependent calpain activation, which initiates myofibril proteins disorganization, and titin conformational changes caused by titin sensitivity to mechanical strain. Further studies are necessary to answer the question.

Normally, MuRF-1 and MuRF-2 expression is more pronounced in fast fibers (II type), while, in mice MuRF-1 and MuRF-2 knockouts, and especially in double knockouts (MuRF-1 and MuRF-2), number of fast soleus fibers is significantly decreased. At that, lack of MuRF-1 noticeably prevented atrophy of II type fibers of tibialis anterior [79]. In double knockouts expression of myozenin-1 (calsarcin-1), an endogenous inhibitor of calcineurin/NFAT signaling pathway was blocked. Obviously, nucleus localization of MuRF-1 and MuRF-2 stimulates calsarcin expression leading to stabilization of fast phenotype of muscle fiber. Mice MuRF-1 knockouts did not show significant atrophy of soleus after 10 days of HS, while fatigue characteristics were more pronounced, posttetanic potentiation was not as

increased as in mice of wild type [80]. The authors suppose possible MuRF-1 influence on intensified processes of phosphorylation of regulatory myosin light chains [81, 82].

Signaling roles of E3 ligases and some of their targets are known now. Thus, MAFbx ubiquitinates MyoD and eIF3 [63, 64]. Taking part in eIF3 ubiquitination MAFbx plays important role in regulation of reciprocal interaction between anabolic and catabolic signaling pathways. Troponin I [65] and myosin heavy chains [66] are the targets of MuRF-1, playing an important role in deterioration of muscle contractility.

Interestingly, ubiquitination of histone deacetylase of II type is supposed to be one of the mechanisms of fast-to-slow transformation of myosin phenotype, which is supported by decrease of slow myosin expression under application of proteasome inhibitor MG132 [83]. At that, exact E3 ubiquitin ligase participating in this phenomenon remains unknown.

Data discussed above showed that direct ubiquitination and indirect participation of E3 ubiquitin ligases in signaling processes makes them important components of central signaling mechanisms in muscle fiber. Unfortunately, today, there is not enough information to understand completely role of E3 ligases in processes of transformation or stabilization of myosin phenotype and concomitant events.

**4.3. Regulation of Ubiquitin Ligases Expression.** In spite of some progress in understanding of activation and possible functions of ubiquitin proteasome system, trigger mechanism stimulating its activity, particularly activity of E3 ubiquitin ligases, remains to be determined.

## 5. NFkB

Transcription regulation factor NFkB, which is a mediator of cytokine  $\text{TNF}\alpha$  (tumor necrosis factor  $\alpha$ ) during cachexia and inflammation, plays important role in skeletal muscle atrophy.  $\text{TNF}\alpha$ , in its turn, induces muscle fibers apoptosis

and specific transcription mechanism, which blocks IGF-1-induced anabolic process [84]. Inactivated NF $\kappa$ B forms complex with I $\kappa$ B in myoplasm. TNF $\alpha$  stimulates I $\kappa$ B kinase, which phosphorylates I $\kappa$ B leading to ubiquitination and degradation of proteins of this family. NF $\kappa$ B then moves to the nucleus and binds to sense sequence of DNA, thus regulating transcription of NF $\kappa$ B-dependent genes. TNF $\alpha$ -induced NF $\kappa$ B activation is known to suppress regulatory muscle factor MyoD at posttranscriptional level [85]. In 2004, Cai and colleagues demonstrated at least partial relation of muscle atrophy caused by cachexia, with activation of MuRF-1, but not with MAFbx [86]. This discovery stimulated study of action mechanism of NF $\kappa$ B system in muscles under disuse.

It was found that atrophy caused by functional unloading in rodents can be partly explained by TNF $\alpha$ -independent activation of NF $\kappa$ B. Seven days of HS in soleus stimulated DNA-binding activity of NF $\kappa$ B and led to an increase of reporter proteins p-50, c-Rel, and nuclear I $\kappa$ B protein Bcl-3 [86, 87]. Akt activation and TNF $\alpha$  expression did not increase as it happened during cachexia. These facts proved mechanism of atypical activation of NF $\kappa$ B during atrophy caused by functional unloading. This mechanism was called *trans*-regulation [87]. Importantly, NF $\kappa$ B pathway was not activated under disuse in fast muscles [87]. Possible targets of *trans*-activation of NF $\kappa$ B under hypokinesia were found recently [88]. Disuse was shown to induce NF $\kappa$ B-dependent increase in expression rate of ubiquitin ligases MAFbx and Nedd4. Analysis of 5'-flanking sites of genes of these ligases allowed finding numerous potential binding sites of NF $\kappa$ B. Moreover, 4EBP1, FoxO3a, and cathepsin L (lysosomal enzyme, which degrades membrane proteins) are also possible targets of NF $\kappa$ B; their expression was increased under disuse atrophy [88]. At the same time, in mice overexpressing IKK $\beta$ , specific component of muscle signaling pathway, 15-fold increase in NF $\kappa$ B activity was shown to stimulate MuRF-1 activity. In the experiments with C2C12 culture of myotubes, 4.6-fold rise of MuRF-1 promoter activity was found, which was blocked by I $\kappa$ B $\alpha$ -SR transfection. Thus, as opposed to disuse-stimulated atrophy, during TNF $\alpha$ -dependent atrophy, induction of MuRF-1 transcription was observed [87]. These data reveal predominant NF $\kappa$ B-mediated activation of MAFbx under disuse, while MuRF-1 is stimulated by NF $\kappa$ B during other kinds of atrophy. Since MuRF-1 is known to be activated under muscle wasting, there must be other mechanisms which activate MuRF-1 expression. Thus, transcription regulators FoxO1 and FoxO3 were shown to activate MuRF-1 expression [89, 90].

## 6. FoxO and Myostatin

Fox factors of transcription regulation have got their name according to their structure (forkhead box) [91], and, in mammals, they are called FoxO (other), because of different structure of their DNA-binding domains; among them FoxO1, FoxO3 a, FoxO4, and FoxO6 [92].

FoxO factors participate in a number of physiological and pathological processes, aging and cancer, for instance [92].

Proteins of this family being phosphorylated by Akt can bind chaperone 14-3-3 in cytoplasm, which leads to loss of their capacity for expression regulation [92]. Model of atrophy on myotube culture revealed decrease of activity of IGF-1/insulin/phosphatidylinositol-3-kinase (PI3 K/Akt) signaling pathway, which caused initiation of FoxO transcription factors and MAFbx induction (Figure 5) [93]. Importantly, FoxO3 directly interacts with MAFbx promoter stimulating expression of the proteases genes, which leads to atrophy of myotubes in culture and animal muscle fibers [93]. Since PI3 K/Akt is known to suppress MuRF-1 expression, so FoxO participates also in regulation of MuRF-1 activity [90]. Recently, FoxO1 was shown to negatively affect expression of myosin heavy chain of type I [94], which confirms important role of FoxO factors in skeletal muscle atrophy.

Balance between protein synthesis and proteolysis involves not only Akt signaling pathway, but also FoxO-dependent cascade, which includes activation of 4EBP1 (protein binding eukaryotic factors 4 E) and inhibition of mTOR. At that, FoxO is regulated by different posttranslational modifications, as phosphorylation, acetylation, mono- and polyubiquitination [94]. Some of these modifications are independent on Akt; hence, they also can be involved into muscle atrophy development under stress.

Akt-FoxO signaling pathway is known to interact with IKK-NF $\kappa$ B system during muscle atrophy development. Thus, inflammatory cytokine TNF $\alpha$  activating NF $\kappa$ B pathway blocks sensitivity to insulin and inhibits IGF-1 pathway [95–97]. Moreover, interaction between these two signaling systems was confirmed by the results of the study on IKK $\beta$  knockout mice, whose were insensitive to muscle atrophy induced by denervation, while demonstrated excessive Akt phosphorylation [98].

Search for triggering mechanism initiating dephosphorylation of Akt and FoxO and stimulating ubiquitin ligases expression under disuse allowed to find process of degradation of IRS-I (insulin receptor-I), which is intermediate in IGF-1/PI3 K/Akt signaling cascade. IRS-I content and its phosphorylation level in Ser<sub>636–639</sub> and Ser<sub>789</sub> sites decreased significantly after 14 days of HS [99]. Later, it was found that IRS-I is degraded by ubiquitin ligase cbl-b, which expression is increased noticeably under disuse [100]. Cbl-b expression is regulated during membrane process of lipid peroxidation.

As was shown previously, FoxO factors mediate ubiquitin ligases activation also through myostatin [101]. The latter blocks IGF-1/PI3 K/Akt pathway, which activates FoxO1, thus stimulating MAFbx expression [101]. Data on regulation of myostatin expression by FoxO1 showed that myostatin signaling pathway is related to Akt-FoxO cascade [102]. Mature myostatin protein forms active dimers, which bind activin receptors on the cell surface stimulating phosphorylation of Smad2 and Smad3 and their interaction with Smad4. Such complex is a transcription regulation factor, which can penetrate the cell and induce expression of any gene flanked with corresponding sequence. In other words, myostatin blocks myoblasts growth in the cell, inhibiting thus the expression of myogenic factors as MyoD and p21 [102]. Myostatin negatively regulates activation and self-renewal of cells and, probably, participates in process of the satellite

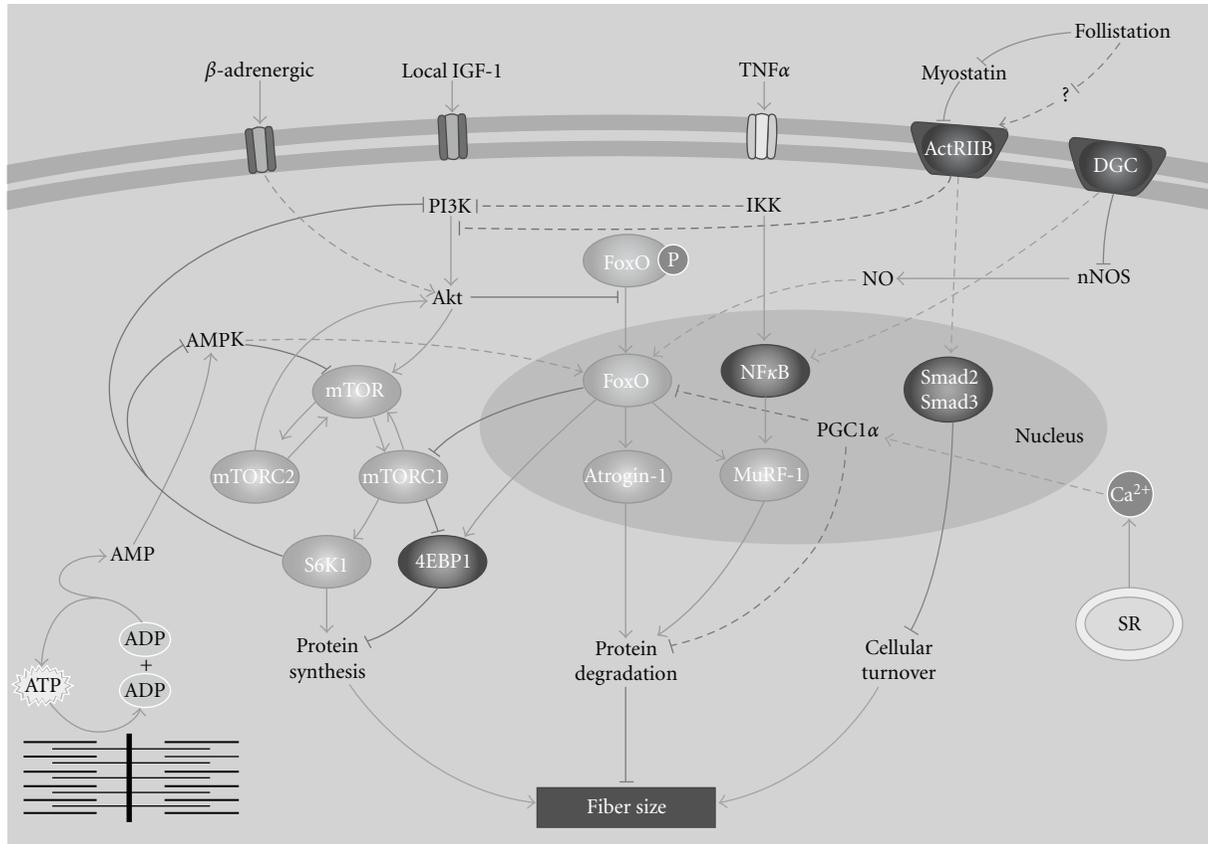


FIGURE 5: Net of signaling systems participating in regulation of fiber size of skeletal muscle under disuse atrophy. Pathways whose molecular function in the cell is not known are dashed [78].

cells silencing [103]. Thus, myostatin activity maintenance under disuse, possibly, promotes maintenance of low level of myofibers renewal and prevents potential increase in satellite cells proliferation and fusion.

At the same time, Smad proteins can recognize CAGAC DNA sequence but possess low affinity to it, so to interact with DNA Smad proteins need some DNA binding co-factors, which can help to recognize and regulate target genes [104]. Such mediators are proteins of the FoxO family [105]. Besides, Smads inhibitor, TGIF, was shown to be activated earlier than muscle mass loss becomes noticeable.

## 7. Autophagy: Lysosomal Proteins Degradation

Lysosomes are the cell organelles responsible for removal of other organelles and aggregated proteins. Autophagy is an integral property of muscle cells, which is confirmed by huge number of autophagosomes in humans with myopathies caused by different diseases or during pharmacological inhibition of lysosomal function by chloroquine, for instance [106]. However, lysosomal enzymes are differently activated during atrophy induced by denervation [107] and unloading [108]. According to biochemical and electron microscope data, lysosomal degradation of proteins is responsible mainly for denervation-induced atrophy [78, 107–109].

These results are in accordance with the data that chloroquine practically cannot block proteolysis during hypokinesia/hypogravity but inhibits it during soleus denervation [110]. Moreover, significant activation of  $Ca^{2+}$ -dependent carboxylic proteases and decrease of total cathepsin B and D activity were observed under disuse stimulated by hindlimb unloading, while denervation-induced disuse caused the highest activity of the cathepsins [17]. At the same time, recently, cathepsin L quantity was shown to be increased during HS [58, 111]. Role of cathepsin L is unknown. Thus, increment of certain cathepsins concentration proves some lysosomal system activation during denervation-induced atrophy but does not have significant contribution of lysosomes to HS-induced atrophy [111]. Cathepsins are known to be inactivated at neutral pH in cytoplasm. Proteolysis with cathepsins is carried out inside the lysosomes; thus, they cannot degrade myofibril proteins despite of their activation. Therefore, lysosomal system contribution into catabolism of myofibril proteins during atrophy is not sufficient [112].

mTOR and PI3 K/Akt signaling systems are also involved in microtubules autophagy [113]. Recently, the results were obtained demonstrating coordination between lysosomal system and UPS during atrophy [114, 115]. Therefore, some genes related with autophagy, as well as MAFbx gene, are controlled by FoxO3 regulatory factor, and thus expression of FoxO1 and FoxO3 can be necessary for lysosomal proteolysis

induction mediated by activation of ubiquitin ligases MAFbx and MuRF-1 in cell culture and in vivo [91, 93]. At that, it is still unknown whether such interaction of these proteolytic systems takes place in skeletal muscles of animals under disuse.

## 8. Caspases

Caspases are known to cleave actomyosin to 14 kDa actin fragments, as was shown in culture of skeletal muscle cells on the model of serum deprivation [116]. Caspase-3 contribution to protein degradation by UPS in culture was 125%. The observed by Du et al. actomyosin cleavage was blocked by specific caspase-3 inhibitor. Cleaved actin fragments were also found in rat muscles after diabetes and chronic uremia [116]. Caspases were shown to be activated during rat hindlimb unloading [117, 118]. Interestingly, at the 5th day of HS, only caspase-3 content increased, while, at the 10th day of disuse, caspases-6 and 9 were activated. These data confirm that caspase-3 activates as through mitochondria-independent, so through mitochondria-dependent pathway during unloading caused by HS. However, the entire mechanism of mitochondria-independent caspase regulation needs further investigation. Physiological role of caspase content increment can be the myonuclei number regulation through apoptosis, which leads to synthesis activity decrease. Though, it was found that caspase-3 increase caused decrease in myonuclei number [119] and stimulated DNA fragmentation at the 14th day of HS [120]. Mechanisms of this phenomenon are also remained unclear.

## 9. Muscle Atrophy Is the Balance between Signaling Systems Involved in Regulation of Protein Synthesis and Proteolysis

Alterations of structure and function of skeletal muscle under functional unloading, so called, muscle plasticity, are caused not only by increment of muscle proteins degradation. It is better to say that disuse atrophy is the result of shift of the balance between protein synthesis and proteolysis towards increase in proteolysis and decrease of synthesis intensity. Main signaling system regulating protein synthesis in muscles is the Akt/mTOR pathway, which is activated when IGF-1 (insulin-like growth factor-1) binds with its receptor on myofiber membrane. This signaling cascade is responsible for stimulation of protein synthesis in skeletal muscle fibers realizing its effect particularly through stimulation of proliferation and fusion of satellite cells (Figure 5) [111]. mTOR (mammalian target of rapamycin) is a part of two multiprotein complexes, one of which is mTORC1 (sensitive to rapamycin). mTORC1 activates S6 K and 4EBP, through which Akt-FoxO signal is realized. mTOR effect on translation process and protein synthesis is realized through TORC1-dependent phosphorylation of ribosomal S6 kinases (S6 K1 and S6 K2) and 4EBP, a repressor of a cap-binding protein eIF4E. S6 K1 is an important component of Akt cascade, which is confirmed by experiments on mice S6 K1 knockouts. Those mice had very small fibers, and

could not respond to activated Akt and IGF-1 [121]. Thus, protein synthesis intensification through PI3 K/Akt/mTOR mechanism is realized by means of activation of S6 K1, eukaryotic factor of translation initiation 4E (eIF4E), and inhibition of translation regulator 4EBP1. At the same time, IGF-1/PI3 K/Akt pathway prevents atrophy development by inhibition (dephosphorylation by Akt) of transcription regulation factors FoxO1-3, stimulating their transition from nucleus to cytoplasm [93]. Activity of IGF-1 plays an important role during functional unloading, since ability of muscle cells to bind insulin increased noticeably under disuse. At that, total quantity of insulin receptors did not change [122]. At the same time, activity of PI3 K/Akt signaling cascade, which plays role of central regulator between insulin and IGF-1 receptor, and activated synthetic pathways in muscle, were shown to be diminished significantly [123, 124]. Moreover, it was clearly demonstrated that c-Jun NH<sub>2</sub>-terminal kinase (JNK) significantly increased after 10 days of rat HS, as in predominantly fast [125, 126], so in predominantly slow muscles [127], on the models of animal HS [125, 127], cast immobilization [126], and denervation [128]. Independently of the model used increment in JNK level evidences that IRS-1 protein is phosphorylated, Akt activity is suppressed, and insulin resistance develops in the wasting muscle. Therefore, activation of PI3 K/Akt signaling pathway is important for disused muscle reloading. Nevertheless, it is not the only way of protein synthesis intensification. Eukaryotic initiation factor 4E (eIF4E), one of the components of eIF4F complex interacts with eIF3 complex, which regulates assembling of 43 S preinitiation complex (PIC) [129]. eIF3 directly captures 40 S ribosomal subunit stimulating its interaction with Met-tRNA-eIF2-GTP complex and with eIF1. Due to interaction of eIF3 and eIF4E-mRNA, mRNA binds 43 S ribosomal complex forming 48 S complex, which initiates synthesis of muscle proteins. Studies revealed 13 different subunits of eIF3 complex with molecular mass varying from 170 to 25 kDa. Five of these subunits form the main nucleus of the complex, and others have regulatory function. Role of one of these subunits, eIF3-f, is not known well, though in yeasts and coronaviruses, decrease of eIF3-f quantity leads to significant reduction of total protein content in dividing cells and to decrement of cytokines IL-6 and IL-8 [130]. IL-6, in its turn, is an essential factor of development of skeletal muscle hypertrophy mediated by proliferating satellite cells [131]. In humans, eIF3-f decrease is related to tumor development, while its overexpression suppresses cell growth and leads to apoptosis stimulation [65].

Small quantity of eIF3-f is found in myoblasts of skeletal muscles. Its concentration increases significantly during terminal differentiation and remains at the same level in adult muscle. Interestingly, eIF3-f binds MAFbx in skeletal muscle, which explains, probably, protein synthesis decrease during muscle atrophy of different nature [129]. MAFbx is known to stimulate polyubiquitination of eIF3-f with its following degradation by 26S proteasome. Therefore, decrease of MAFbx will lead to maintaining of eIF3-f level.

eIF3-f directly interacts with mTOR and S6 K1 stimulating assembling of preinitiation complex of translation of specific mRNA encoding proteins, which participate in

muscle hypertrophy. According to Csibi's data [129], inactive hypophosphorylated form of S6 K1 is physically bound to eIF3-f in the site of MAFbx and ubiquitin binding. Hence, MAFbx-initiated eIF3-f degradation must lead to S6 K1 inhibition during atrophy. Though it is still unknown whether MAFbx interacts with free-eIF3-f molecule or with the molecule bound to S6 K1. Thus, it is difficult to suppose in which direction shifts the balance between eIF3-f binding with the two ligands under disuse and which factors can affect this balance.

MAFbx has been considered as active proteolytic system member under functional unloading, while recent data confirm predominant role of MAFbx in suppression of protein synthesis at the stage of mRNA translation in case of its interaction with eIF3-f, or at the level of FoxO and MyoD activity inhibition [64, 129]. Thus, MAFbx should be considered as muscle atrophy marker rather than an index of proteolysis.

## 10. Ways to Suppress Expression of Ubiquitin Ligases under Functional Unloading

Chronic passive stretch of the muscle is supposed to be one of the most effective experimental approaches to prevent muscle atrophy [132]. First experiments with stretch combined with HS revealed marked protein synthesis intensification, while proteolysis, at least, during first 7 days, remained unchanged [132]. Meanwhile, our study of stretch combined with HS for 7 days showed twofold (and after 14 days of disuse with stretch threefold) increase in MuRF-1 and MAFbx expression in soleus [75]. At the same time, these effects were accompanied by significant rise of IGF-1 expression in soleus, which allows maintaining of proteolysis-synthesis balance in the stretched unloaded muscle with high UPS expression level. IGF-1 expression activation itself can promote decrease in the expression rate of ubiquitin ligases through the induction of Akt phosphorylation, as was described in [90].

Moreover, it was shown also that the stretch-induced dynamic redistribution of p94 is dependent on its protease activity and essential to protect muscle from degeneration, particularly under physical stress [133]. Though, we do not know whether the same phenomenon takes place during the unloaded muscle stretch.

Another possible way of protein synthesis maintaining in unloaded muscle can be injection of amino acids. Recently, it was shown that per oral administration of leucine amino acid during rat soleus immobilization significantly decreased expression rate of E3 ubiquitin ligases [134]. Mechanism of this phenomenon remains unclear.

Dependence of FoxO3 migration to the nucleus and inhibition of expression of E3 ubiquitin ligases on protective systems of myofibers was found in the experiments with plasmid transfection encoding heat shock protein 70 (HSP70). Four days of immobilization after such transfection revealed decrement of E3 ubiquitin ligases expression mediated by FoxO3 dephosphorylation [89]. We observed decrease of MuRF-1 and MAFbx expression in rat soleus under HS combined with application of NO donor, L-arginine. Evidently,

experimental intensification of NO production inhibited ubiquitin ligases expression [40].

## 11. Conclusion: Hypogravitational Atrophy of Skeletal Muscle Is a Sustainable Adaptation of Signaling Systems

Signaling systems in the cell and in fibers of skeletal muscle, in particular, are joined into extremely complex net. Change of one of its components will certainly lead to alteration of many others. At that, activity of all chains involved in the net is directed differently; therefore, any imbalance of the system should initiate processes of homeostasis maintaining. Functional unloading leads to atrophy of skeletal muscles, which manifests in increased proteolysis leading to decrease of fiber size, muscle mass loss, function deterioration, loss of  $\text{Ca}^{2+}$  sensitivity, and so forth. However, under disuse, balance between protein synthesis and proteolysis shifts so that muscle adapts to the altered circumstances. This conclusion is supported by accumulated knowledge about activity of signaling systems, which take part in proteolysis and synthesis of muscle proteins, as NFkB, for instance. Thus, under disuse, NFkB is activated independently of  $\text{TNF}\alpha$  (decreasing IGF-1-dependent pathway), which may be important, because, in this case, a number of factors blocking PI3 K/Akt pathway and protein synthesis are activated. Thus, unchanged Akt level allows keeping phosphorylation level of FoxO3 and restraining the latter in myoplasm.

At the same time, NFkB does not stimulate MuRF-1 activity during hypokinesia/hypogravity [89], which can limit in some way total level of ubiquitin ligases activity. Consequently, predominant role of MAFbx in suppression of synthetic processes in muscle and NFkB transactivation under disuse prove adaptation of the muscle to functional unloading, rather than its negative reaction to lack of basement.

Thus, it is clear now that skeletal muscles do not degrade under disuse, they adapt to altered circumstances in a way different from that of atrophy development caused by other stimuli. Mechanism of development of skeletal muscle adaptation is extremely complicated and needs further studies to reveal key regulators, which can be affected further in order to prevent development or decrease of muscle atrophy intensity.

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