Proteasome-dependent Activation of Mammalian Target of Rapamycin Complex 1 (mTORC1) Is Essential for Autophagy Suppression and Muscle Remodeling Following Denervation*

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Abstract:

Drastic protein degradation occurs during muscle atrophy induced by denervation, fasting, immobility, and various systemic diseases. Although the ubiquitin-proteasome system is highly up-regulated in denervated muscles, the involvement of autophagy and protein synthesis has been controversial. Here, we report that autophagy is rather suppressed in denervated muscles even under autophagy-inducible starvation conditions. This is due to a constitutive activation of mammalian target of rapamycin complex 1 (mTORC1). We further reveal that denervation-induced mTORC1 activation is dependent on the proteasome, which is likely mediated by amino acids generated from proteasomal degradation. Protein synthesis and ribosome biogenesis are paradoxically increased in denervated muscles in an mTORC1-dependent manner, and mTORC1 activation plays an anabolic role against denervation-induced muscle atrophy. These results suggest that denervation induces not only muscle degradation but also adaptive muscle response in a proteasome- and mTORC1-dependent manner.

Protein turnover, the net balance between protein degradation and protein synthesis, in the muscle is regulated by the metabolic demands of muscle itself and of the entire body. Muscle atrophy occurs under catabolic conditions such as denervation, fasting, immobility, and various systemic diseases. Degradation of myofibrillar components is mainly mediated by the ubiquitin-proteasome pathway (1). In denervation-induced atrophy, the two E3 ubiquitin ligases, muscle RING finger 1 (MuRF1) and muscle atrophy F-box (MAFb or Atrogin-1), are markedly induced within 1 day and reach a peak at 3 days after denervation (2). In knock-out mice lacking either enzyme, the rapid loss of muscle mass upon denervation is significantly reduced (3, 4).

However, the contribution of autophagy, another major proteolytic system by which cytoplasmic materials are enclosed by the autophagosome and degraded in the lysosome, remains largely unknown. Transcription of several autophagy-related genes such as LC3b, Bnip3, GABARAPI, Atg12, Atg4B, and Beclin 1 is up-regulated in denervated muscles through FoxO3 activation (5, 6). Using an in vivo microtubule-associated protein light chain 3 (LC3) turnover assay, Ju et al. (7) demonstrated that autophagic flux is increased by denervation (8). However, muscle-specific deletion of Atg7 not only fails to alleviate but in fact exacerbates denervation-induced atrophy. Similarly, administration of lysosomal inhibitors does not reduce overall proteolysis during atrophy (9), although cathepsins can be induced after denervation (2, 9).

Whether denervation stimulates only protein catabolism has been questioned. Several studies have shown that protein synthesis is also increased in denervated hindlimb muscle (10) and diaphragm muscle (11). Activation of the mammalian target of rapamycin complex 1 (mTORC1) following denervation has also been suggested (12–14), although mTORC1 is generally important for maintenance of muscle mass (15, 16). However, the mechanism underlying the paradoxical up-regulation of protein synthesis and mTORC1 activation and the physiologic significance of these phenomena have not been investigated.

In this study, we demonstrate that autophagy is rather suppressed in denervated muscles by a constitutive activation of mTORC1. Furthermore, mTORC1 is activated via a proteasome-dependent activation of mTORC1. We further reveal that mTORC1 activation plays an anabolic role against denervation-induced muscle atrophy. These results suggest that denervation induces not only muscle degradation but also adaptive muscle response in a proteasome- and mTORC1-dependent manner.

Background: The overall protein metabolism during denervation atrophy remains unclear.

Results: Autophagy is suppressed, whereas ribosome and protein synthesis are up-regulated in denervated muscles by proteasome-dependent activation of mTORC1.

Conclusion: Denervation does not simply induce muscle degradation but also promotes proteasome- and mTORC1-dependent muscle remodeling.

Significance: This information is beneficial for understanding the pathophysiology of other types of muscle atrophy.

Keywords: muscle atrophy, denervation, muscle proteasome, mTORC1, autophagy

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mediated increase in intramuscular amino acids, which occurs following denervation. Finally, we reveal that mTORC1 activation is critical for adaptive muscle remodeling in denervated muscles through up-regulation of protein and ribosome synthesis.

**EXPERIMENTAL PROCEDURES**

**Mice**—Autophagy-indicator GFP-LC3 mice were previously reported (17). C57BL/6j mice were purchased from Sankyo Labo Service Corp. (Tokyo, Japan) and fed a standard mouse chow and water. Most mice used in this study were between 10 and 14 weeks old. Sciatic denervation was performed by anesthetizing the mice with an intraperitoneal injection of 2,2,2-tribromoethanol (Avertin®, Sigma), shaving, and making a 0.5-cm incision at middle thigh level on the lateral side of the right hindlimb, separating the muscles at the fascia, lifting out the sciatic nerve with surgical forceps, removing a 0.5-cm piece of sciatic nerve, and finally closing the incision with surgical clips. Surgery was also performed on the left hindlimb, and the sciatic nerve was visualized in the same way but left intact (sham-operated). Muscles of the left hindlimb were used as internal controls for the denervated tissues. For inhibitor (sham-operated). Muscles of the left hindlimb were used as internal controls for the denervated tissues. For inhibitor

**In Vivo SUnSET**—For in vivo measurements of protein synthesis, mice were given an intraperitoneal injection of 0.04 μmol/g puromycin exactly 20 min before tissue sampling. Extracted tissues were stored and processed for immunoblot analysis as described elsewhere (22).

**Proteasomal Activity Assay**—Crude muscle extracts were prepared as described previously (23). To measure the chymotrypsin-like activity of the 20 S proteasome, 50 μg pf protein was mixed with an assay buffer containing 100 μM fluorogenic peptide substrate succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Peptide Institute, Inc.) in 50 mM Tris-HCl (pH 8.0) and 1 mM dithiothreitol. After incubation for 30 min at 37 °C, hydrolysis of the synthetic peptides was measured at excitation and emission wavelengths of 355 and 460 nm, respectively, using an ARVO MX Plate Reader (PerkinElmer Life Sciences). Epoxomicin-sensitive activity was considered to be proteasome-specific.

**Measurement of Amino Acid Concentration**—Muscle samples were weighed while frozen and homogenized in 5 volumes of ice-cold 5% sulfosalicylic acid. After centrifugation at 10,000 × g for 15 min at 4 °C, free amino acids in the supernatant were measured using an L8500 amino acid analyzer (Hitachi, Ltd.).

**Quantitative RT-PCR**—Total RNA was extracted from skeletal muscle using ISOGEN (Nippon Gene, Tokyo, Japan) following the manufacturer’s instructions. Complementary DNA was generated with ReverTra Ace (Toyobo) and was analyzed by quantitative real time RT-PCR using the SYBR Green assay (Takara). All data were normalized to GAPDH expression. Sequences of primers used were as follows: Rps6, 5'-ccgccacggtc-tcaag-3' and 5'-tcggacattcataacccctct-3'; Rpl7, 5'-aggatttcccccagacgagaa-3' and 5'-tgggaacacggtgcctc-3'; and Gapdh, 5'-cccagctcacatgaa-3' and 5'-catgctggcatgctctt-3'.

**Statistical Analysis**—All numerical data including error bars represent the mean ± S.E. Statistical comparisons were made using the paired Student’s t test.

**RESULTS**

**Muscle Autophagy Is Suppressed by Denervation**—To examine autophagic activity in denervated muscles, sciatic nerve transection was performed on the right side of transgenic mice.
expressing the autophagosome indicator GFP-LC3. Surgery was also performed on the left side, but the sciatic nerve was left intact, thus serving as an internal control (sham-operated). One or 2 days after this procedure, basal levels of GFP-LC3 puncta representing autophagosomes were observed in sham-operated gastrocnemius (GAS) muscles (mainly consisting of fast twitch fibers). Contrary to our expectations, the number of GFP-LC3 puncta was not increased but rather decreased in denervated muscles (Fig. 1A).

We also tested whether autophagy was also suppressed in denervated muscles under starvation conditions (a known trigger of autophagy). When mice were starved for the entire 48-h period after the operation or for 24 h before sampling, numerous GFP-LC3 puncta were detected in sham-operated muscles; however, there were only a few puncta in denervated muscles (Fig. 1A). Although normal levels of starvation-induced autophagy were observed in denervated muscles at 24 h after denervation, autophagy became suppressed even under starvation conditions from the 2nd day post-denervation (Fig. 1A). Autophagy was also suppressed in denervated extensor digitorum longus (EDL) muscles (representative of fast twitch muscles) and soleus (SOL) muscles (representative of slow twitch muscles).

**FIGURE 1.** Muscle autophagy is suppressed by denervation. A, representative images of GFP-LC3 puncta observed in gastrocnemius muscles of GFP-LC3 transgenic mice after denervation (n = 4). Mice were fed (Fed) for 24 or 48 h, starved (Stv) for 48 h, fed for 24 h, and then starved for another 24 h or starved for 24 h after surgery. Scale bar, 10 μm. Ope, time of operation. B, representative images of GFP-LC3 puncta observed in GAS, EDL, and SOL muscles of GFP-LC3 transgenic mice after denervation (n = 5). Mice were fed (Fed) for 24 h and starved (Stv) for another 24 h after surgery. Scale bar, 10 μm. Ope, time of operation. C, representative images of GFP-LC3 dots observed in gastrocnemius muscles of GFP-LC3 transgenic mice 3, 7, 14, 21, and 28 days after denervation. Mice were starved for 24 h before sampling to induce autophagy. Scale bar, 10 μm. POD, post-operative day.
muscles) (Fig. 1B). The suppression of autophagy lasted at least until 1 month after denervation (Fig. 1C). These results suggest that autophagy is generally suppressed in denervated muscles.

A decrease in the number of the GFP-LC3 puncta in denervated muscles might represent rapid turnover of autophagosomes. To rule out this possibility, we measured the autophagic flux using the microtubule-depolarizing agent colchicine to inhibit consumption of autophagosomes (7). Administration of colchicine increased the number of GFP-LC3 puncta in sham-operated muscles but not in denervated muscles (Fig. 2). This result suggests that the autophagic flux is indeed suppressed in denervated muscles.
Upon autophagy induction, LC3-I (cytosolic form) is converted to LC3-II (membrane-bound form) as a result of conjugation with phosphatidylethanolamine (21, 24). Denervation caused accumulation of LC3-I under starvation conditions, although the levels of LC3-II were not significantly changed in GAS and EDL muscles (Fig. 3, A and B, and supplemental Fig. S1). In SOL muscles, denervation increased the amount of both LC3-I and II (Fig. 3B and supplemental Fig. S1B), which may reflect transcriptional up-regulation of LC3 (5, 6). The ratio of LC3-II to LC3-I was lower in denervated muscles, suggesting a reduction in the conversion of LC3-I to LC3-II. These results are consistent with the fluorescence microscopy data showing that muscle autophagy is suppressed by sciatic nerve transection.

Denervation Induces Constitutive mTORC1 Activation in Muscle—Autophagy can be regulated by a number of factors. Because mTORC1 is a major suppressor of autophagy (25, 26), we investigated whether mTORC1 activity was modulated by denervation. The activity of mTORC1 was assessed by phosphorylation levels of its substrate, S6 kinase 1 (S6K1) (27, 28). Under conditions of feeding ad libitum, phosphorylation of S6K1 and S6 were detected at basal levels and further increased in denervated GAS muscles (Fig. 3A). After starvation for 24 h, S6K1 and S6 were dephosphorylated almost completely in sham controls but were still phosphorylated in denervated GAS muscles (Fig. 3A). The phosphorylation level of eIF4E-binding protein 1 (4E-BP1), another mTORC1 substrate, was also increased in denervated muscles, although the difference was less apparent before starvation.

We further investigated the activity of mTORC1 in EDL and SOL muscles. Under starvation conditions, 4E-BP1 and S6 were phosphorylated in denervated EDL and SOL muscles (more prominent in fast twitch EDL muscles) (Fig. 3B). Moreover, in accord with long term observation of the suppression of autophagy, we observed mTORC1 activation in the muscle at least for 1 month post-denervation (Fig. 3C). These results suggest that mTORC1 is constitutively activated after denervation.
which could be the reason why autophagy is suppressed in denervated muscles.

**Denervation-induced mTORC1 Activation Is Proteasome-dependent**—We next addressed the mechanism underlying denervation-induced mTORC1 activation. mTORC1 can be activated by amino acids and growth factors (27–29). Because denervation up-regulates ubiquitin-proteasome-mediated muscle proteolysis (3, 4), which can generate high levels of amino acids (30), we hypothesized that mTORC1 could be activated by a proteasome-dependent release of amino acids in denervated muscles.

We verified this hypothesis by *in vivo* administration of bortezomib, a proteasome inhibitor (31). Intravenous injection of bortezomib decreased the chymotrypsin-like activity of the muscle proteasome by up to 90% (Fig. 4A) and effectively increased the accumulation of ubiquitin-conjugated proteins in muscle 2.4-fold (supplemental Fig. S2). Because bortezomib treatment also slightly reduced the amount of food intake, we starved mice in both the bortezomib-treated and -untreated groups for 24 h before sampling to avoid differences in the nutritional conditions between the two groups. In this setting, phosphorylation of 4E-BP1, S6K1, and S6, which were increased by denervation in saline-treated mice, were profoundly suppressed in bortezomib-treated mice (Fig. 4B). We found virtually no differences in the phosphorylation levels between denervated and sham-operated muscles in bortezomib-treated mice. This result suggests that proteasome activity is required for the activation of mTORC1 in muscle after denervation. It was previously shown that REDD1, an mTORC1 repressor, has a very short half-life (~5 min) (32), but we did not observe a significant change in the REDD1 level as a result of bortezomib treatment also slightly reduced the amount of food intake,3 we starved mice in both the bortezomib-treated and -untreated groups for 24 h before sampling to avoid differences in the nutritional conditions between the two groups. In this setting, phosphorylation of 4E-BP1, S6K1, and S6, which were increased by denervation in saline-treated mice, were profoundly suppressed in bortezomib-treated mice (Fig. 4B). We found virtually no differences in the phosphorylation levels between denervated and sham-operated muscles in bortezomib-treated mice. This result suggests that proteasome activity is required for the activation of mTORC1 in muscle after denervation. It was previously shown that REDD1, an mTORC1 repressor, has a very short half-life (~5 min) (32), but we did not observe a significant change in the REDD1 level as a result of bortezomib treatment (31).

Accordingly, administration of bortezomib to GFP-LC3 transgenic mice restored the number of GFP-LC3 puncta in denervated muscles up to a level comparable with that of sham control mice (Fig. 4C). Bortezomib also diminished the differences in the levels of LC3 between sham-operated and denervated muscles (Fig. 4B). Thus, denervation-induced mTORC1 activation and autophagy suppression are proteasome-dependent.

**Intracellular Amino Acids Levels Increase in Denervated Muscles**—Two days after sciatic nerve transection, intramuscular concentrations of leucine and glutamate were increased by ~25 and ~100%, respectively, in mice that were fed (Fig. 5A and supplemental Fig. S3A). Levels of some amino acids (e.g., arginine, histidine, lysine, and glutamine) were decreased by an unknown reason. These results are generally consistent with previously reported observations (33, 34).

To verify the role of the proteasome in amino acid production, we measured intramuscular amino acid levels with or without bortezomib treatment. Because of the aforementioned reason, we could only test the effect of bortezomib under starved conditions. We still observed an 80% increase in glutamate in denervated muscles when the mice were starved for 24 h from post-operative day 1 (Fig. 5B). The concentrations of branched-chain amino acids (BCAAs) such as leucine, isoleucine, and valine were slightly increased by denervation although the changes did not reach statistical significance (Fig. 5B and supplemental Fig. S3B). In bortezomib-treated mice, the levels of these amino acids were reduced on both sham-operated and denervated sides, and the difference between them was no longer significant (Fig. 5B and supplemental Fig. S3B). These data suggest that the proteasome is the cause of the difference in amino acid concentrations between innervated and denervated muscles.

As leucine is a well known stimulator of mTORC1 (27–29, 35), intramuscular elevation of leucine may be a trigger for mTORC1 activation in denervated muscles. In addition, glutamate might stimulate mTORC1. When differentiated C2C12 myotubes were subjected to serum and amino acid starvation for 3 h and stimulated with 4× physiological concentrations of glutamate (0.44 mM), leucine (0.6 mM), and alanine (1.64 mM) for 30 min, Glu, growing condition; Svr, starved condition. Tissue amino acid concentrations are expressed as nanomoles/mg of wet weight. Data are mean ± S.E. *p < 0.05; n.s., not significant; S, sham control; D, denervation (A and B).

**mTORC1 Activation by Denervation**

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**FIGURE 5. Amino acids derived from proteasome-mediated proteolysis activate mTORC1 in denervated muscles.** A, intramuscular concentration of BCAAs, leucine, and glutamate in sham-operated and denervated gastrocnemius muscles under fed conditions at 48 h after surgery. BCAAs indicate the sum of the concentrations of Leu, Ile, and Val. B, intramuscular concentration of BCAAs and glutamate in the medial half of gastrocnemius muscles of starved mice used in Fig. 3B. C, immunoblot analysis showing the effect of glutamate addition on mTORC1 activity. C2C12 myotubes were subjected to serum and amino acid starvation for 3 h and stimulated with 4× physiological concentrations of glutamate (Glu, 0.44 mM), leucine (Leu, 0.6 mM), and alanine (Ala, 1.64 mM) for 30 min. Gw, growing condition; Svr, starved condition. Tissue amino acid concentrations are expressed as nanomoles/mg of wet weight. Data are mean ± S.E. n = 3 mice. *, *p < 0.05; n.s., not significant; S, sham control; D, denervation (A and B).

3 P. N. Quy and N. Mizushima, unpublished observations.
22). Using this method, we revealed that denervation induced an increase of ~40% in the amount of puromycin-labeled peptides (Fig. 6A). Daily injection of rapamycin inhibited puromycin incorporation in both innervated and denervated muscles, but the effect was larger in denervated muscles (Fig. 6A), suggesting that protein synthesis was increased in denervated muscles at least partially in an mTORC1-dependent manner.

Administration of rapamycin accelerated the atrophy process in denervated muscles. Seven days after denervation, muscle cell size in the vehicle-treated group was decreased by ~20%. By contrast, daily injection of rapamycin resulted in a greater decrease in muscle fiber size up to ~40%, suggesting that activated mTORC1 has an anabolic role against denervation-induced muscle atrophy (Fig. 6B).

mTORC1 activation can also stimulate ribosome biogenesis through translation of ribosome proteins and transcription of rRNA genes (27, 36). Indeed, it has been reported that transcripts of ribosomal subunits are increased in denervated muscles (2, 37). We confirmed that the amount of ribosomal proteins S6 and L7, members of the 40 S and 60 S ribosomal subunit, were increased by 80 and 50%, respectively, in denervated muscles at 2 days post-operation (Fig. 7, A and B). Real time PCR analysis showed that denervation up-regulated the relative amounts of Rps6 and Rpl7 transcripts by 2- and 2.5-fold, respectively (Fig. 7C). Rapamycin treatment reduced the increase in both protein (Fig. 7B) and mRNA (Fig. 7C) levels of these ribosomal proteins in denervated muscles, suggesting that the activation of mTORC1 increases the expression of ribosomal proteins, probably at both transcriptional and translational steps. Taken together, these data suggest that mTORC1 activation contributes to adaptive metabolic changes in denervated muscles.

**DISCUSSION**

Proteasome-derived Amino Acids Activate mTORC1 and Suppress Autophagy in Denervated Muscles—In this study, we show that autophagy is suppressed in denervated muscles due to constitutive activation of mTORC1 (Fig. 8). This suggests that autophagy has almost no role in the atrophic process following denervation. This is consistent with a previous report that genetic ablation of muscle autophagy does not protect muscle atrophy (8). These authors even observed that autophagy suppression causes more severe atrophy (8). It may be caused by impaired cellular function due to constitutive blockade of intracellular quality control by basal autophagy.

It has been suggested that phosphorylation levels of S6K1 and 4E-BP1, which are indicators of mTORC1 activity, are up-regulated in denervated hindlimb and diaphragm muscles, although the mechanism of this has not been investigated (12–14). From our findings, we propose that the release of amino acids produced by proteasomal proteolysis is the major cause of
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The constitutive mTORC1 activation (Fig. 8). This positive effect on mTORC1 is very strong, even canceling the negative impact of starvation. Activation of mTORC1 and suppression of autophagy are not observed 1 day after denervation (Figs. 1 and 3). This result suggests that it takes ~2 days to accumulate intracellular amino acids to a sufficiently high level. We do not completely rule out the possibility that proteasome inhibition suppresses mTORC1 via other pathways. For example, bortezomib may cause accumulation of proteasomal substrate proteins with short half-lives. However, we do not observe such an effect at least on the typical mTORC1 repressor REDD1 (Fig. 4B). Finally, the two inhibitors we used, rapamycin and bortezomib, may not be strictly specific to mTORC1 and the proteasome; these results may need to be confirmed in the future using genetic techniques.

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In line with the findings of previous studies, we observed that intramuscular levels of all amino acids were not equally elevated; levels of glutamate and BCAAs tended to increase in denervated muscles (Fig. 5) (33, 34). The elevation of glutamate in denervated muscle might reflect the increase in BCAAs, because BCAAs are extensively catabolized into glutamate and α-keto acids by branched-chain aminotransferase, which is active in skeletal muscle (38–40). It is well known that mTORC1 can be activated by leucine, which may also be the case in denervated muscles. Alternatively, the levels of glutamate, which are considerably increased after denervation, may contribute to mTORC1 activation directly, as suggested above. There may be another indirect mechanism. For example, we observed in denervated muscles a marked increase in the mRNA level of SLC7A5,3 which is an antiporter that exchanges intracellular glutamine and extracellular leucine/essential amino acids and can therefore activate mTORC1 (41). Furthermore, we observed a denervation-induced increase in the level of glutamine synthetase,3 which is the only specific enzyme capable of glutamine synthesis in muscle (42). Based on these data, the increase in glutamate may eventually lead to import of leucine from the extracellular space and mTORC1 activation even under starvation conditions.

mTORC1 Activation Induces Anabolic Responses in DenervatedMuscles—It appears that denervation-induced atrophy is not a simple catabolic process. Consistent with mTORC1 activation, we observed that protein synthesis is up-regulated in denervated muscles using the recently developed SUnSET method; this finding supports previously published observations using radioisotope-labeled amino acids (10, 11). Furthermore, it has been shown that denervation increases the mRNA expression of a wide range of genes involved in protein translation such as ribosomal subunits and translation initiation and elongation factors (2, 37, 43). Thus, our study confirms that denervated muscles show both catabolic and anabolic features.

The increased protein synthesis can be at least partially suppressed by rapamycin treatment, suggesting that mTORC1
activation contributes to the anabolic process in denervated muscles. This could be an adaptive response to denervation because rapamycin treatment causes more severe atrophy in denervated muscles compared with innervated muscles (Figs. 6 and 8). However, how mTORC1-dependent protein synthesis produces a beneficial effect in denervated muscle remains unclear. mTORC1 is important for ribosome biogenesis at both transcriptional and translational steps (27, 36). In this study, we have shown that the increase in expression of ribosome sub-units depends on mTORC1 (Fig. 7). Ribosome biogenesis may generally support the anabolic responses of denervated muscles. Furthermore, a recent proteomic analysis of denervated tibialis anterior muscles showed that more than 200 proteins are up-regulated at certain time points after denervation (44). These proteins include many metabolic enzymes, structural proteins, and molecular chaperones as well as ribosomal proteins. mTORC1 activation may also contribute to the synthesis of these proteins.

In summary, we found that after denervation, autophagy is suppressed by constitutive activation of mTORC1, which is caused by amino acids derived from proteasomal proteolysis. mTORC1 activation might be an adaptive response to denervation to aid protein synthesis and ribosome biogenesis.

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