Review Article

The Functional Role of Calcineurin in Hypertrophy, Regeneration, and Disorders of Skeletal Muscle

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Skeletal muscle uses calcium as a second messenger to respond and adapt to environmental stimuli. Elevations in intracellular calcium levels activate calcineurin, a serine/threonine phosphatase, resulting in the expression of a set of genes involved in the maintenance, growth, and remodeling of skeletal muscle. In this review, we discuss the effects of calcineurin activity on hypertrophy, regeneration, and disorders of skeletal muscle. Calcineurin is a potent regulator of muscle remodeling, enhancing the differentiation through upregulation of myogenin or MEF2A and downregulation of the Id1 family and myostatin. Foxo may also be a downstream candidate for a calcineurin signaling molecule during muscle regeneration. The strategy of controlling the amount of calcineurin may be effective for the treatment of muscular disorders such as DMD, UCMD, and LGMD. Activation of calcineurin produces muscular hypertrophy of the slow-twitch soleus muscle but not fast-twitch muscles.

1. Introduction

Calcium serves as a second messenger in signal transduction pathways, using spatiotemporal patterns of intracellular calcium to generate oscillatory changes in calcium concentrations. It is well established that the elevations in intracellular calcium levels in skeletal muscle that are essential for contractile activity also give rise to muscle-specific gene expression through downstream transcriptional pathways [1, 2]. Changes in intracellular Ca^{2+} concentrations regulate the physiological activities of calmodulin. Calmodulin is a multifunctional signal transducer that undergoes conformational changes before activating a wide range of binding substrates, mainly downstream phosphatases (calcineurin) and kinases (CaMKs) [3]. The serine/threonine phosphatase calcineurin plays a major role in a variety of physiological and pathological processes, including immune responses, neuronal plasticity, and cardiac development and hypertrophy [4]. For example, transgenic mice overexpressing calcineurinA developed a profound hypertrophic responses

and heart failure that mimicked human heart disease [5]. Bueno et al. [6] demonstrated that calcineurin $A\beta$ null mice showed a reduced basal heart size. In addition, the absence of NFATc2 (the nuclear factor of activated T cells c2) has been shown to inhibit pathological (biomechanical stress) but not physiological (voluntary exercise training) cardiac enlargement [7]. Although the downstream pathway of calcineurin is not completely clear [8], calcineurin signaling seems to play an important role in cardiac muscle. Many excellent reviews [8, 9] have indicated a central role for calcineurin signaling in determining fiber types and myosin heavy chain (MHC) (IIB → IID → IIA → I). However, few systematic and descriptive reviews have dealt with the role of calcineurin in regulating the hypertrophy and regeneration of skeletal muscle in mature mammals. This review aims to outline the functional role of calcineurin in the hypertrophy and regeneration of skeletal muscle. In addition, it discusses the present situation and future therapeutic applications for modulating calcineurin levels to alleviate muscular disorders.

2. Structure of Calcineurin

Calcineurin enzymatic activity requires a catalytic subunit (CnA, 59 to 62 kDa) and a calcium-binding regulatory subunit (CnB, 19 kDa), which comprise several isoforms coded by different genes or generated by alternative splicing. The CnA subunit includes domains for catalytic activity, CnB interaction, and calmodulin-binding. A C-terminal autoinhibitory domain, which blocks the catalytic site, is removed in response to an increase in calcium. Three calcineurin genes have been described; cnAα and CnAβ expressed ubiquitously, and CnAγ restricted to brain and testis. Two CnAβ isoforms, CnAβ1 and CnAβ2, which differ in their C-terminal domain, are encoded by alternatively spliced transcripts [10]. The typical autoinhibitory domain present in CnAβ2 and other calcineurin isoforms is absent from CnAβ1, in which an unrelated C-terminal domain is generated by the translation of intronic sequences [11]. This novel domain is preserved in CnAβ1 orthologues from different species [11], especially in higher vertebrates, suggesting an evolutionarily conserved role for this calcineurin variant. When activated by Ca^{2+}-calmodulin binding, calcineurin affects gene expression by dephosphorylating specific substrates, including the four calcineurin-dependent members of NFAT gene family, NFATc1, NFATc2, NFATc3, and NFATc4. Following dephosphorylation, NFAT translocates from the cytoplasm to the nucleus and activates target genes in cooperation with other transcription factors [12]. Calcineurin activity and the ability to activate NFAT are directly antagonized by the immunosuppressive agents FK 506 and cyclosporine A (CsA) through complexes with cyclophillins and FK506-binding proteins, respectively [13, 14], or by endogeneous protein inhibitors, such as cain (also known as cabin-1) and MCIP-1 (myocyte-enriched calcineurin interacting protein 1), the latter being particularly abundant in slow-twitch muscle but not detectable in fast-twitch glycolytic muscles [15, 16]. More recently, reporter assays using cultured myoblasts indicated that the transcriptional activation of NFAT by calcineurin was also inhibited by calsarcin-2 [17].

3. Muscle Regeneration

Skeletal muscle satellite cells are generally in a quiescent state in adult muscle, but when minor damage or injury occurs, signals are generated within the muscle that activate these satellite cells, stimulating them to migrate to the site of an injury where they proliferate, differentiate, and fuse with the damaged fibers or form new fibers [18, 19]. Studies in vitro have documented many factors, primarily protein growth factors, which can modulate satellite cell activity [18, 19]. In particular, insulin-like growth factor I (IGF-I), whose expression is known to be upregulated in regenerating muscle in vivo [20], positively regulated the proliferation and differentiation of satellite cells/myoblasts in vitro via different pathways. Calcineurin is a major candidate for a component in the pathway downstream of IGF-I as well as Akt. In fact, the inhibition of calcineurin completely blocked the growth of myotubes on treatment with IGF-I in vitro [21]. Local expression of IGF-I in muscle protected both motor

neurons and innervating muscle fibers in a mouse model of amyotrophic lateral sclerosis (the SOD1^{G93A} transgenic mouse) possibly due to the enhancement of CnAβ1 [22]. Since activated calcineurin promotes the transcription and activation of myocyte enhance factor 2(MEF2), myogenin, and MyoD [23–25], calcineurin seems to control satellite cell differentiation and myofiber growth and maturation, all of which are involved in muscle regeneration. In fact, our previous Western blot analysis [26] showed a long-term (1–14 days post bupivacaine injection) increase in the amount of calcineurin protein in the regenerating muscle of adult rats. Immunofluorescence microscopy revealed marked immunolabeling of calcineurin in many myoblasts and myotubes that expressed MyoD and myogenin at an active differentiation phase (4–6 days post injection). In addition, our biochemical approach demonstrated that the amount of calcineurin coprecipitating with NFATc1 and GATA-2 and NFATc1 coprecipitating with GATA-2 gradually increased in the regenerating muscle. Furthermore, we showed that the inhibition of calcineurin by CsA induced extensive inflammation, marked fiber atrophy, the appearance of immature myotubes, and calcification in the regenerating muscle compared with placebo-treated mice [26]. Several other studies indicated such defects in skeletal muscle when calcineurin was inhibited [27, 28], whereas transgenic activation of calcineurin is known to markedly promote the remodeling of muscle fibers after damage [11, 29].

Myostatin is a member of the transforming growth factor-β (TGF-β) family that negatively regulates skeletal muscle growth, with its inhibition shown to enhance muscle size. Mice, which are null for (knock-out) or display inactivating mutations of myostatin, exhibit obvious skeletal muscle hyperplasia and hypertrophy [30, 31]. Recent evidence [32–34] has also identified myostatin as well as Id1 [22], Id3 [22], and Egr-1 [25] as a possible downstream negative hypertrophic effector target of the calcineurin-NFAT pathway. In addition, using a pharmacological approach (intraperitoneal CsA treatment), our recent study [35] demonstrated that the inhibition of calcineurin enhanced the expression of myostatin and Smad3 mRNA in regeneration-defective TA muscle after an injection of bupivacaine. An increase in myostatin levels is closely linked with muscle atrophy after unloading in mice and humans [36–38] and with severe muscle wasting in HIV patients [39]. Myostatin has been shown to interact with Smad2 and Smad3 [40, 41], and the activation of the myostatin pathway inhibits myogenic differentiation through a downregulation of MyoD expression [40, 41]. The possibility that myostatin is a downstream mediator of calcineurin signaling has been indicated by recent experiments with two different transgenic mice [42]. Muthuri et al. [42] found that myostatin mRNA levels in skeletal muscle were significantly lower in mice expressing high levels of calcineurin and significantly higher in mice displaying inhibited calcineurin signals. Moreover, levels of calcineurin mRNA were higher in null myostatin transgenic mice than wild-type mice [42]. On the other hand, using MCK-CnAα transgenic mice, Stupka et al. [29] demonstrated that calcineurin activates two differentiation-enhancing molecules, myogenin, and MEF2A, during muscle

regeneration. Indeed, calcineurins pharmacological inhibition caused a decline in the transcription and activation of MEF2, myogenin and MyoD during myogenic differentiation in vitro [23–25]. However, the notion that muscle regeneration is promoted by a myogenic transgene with CnAα is controvertial [11, 29]. A more recent study using a similar mouse model found that transgenic expression of CnAα excessly stimulated the inflammatory response after muscle damage and prevented prompt muscle regeneration [11].

The induction of MAFbx/Atrogin-1 expression by Foxo has been shown to inhibit calcineurin activity [43, 44]. More recently, the calcineurin variant CnAβ1 was suggested to block the nuclear localization of Foxo protein and the expression of several Foxo-targeted genes (MuRF1, Gadd45a, Pmaip1, and atrogin) in C2C12 myoblasts [11]. In addition, transgenic upregulation of CnAβ1 expression promoted the remodeling of cardiotoxin-treated damaged muscle fibers [11]. Foxo factors play a crucial role in skeletal muscle atrophy through the induction of MAFbx/Atrogin-1 and MuRF1 [45, 46]. Interaction between CnAβ1 and Foxo in muscle regeneration is an attractive notion, although it has not been demonstrated in adult skeletal muscle in vivo.

4. Muscle Hypertrophy

The major extracellular mediator of skeletal muscle hypertrophy is thought to be IGF-I which binds to its receptor to initiate a cascade of signaling pathways via phosphoinositide 3-kinase (PI3-K/Akt/mammalian target of rapamycin (mTOR)) [47–49]. However, several lines of evidence suggest that IGF-I also mediates hypertrophy through calcineurin/NFAT signaling pathways. Overexpression of IGF-I in murine C2C12 myoblasts [21] and rat L6MLC cells [50] induced hypertrophy of myotubes, which was abolished by treating the cells with CsA. Dunn et al. [51] proposed that calcineurin signaling regulates the hypertrophy of muscle fiber in mature rats. They concluded that the enlargement of fibers in the plantaris muscle after mechanical overloading was completely blocked at both 2 and 4 weeks post surgery by subcutaneous treatment with CsA at 25 mg/Kg twice per day. However, several lines of evidence exclude a functional role for calcineurin in the hypertrophy of muscle fiber in vivo [52–55]. For example, different to the positive effect of rapamycin, the calcineurin inhibitors CsA and FK506 for up to 30 days did not block the hypertrophy of plantaris muscle that followed surgical removal of the soleus, medial, and lateral gastrocnemius muscles in the rat [53]. Consistent with these findings, others have demonstrated that even a tenfold increase in the expression of activated calcineurin in transgenic mice did not induce muscle hypertrophy [53] in spite of an increase in the proportion of slow muscle fibers due to the influence of CnA. In addition, Parsons et al. [54] indicated that neither CnAα nor CnAβ null mice showed any growth-related alterations in skeletal muscle, and fiber size or number was not altered in glycolytic/fast muscle types (tibialis anterior, gastrocnemius, quadriceps, etc.). Furthermore, no change in the size of several fast-type

muscles has been observed in mice with a transgenic upregulation of calcineurin [56], although a transgenic mouse with a constitutively active form of Akt exhibited rapid and significant hypertrophy of fast-type muscles [57]. However, Talmadge et al. [56] also demonstrated that overexpression of calcineurin induced marked hypertrophy of slow-and fast-twitch fibers of the slow-type soleus muscle. In addition, fiber size in the soleus muscle was markedly reduced by a null mutation of CnAβ different to CnAα [54]. Muscle-specific overexpression of MCIP1, an inhibitor of calcineurin, using the Flox-On approach resulted in a marked reduction (about 30%) in cross-sectional area of the soleus muscle [58]. Moreover, our recent study [59] using ICR mice did not detect any apparent hypertrophy of fibers in the soleus muscle after mechanical overloading on treatment with CsA. Other researchers also suggest growth-retarded effects in the soleus muscle caused by calcineurins inhibition during recovery from hindlimb unloading [60–62]. These lines of evidence seem to indicate a selective influence of calcineurin or Akt on the size of antigravity/slow-type soleus and fast-type muscles, respectively. Table 1 shows an overview of the effect of calcineurin activation or inhibition on fiber growth (hypertrophy) of skeletal muscle in vivo.

Although calcineurin activity appears critical to mediating the hypertrophy of slow-type muscle, the downstream effector genes or targets in this process have yet to be clearly defined. Various downstream mediators of calcineurin-dependent signaling have been proposed [34], including NFAT and MEF2 proteins as well as GATA transcription factors. These factors are known to costimulate the transcriptional response of certain hypertrophic marker genes in the heart [63, 64] and affect IGF-I-related growth of skeletal myocytes in vitro [50]. GATA-2 expression is upregulated [65] and NFAT more extensively dephosphorylated [63] in hypertrophying fast-type plantaris muscle in vivo. In addition, mice with targeted inactivation of NFATc2 or NFATc3 exhibited reduced muscle size as well as fiber type abnormalities [66] or defects in muscle formation [67], respectively. Cultured C2C12 murine myoblasts expressing activated calcineurin showed increased enzymatic activity in association with NFATc3's nuclear translocation during the initiation of myogenic differentiation [23]. Given these findings, NFAT and GATA seem to play an important role in the normal growth and hypertrophy of skeletal muscle. Therefore, NFAT-GATA complexes may be mediators of calcineurin signaling during the hypertrophic process in soleus muscle. In contrast, a more recent study in our laboratory [58] suggested that MEF2C, not MEF2D or myogenin, regulates the hypertrophic process in slow-twitch soleus muscle subjected to mechanical overloading (MOV). Hypertrophy-defect soleus muscle after mechanical overloadingby calcineurin inhibition contained less MEF2C protein than a placebo-treated control. In addition, this growth-failed soleus muscle showed less extensive immunoreactivity to MEF2C in the subsarcolemmal region in a group of myotubes and/or myofibers during an active-differentiation period (4 days postsurgery) [59]. Two recent findings [68, 69] clearly showed that MEF2C is required for thick filaments to form in nascent muscle fibers and for the integrity of

TABLE 1: Effect of calcineurin activation or inhibition on fiber growth (hypertrophy) of skeletal muscle in vivo.

References	Species	Experimental system	Modulating method of calcineurin activity	Outcomes
Dunn et al. 1999 [51]	Mouse	Mechanical overloading	Pharmacological inhibition for 4 weeks (CsA; 25 mg/Kg twice daily)	Inhibition of fiber hypertrophy of the plantaris muscle
Bodine et al. 2001 [53]	Mouse	Mechanical overloading	Pharmacological inhibition for 30 days (CsA; 15 mg/Kg once daily)	No inhibition of fiber hypertrophy of the plantaris muscle
Sakuma et al. 2008 [59]	Mouse	Mechanical overloading	Pharmacological inhibition for 2 weeks (CsA; 25 mg/Kg once daily)	Inhibition of fiber hypertrophy of the soleus muscle
Parsons et al. 2003 [54]	Mouse		Gene-knock out (complete inactivation of cna α or cna β)	Slow-twitch soleus muscle; hypertrophy (cna α KO), atrophy (cna β KO), Several fast-twitch muscles (gastrocnemius, tibialis anterior, triceps brachii); no change (both cna α and cna β KO)
Talmadge et al. 2004 [56]	Mouse		Transgenic manipulation (high-level expression of a constitutively active form of calcineurin)	Slow-twitch soleus muscle; marked hypertrophy Several fast-twitch muscles (extensor digitorum longus, tibialis anterior, plantaris); no change
Oh et al. 2006 [58]	Mouse		(Transgenic manipulation (Flox-On approach), upregulation of calcineurin inhibitor (MCIP1))	Atrophy of the soleus muscle
Mitchell et al. 2002 [60]	Mouse	Reloading after hindlimb suspension	Pharmacological inhibition for 2 weeks (CsA; 12.5, 25, and 50 mg/Kg once daily)	Inhibition of fiber growth (recovery) of both the soleus (25 and 50 mg/Kg) and plantaris (12.5, 25, and 50 mg/Kg) muscles
Miyazaki et al. 2006 [61]	Mouse	Reloading after hindlimb suspension	Pharmacological inhibition for 4 weeks (FK506; 3–5 mg/Kg once daily)	Inhibition of fiber growth (recovery) of the soleus muscle
Oishi et al. 2008 [62]	Rat	Reloading after hindlimb suspension	Pharmacological inhibition for 2 weeks (CsA; 25 mg/Kg twice daily)	Inhibition of fiber growth (recovery) of the soleus muscle

the sarcomere and M-line during postnatal muscle growth, by directly regulating several muscle structural genes such as the genes for myomesin, MHC, and myosin light chain. Further study will be required to identify downstream modulators of calcineurin signaling during hypertrophy of slow-twitch soleus muscle. Figure 1 provides an overview of molecular pathway of calcineurin to regulate hypertrophy and regeneration of skeletal muscle.

5. The Ameliorating Role of Calcineurin in Muscular Disorders

Calcineurin signaling is considered to regulate the degenerative symptoms of various muscular disorders [74–84]. In animal models, pharmacological inhibition of calcineurin in regenerating muscles of young mdx dystrophic mice,

a model of DMD, severely compromised muscle structure and function [76]. Moreover, transgenic mice overexpressing the activated form of CnA have been used to determine whether signaling through CnA improves the dystrophic pathology due to alterations in the expression of utrophin A, a therapeutically relevant protein that can compensate for the lack of dystrophin, and improved muscle membrane integrity [75]. Breeding CnA transgenic mice with mdx counterparts led to offspring (mdx-CnA) with both genes altered in their skeletal muscle, but displaying a marked improvement in the dystrophic phenotype. Muscles from transgenic mdx-CnA mice showed improvements in various markers of muscle damage, including a decrease in central nucleation (indicating denervation or fiber regeneration), a decrease of variation in fiber size, a decrease in the intracellular presence of immunoglobulin M (a marker of
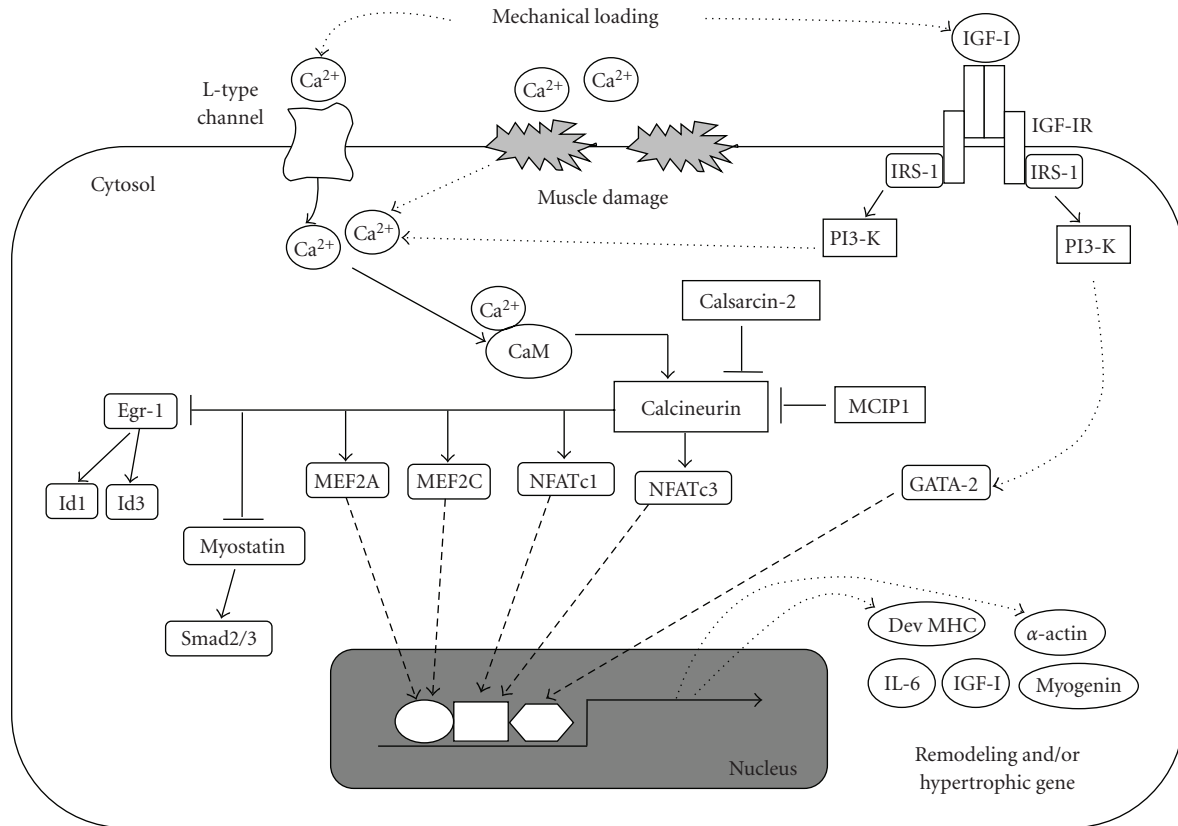
FIGURE 1: Schematic diagram of calcineurin signaling to regulate hypertrophy and regeneration of skeletal muscle. Mechanical loading of skeletal muscle increase intracellular Ca^{2+} levels via the influx (L-type Ca^{2+} channel) of Ca^{2+} from the extracellular space and its efflux from the sarcoplasmic reticulum. The damage of muscle fiber membrane after treatment with myotoxin also elicits an increase in intracellular Ca^{2+} levels via the influx of Ca^{2+} from the extracellular space. Binding of the Ca^{2+}/CaM complex to the calcineurin regulatory subunit leaded to its activation. Activated calcineurin dephosphorylates NFATc1 [26, 65, 70], NFATc3 [23], MEF2C [59], and MEF2A [29, 70] resulting in their translocation from the cytoplasm to the nucleus. These transcription factors induce the expression of hypertrophic and/or remodeling genes such as Dev MHC [29], α-actin, IGF-I [71], myogenin [25], and IL-6 [72]. In addition, activated calcineurin inhibits the functional role of Egr-1 [25] and myostatin [32–34]. Mechanical overloading upregulates gene expression of IGF-I. IGF-I modulates calcineurin signaling via increasing intracellular Ca^{2+} levels [25] and activating GATA-2 [50, 73]. MCIP1 [58] and calsarcin-2 [17] are potent inhibitors of calcineurin signaling. MEF2A: Myocyte enhancer factor 2A; NFATc1: nuclear factor of activated T cells c1; MCIP1: Modulatory calcineurin-interacting protein 1; IRS-1: Insulin receptor substrate-1; PI3-K: Phosphatidylinositol 3-kinase; CaM: Calmodulin; Dev MHC: Developmental myosin heavy chain; IL-6: Interleukin-6.

sarcolemmal integrity), a decrease in the uptake of Evans blue dye by muscle fibers in vivo (indicating sarcolemmal microdamage), and a decrease in numbers of infiltrating immune cells revealed by Mac-1 antibody staining (a marker of an inflammatory response) [75]. Thus, an attenuation of the muscle pathology-associated dystrophin deficiency is observed when utrophin A is upregulated via activation of CnA-NFAT signaling. In fact, Chakkalakal et al. [74] showed that calcineurin/NFATc1 signaling as well as peroxisome proliferator γ co-activator-1α (PGC-1α)/GA-binding protein (GABPα) [77] can stimulate the transcriptional activity of utrophin A. Subsequent similar crossbreeding experiments by others [81, 83], leading to mdx-CnA mice with potentiated CnA-NFAT activation, showed related improvements in the contractile function and attenuation of contractile-induced injury in muscles from these animals comparted with mdx counterparts.

On the other hand, transgenic upregulation of the CaM-binding protein (CaMBP), a small peptide inhibitor for calmodulin, exacerbated the dystrophic phenotype in mdx mouse muscle. mdx/CaMBP mice revealed an impairment of both the Ca^{2+}/CaM-regulated enzyme calcineurin and a Ca^{2+}/calmodulin-dependent kinase [78]. These mice exhibited significant reductions in utrophin A attributable to the marked decrease in nuclear accumulated NFATc1 and MEF2C and in CABPα mRNA expression. In contrast, pharmacological and genetic inhibition of calcineurin signaling was suggested to be effective in a mouse model and in patients with several other muscular disorders such as limb-girdle muscular dystrophy (LGMD), Ullrich congenital muscular dystrophy (UCMD), and collagen VI myopathies [81, 82, 84]. For example, in an open pilot trial, oral CsA treatment for 1 month markedly ameliorated mitochondrial dysfunction and reduced the frequency of apoptotic nuclei

in muscle fibers in patients with collagen VI myopathies [84]. In addition, genetic deletion of the loxP-targeted calcineurin B1 or CnAβ gene resulted in enlarged muscle fibers and decreases in the frequency of centrally nucleated fibers and of fibrosis, and in the amount of hydroxyproline in scgd−/− mice [82]. These findings clearly showed that inhibition of calcineurin signaling reduced skeletal muscle degeneration and the histopathology of LGMD. Since the therapeutic effectiveness of the pharmacological attenuation or activation of calcineurin signaling differs entirely among muscular disorders, careful attention should be paid to this application.

6. Conclusions

This review summarised and highlighted current understanding of the role of calcineurin in the regulation of hypertrophy, regeneration, and disorders of skeletal muscle. Although several lines of evidence exclude a functional role for calcineurin in the hypertrophy of muscle fiber in vivo, recent findings have suggested that the hypertrophy of slow-twitch soleus muscle is regulated by calcineurin signaling. A possible downstream modulator of the calcineurin pathway during muscle regeneration may be MEF2A, myostatin, or Foxo. The strategy of controlling the amount of calcineurin may be effective in the future treatment of muscular disorders.

Acknowledgment

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