Similar to skeletal muscle, the hypoglycosylation and reduced laminin binding activity of α-DG were demonstrated in Large^{myd} mouse brain [53]. Because a constitutive disruption of dystroglycan results in embryonic lethality of mouse [6], Moore et al. utilized Cre-loxP system and disrupted the dystroglycan gene selectively in brain [59]. Because DG mainly localizes to the foot-process of astrocyte that constitutes glia limitans at the pial surface, they used GFAP promoter to drive the expression of cre-recombinase in astrocyte. Surprisingly, this mouse exhibited extremely similar neuropathological findings to those of FCMD, MEB, WWS and Large^{myd} mouse [59]. Furthermore, it was demonstrated that laminin binding activity is lost in the brain and glia limitans-basal lamina was severely disrupted in the brain of this mouse [59]. Recently, the same brain malformation, including disrupted glia limitans-basal lamina, was demonstrated in mice with specific loss of α-DG in epiblast. In these mice, the laminin binding activity of α-DG was severely reduced in brain [60]. These observations strongly support the idea that the functional defect in α-DG is central to the pathogenesis of brain malformation in CMD. Interestingly, DG is also expressed by oligodendrocytes and plays a role, by interacting with laminin, in regulating terminal stages of myelination, such as myelin membrane production, growth, or stability in central nervous system [61].

It should also be noted that dystroglycan is expressed in neurons in addition to glial cells in several locations including hippocampus [62, 63]. Because cre-recombinase is transiently expressed in neuronal progenitor cells in the GFAP-DG null mouse, dystroglycan is ablated also in neurons in the mouse. Interestingly, it was shown that hippocampal long-term potentiation is blunted possibly by a postsynaptic mechanism in the GFAP-DG null mouse [59]. These results raise an intriguing possibility that neuronal dystroglycan is playing an important role in learning and memory and the mental retardation seen in FCMD, MEB and WWS patients may be in part attributed to the dysfunction of the neuronal dystroglycan. It has been reported that DG localizes inhibitory synapses and α-DG binds to neurexin [12], whereas β-DG interacts with synaptic scaffolding molecule (S-SCAM) [64].

VERSATILE FUNCTION OF DG IN PERIPHERAL NERVE

In peripheral nerve, DG is localized to outer membrane of Schwann cells and binds to laminin 2 in the endoneurial basal lamina [65]. Mutation of laminin α2 chain results in congenital muscular dystrophy type 1A (MDC1A). It is known that in MDC1A patients as well as in its rodent model, dy/dy mouse, dysmyelination of peripheral nerve develops in addition to muscular dystrophy. Myelinating Schwann cells express dystroglycan and integrin (α6β1 and α6β4) as laminin receptors [66]. Recently, conditional knockout mouse of β1 integrin in peripheral nerve revealed that β1 integrin is essential for the radial sorting of axon, a process in which a Schwann cell wraps a single axon with 1:1 relationship [67]. DG is another candidate for a laminin 2 receptor involved in myelination, because it is co-expressed with laminin 2 during peripheral nerve myelination and regeneration after nerve crush injury [68]. Therefore, it has been speculated that the α-DG-laminin interaction is necessary for the myelin formation and its maintenance in peripheral nerve.

To test this hypothesis, we have generated mice deficient in DG selectively in Schwann cells by crossing DG floxed mice with P0 promoter driven cre-recombinase expressing transgenic mice [69]. Interestingly, the peripheral nerves of this mouse exhibited broad

spectrum of morphological abnormalities. Myelin sheath was extensively folded, which is consisted of multiple external myelin loops. Polyaxonal myelination, in which single myelin sheath wraps multiple axons, and single Schwann cells including multiple myelinated axons were observed. The latter finding indicates that radial sorting mechanism is disturbed in this mouse [69]. In addition to these myelination defects, formation of the node of Ranvier was abnormal. Microvillis in node, where DG localizes, was disorganized and blunted in this mouse. In consistent to this structural defect, clustering of Na^+ channel was strikingly reduced at the node of Ranvier [69]. Recently, we also observed defective myelination of peripheral nerve in fukutin deficient chimeric mouse, a model mouse of FCMD. In this mouse, myelinated fiber density is decreased and numerous clusters of non-myelinated fibers, which indicate defective radial sorting, are observed [70]. Interestingly, neuromuscular junction is fragmented in appearance and clustering of acetylcholine receptor is defective in this mouse [70]. The laminin binding activity of α-DG is severely decreased in the skeletal muscle as well as neuromuscular junction, indicating that the α-DG-laminin interaction is crucial for normal myelin sheath and neuromuscular junction formation and/or their maintenance.

Apart from the extracellular linkage of DG, cytoplasmic linkage is also associated to dysmyelination of peripheral nerve. Cytoplasmic domain of β-DG binds dystrophin related protein 2 (DRP2), and DRP2 in turn interact with L-periaxin in Schwann cells [71]. It has been reported that L-periaxin deficient mice exhibit peripheral neuropathy [72]. Consistent with this observation, mutations in L-periaxin gene were identified in patients with hereditary peripheral neuropathy, Charcot-Marie-Tooth disease type 4F and Dejerine-Sottas disease [73, 74]. Taken together, disruption of both the extracellular and intracellular linkage mediated by DG leads to impaired myelin formation in peripheral nerve.

DG is a target of infectious diseases. *Mycobacterium leprae* (*M. leprae*), the causative organism of leprosy, a pathogen that preferentially infects Schwann cells. The LG like domain of laminin α2 chain is an initial target of *M. leprae* and they attach to the domain via 21kD laminin binding protein and phenolic glycolipid 1, molecules in the bacterial cell wall [75]. Then, α-DG on the plasma membrane of Schwann cells serves as a receptor for *M. leprae* to invade in Schwann cells [76]. Interestingly, it was shown that *M. leprae* cause demyelination by a contact depending mechanism in the absence of immune cells [77]. Lymphocytic choriomeningitis virus (LCMV) and several other arenaviruses including Lassa fever virus also utilize α-DG as a receptor [78]. Large dependent O-mannosyl glycosylation of α-DG is crucial for the virus-host interaction [79, 80]. Interestingly, infection of arenavirus to Schwann cell perturbates laminin-α-DG linkage, resulting in myelination defect in peripheral nerve [81].

CONCLUSION

A broad spectrum of neuromuscular diseases is now attributed to dysfunction of dystroglycan. Especially, recent progress in molecular genetics and biochemistry in this area revealed that defective glycosylation of α-DG underlies the pathogenesis of muscular dystrophy. The defective glycosylation results in both degeneration of the skeletal muscle and migration defects of neuron in the brain via disruption of DG-laminin linkage. Apart from these tissues, defective DG function also affects myelination and radial sorting of the

peripheral nerve. Both the extracellular interaction through α-DG-laminin and the intracellular interaction through β-DG-DRP2-L-periaxin are essential for these basic biological processes in peripheral nerve. Intact DG function is also necessary for the formation of neuromuscular junction and the clustering of Na^+ channel at the node of Ranvier. When we seek therapeutic procedures for this wide variety of neuromuscular disorders, amelioration or up-regulation of DG function could be a most suitable molecular target. In this regard, putative glycosyltransferase Large is now known to restore the defective DG function, thus it is an intriguing idea to apply this effect of Large to the therapy for α-dystroglycanopathy. Further experiments are necessary to shed further lights on the pathomechanism and to facilitate the development of therapeutic strategy for these disorders.

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Neuronal NOS as a regulator of muscle mass

Naoki Ito[1, 2] and Yuko Miyagoe-Suzuki[2]

[1]Department of Biological Information, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, and [2]Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-higashi, Kodaira, Tokyo 187-8502, Japan.
E-mail: miyagoe@ncnp.go.jp

Abstract

Skeletal muscle mass is regulated by the balance between protein synthesis and protein degradation. Although a number of molecules and signal transduction pathways are reported to be involved in the regulation of muscle mass, in this review, we focus on neuronal nitric oxide synthase (nNOS). nNOS is largely localized at the sarcolemma as a member of the dystrophin glycoprotein complex (DGC), and it produces nitric oxide (NO) to regulate diverse biological processes in skeletal muscle, including activation of satellite cells, muscle contraction, glucose transport, mitochondrial energy generation, and vasodilation. Recent studies suggest that nNOS also participates in both muscle hypertrophy and muscle atrophy as a signaling molecule. Elucidation of the mechanisms by which nNOS controls muscle mass would help us develop a treatment for muscle atrophy due to aging, disuse, and disease.

Key words: neuronal NOS; nitric oxide; skeletal muscle; muscle hypertrophy; muscle atrophy

Introduction

NO is a highly diffusible free radical, and it participates in both physiological and pathological processes in skeletal muscle: NO functions as a molecular mediator in blood flow (37, 49), inflammatory response (28), activation of satellite cells (3, 57), myoblast fusion (24, 30), contractile activity (22, 32), glucose uptake (5), and was also proposed to participate in the pathogenesis of muscular dystrophy (4, 37, 56).

Nitric oxide synthases (NOSs) are enzymes that produce nitric oxide (NO) by conversion of L-arginine to L-citrulline (1). The NOS family consists of three isoforms, neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS), which are also known as NOS1, NOS2 and NOS3, respectively (1). eNOS is constitutively expressed in the vascular endothelium and regulates blood flow. In muscle fiber, eNOS is localized to mitochondria (10). Expression levels of iNOS are usually quite low in skeletal muscle, but are

increased in association with inflammatory responses (51). nNOS is abundantly expressed at the sarcolemma of fast-twitch muscle fibers, especially at neuromuscular junctions, and myotendinous junctions, and it is the prime source of the NO released from skeletal muscle (9, 22). nNOS in myoblasts has a similar structure to that in brain, but nNOS in mature myofibers contains a 34-amino- acid insert that arises from alternative splicing between exons 16 and 17 of nNOS pre-RNA (41). This muscle-specific nNOS isoform is called nNOSμ. Whether the 34-amino acid insert endows nNOSμ with a unique function remains to be determined. In addition to nNOSμ, another isoform of neuronal NOS, mitochondrial NOS (mtNOS, also known as nNOSα), is reported to be expressed in mitochondria in skeletal muscle and to regulate the functions of mitochondria (14, 18).

Recent studies suggest that NO derived from nNOS is also involved in both muscle hypertrophy and atrophy. Because the expression and production of NO are increased by mechanical stimulation of muscle, such as exercise, mechanical loading, and passive stretching, nNOS is a candidate molecule for a mechanical sensor. In this review, first we will give an overview of the functions of nNOS in skeletal muscle, and then discuss how nNOS regulates muscle mass.

nNOS is a member of the dystrophin-glycoprotein complex

In skeletal muscle, dystrophin and dystrophin-associated proteins form the dystrophin glycoprotein complex (DGC) at the sarcolemma (Fig. 1). The DGC links the cytoskeleton to the extracellular matrix, protecting the muscle membrane from contraction-induced damage (29) and mediating signal transduction (8). A lack of dystrophin results in the loss of DGC from the sarcolemma, and makes muscle fibers susceptible to mechanical damage, leading to muscle damage and degeneration.

In skeletal muscle, a major fraction of nNOS exists as a member of the DGC. nNOS is reported to interact directly with α1-syntrophin through its N-terminal PDZ domain (8, 21, 54) (Figure 1), and in fact, nNOS is selectively absent from the sarcolemma in α1-syntrophin knockout mice (21). In cell fractionation experiments, nNOS activity is recovered mainly in a pellet fraction, but it is easily released by washing with 1M KCl (27). Therefore, compared with other members of the DGC, the interaction between nNOS and α1-syntrophin seems to be relatively weak. In Duchenne muscular dystrophy (DMD) patients and *mdx*, a mouse model of DMD, both nNOS and α1-syntrophin are absent from the sarcolemma, and nNOS is diffusely distributed in the cytoplasm at reduced levels (9). In several experimental models including α-dystrobrevin-deficient mice (17) and sarcoglycan-deficient mice (13), α1-syntrophin was found to be normally localized at the sarcolemma, but nNOS was translocated to the cytosol. These findings suggest that localization of nNOS to the sarcolemma was not regulated simply by interaction with α1-syntrophin but also by other factors.

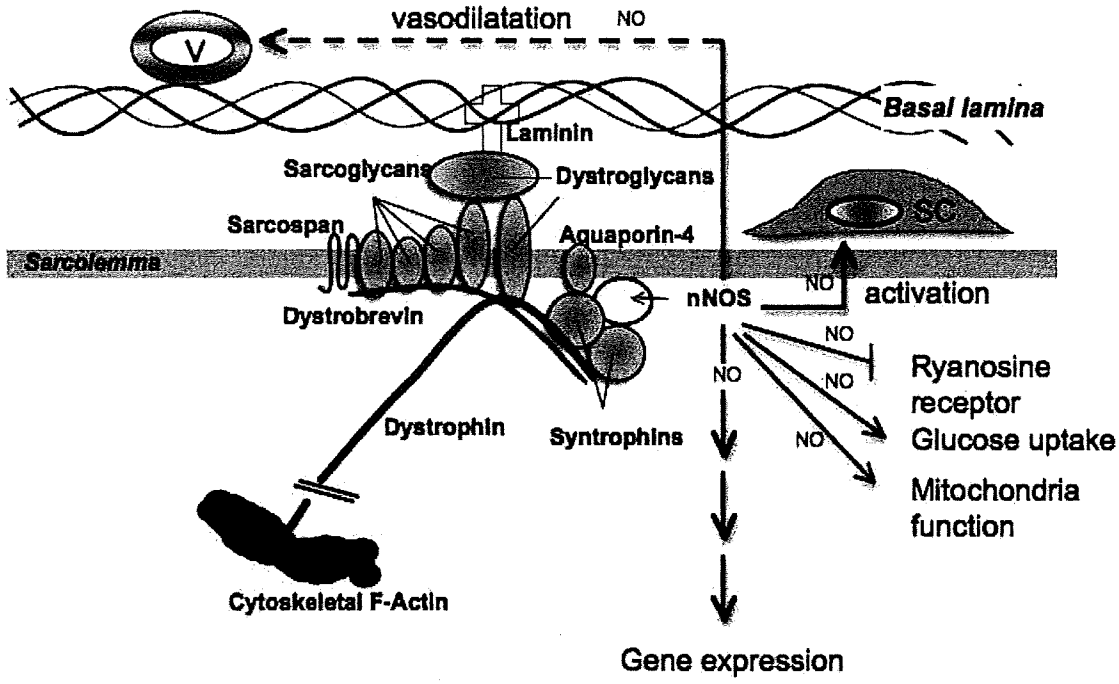

Figure 1: nNOS, a member of the dystrophin-glycoprotein protein complex (DGC) in skeletal muscle, is a versatile regulator of muscle physiology. nNOS in skeletal muscle is associated with α1-syntrophin in the DGC complex at the sarcolemma, and is especially concentrated at neuromuscular junctions and myotendinous junctions. Nitric oxide (NO) gas produced just under the sarcolemma is thought to diffuse and reach its targets: blood vessels (V), and satellite cells (SC). nNOS-derived NO also regulates glucose uptake, muscle contraction, and mitochondria function.

The redistribution of nNOS from the sarcolemma to the cytosol is also seen during hind limb suspension and cytosolic nNOS activity was found to be elevated in the atrophying muscle (46). Thus, at least in some circumstances, the translocation of nNOS is not merely secondary to the DGC defect, but might be a critical step for activation of nNOS.

nNOS participates in a variety of biological process in skeletal muscle

nNOS and blood flow:

One of the best-characterized functions of nNOS in skeletal muscle is the autoregulation of local blood flow. In healthy organisms, muscle increases its blood flow in response to exercise or muscle contraction to meet the metabolic demands. In nNOS-knockout mice, *mdx* mice, and DMD patients, exercise-induced vasomodulation is impaired (37, 49). Thomas *et al.* (50) suggested that the impairment was due to the loss of nNOS from the sarcolemma, because

the sarcolemmal localization of nNOS helps the gas, which has a short half-life, to reach and act on nearby blood vessels.

Recently, Asai *et al.* (4) showed that functional ischemia during muscle contraction greatly exacerbates muscle fiber damage in *mdx* muscle. They also reported that a phosphodiesterase-5 inhibitor improved blood flow in contracting *mdx* muscle and greatly ameliorated muscular dystrophy. Functional ischemia alone, however, did not cause severe muscle damage in wild-type mice. Therefore, impaired vasomodulation caused by the lack of nNOS is an auxiliary event rather than a direct cause of the disease.

nNOS and inflammation:

The function of nNOS in muscle fibers is not restricted to autoregulation of the blood flow. nNOS-derived NO is reported to function as an anti-inflammatory and cytoprotective molecule. Tidball and co-workers (28) showed that nNOS prevented membrane injury caused by neutrophils, and normalization of NO production in *mdx* muscle by a nNOS transgene reduced the macrophage concentration in *mdx* muscle, protected muscle from membrane injury, and greatly alleviated its pathology (56).

nNOS and muscle satellite cells:

Several reports suggest that nNOS is involved in the activation and fusion of satellite cells, and muscle regeneration. Muscle satellite cells are myogenic stem cells that are present in a quiescent state between the basement membrane and sarcolemma. Several types of stimulation, such as muscle damage and muscle contraction, activate satellite cells to participate in muscle regeneration. Anderson and co-workers reported that NO mediates the activation of satellite cells, and contributes to muscle regeneration (2, 3, 57). Tatsumi *et al.* (47, 48) showed that mechanical stimuli activate satellite cells through the release of hepatocyte growth factor (HGF), and this process was regulated by NO. Lee *et al.* (24) suggested that NO was necessary for myoblast fusion. Pisconti *et al.* (30) showed that NO directly stimulates myoblast fusion through the up-regulation of follistatin. The action of NO is reported to be mediated through activation of guanylate cyclase and generation of cyclic guanosine monophosphate (cGMP) (30).

Others:

nNOS is also implicated in glucose uptake, mitochondrial function, and excitation-contraction coupling (5, 26, 36). For details, please read the comprehensive review article written by Stamler and Meissner (44).

Roles of nNOS in muscle hypertrophy

Insulin-like growth factor I (IGF-I) is among the best-studied of the molecules that promote muscle hypertrophy (16, 20, 39). IGF-I activates PtdIns-3-OH kinase (PI3K), which in turn activates Akt. Akt then activates the mammalian target of rapamycin (mTOR)/p70S6 kinase (p70S6K) pathway, and on the other hand, inactivates the glycogen synthase kinase 3β (GSK3β) pathway and members of the forkhead box O (FoxO) family. Akt/mTOR-S6K suppresses protein breakdown and promotes protein synthesis (7, 34, 35), whereas GSK3β and FoxO negatively regulate muscle mass (38, 55).

These molecular events might successfully explain what is happening in muscle hypertrophy, but how mechanical signals activate the IGF-1/PI3K/Akt signaling pathway is not well understood. Because the expression and activity of nNOS are up-regulated by exercise (33), mechanical loading, electrical stimulation of muscle, and passive stretching (31, 52), it is reasonable to speculate that NO functions as a sarcolemma-associated mechanical sensor and activates the IGF-1/PI3K/Akt signaling pathway. Criswell and co-workers reported that inhibition of NOS by L-NAME (a wide inhibitor of NOS) during functional overload attenuated the increase of muscle mass (the control showed 88% increase, whereas L-NAME treated muscle showed only 39% increase) and fiber type transition (42, 43). The same research group showed that NO up-regulated contractile protein mRNAs including α-actin and type 1 myosin heavy chain (40). Steensberg *et al.* (45) reported that L-NAME treatment inhibited exercise-induced mRNA expression of IL-6, HO-1 and PDK4. Chang *et al.* (12) showed that nNOS is concentrated at myotendinous junctions, which are specialized for force transduction across the muscle cell membrane. Tidball *et al.* (53) showed that talin and vinculin mRNA expressions induced by mechanical stimulation are mediated by NO. Moreover, Koh and Tidball (23) showed that NO positively modulates sarcomere addition in immobilized-remobilized muscles. Together, all these data strongly suggest that nNOS is a mechanotransducer in skeletal muscle.

Roles of nNOS in muscle atrophy

Bed rest, immobilization, denervation, hind limb suspension, and space flight all increase the rates of protein degradation in skeletal muscle, leading to muscle atrophy. The accelerated protein breakdown was reported to result from mainly the activation of the ubiquitin proteasome system (6), but recent studies revealed that the autophagy system is also activated in atrophying muscle (25). The ubiquitin proteasome pathway in skeletal muscle is mainly regulated by FoxO transcription factors (38) and NF-κB (11). These transcription factors up-regulate muscle-specific E3 ubiquitin ligases, MuRF-1 and atrogin-1/MAFbx (6, 11, 38). Suzuki *et al.* (46) recently reported that nNOS acts as an upstream regulator of FoxO

transcription factors in unloading-induced muscle atrophy. During hind limb suspension, nNOS was translocated from the sarcolemma to the cytosol and produced high levels of NO. Using nNOS-null mice, the authors further demonstrated that cytosolic nNOS activates FoxO3a, which in turns up-regulates MuRF-1 and atrogin-1/MAFbx during hind limb suspension (Figure 2). Although the data suggest that nNOS is one of the mechanosensors in skeletal muscle, and is a potential therapeutic target for unloading-induced muscle atrophy, the mechanism by which nNOS is activated in the cytosol remains unknown. It is also to be determined how nitric oxide activates FoxO transcription factors. One plausible mechanism by which NO controls its target molecules is S-nitrosylation. NO reacts with thiol of protein cysteine residues to form S-nitrosothiol. This posttranslational modification affects the functions of a wide range of proteins, and regulates protein-protein interactions and DNA-binding activity of transcription factors (19). It remains to be tested whether NO S-nitrosylates FoxO transcription factors during unloading.

Recent studies show that FoxO3 is necessary and sufficient for induction of autophagy in skeletal muscle (25, 58). Whether nNOS also activates the autophagy- lysosome system through activation of FoxO3 in unloading-induced muscle atrophy is yet to be determined.

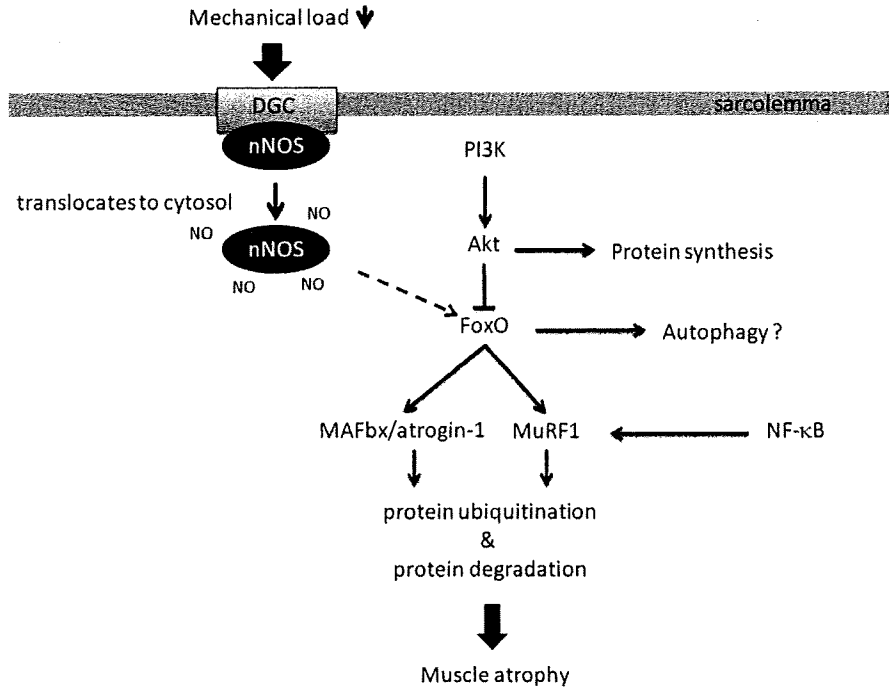

Figure 2: In muscle atrophy, nNOS translocates from the sarcolemma to the cytosol, and activates FoxO. When the mechanical load is reduced, sarcolemmal nNOS disappears and cytoplasmic nNOS activates FoxO, which in turn up-regulates the muscle-specific E3 ligases MuRF1 and MAFbx/atrogin-1 and suppresses Akt activity. Similar dislocation of nNOS is observed in denervated muscle (46).

Discussion

Skeletal muscle changes its fiber size, fiber type, and the ability to generate force in response to modifications of muscle use, availability of nutrients, cytokines/growth factors, and hormones. The mechanisms by which skeletal muscle adapts to functional demands are mainly explained by the Akt signaling pathway; Akt activates its downstream target mTOR, and inactivates GSK3β and FoxO transcription factors to regulate the balance between synthesis and breakdown of protein *in vitro* and *in vivo* (7, 34, 39).

In recent years, many reports suggested that nNOS also regulates skeletal muscle mass. nNOS is normally targeted to the sarcolemma, and is particularly concentrated at the myotendinous junction (12). The expression and activity of nNOS are increased by muscle contraction. Several reports showed that NO produced by nNOS regulated gene expression and modulated muscle mass in both unload and overload models. These observations suggest that nNOS is a mediator that converts mechanical signals into biological responses in skeletal muscle. Because nNOS activity is mainly regulated by the levels of intracellular Ca^{2+} and the L-type Ca ion channel DHPR is located close to the dystrophin complex (15), the direct association of nNOS with the DGC at the sarcolemma might be necessary for its function in mechanotransduction. It remains to be determined how nNOS senses mechanical signals and regulates the Akt/mTOR signaling pathway.

Conclusion

nNOS is a versatile regulator of muscle physiology, and a number of studies indicate that nNOS contributes to both muscle hypertrophy and atrophy. Understanding the molecular mechanisms by which nNOS regulates muscle mass could lead to successful intervention in muscle atrophy caused not only by disuse, but also by diseases and aging.

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