Molecular Mechanisms of Skeletal Muscle Atrophy in a Mouse Model of Cerebral Ischemia

Marine Maud Desgeorges, PhD; Xavier Devillard, PhD; Jérôme Toutain, MSc; Didier Divoux, MSc; Josiane Castells, BSc; Myriam Bernaudin, PhD; Omar Touzani, PhD; Damien Gilles Freyssenet, PhD

Background and Purpose—Loss of muscle mass and function is a severe complication in patients with stroke that contributes to promoting physical inactivity and disability. The deleterious consequences of skeletal muscle mass loss underline the necessity to identify the molecular mechanisms involved in skeletal muscle atrophy after cerebral ischemia.

Methods—Transient focal cerebral ischemia (60 minutes) was induced by occlusion of the right middle cerebral artery in C57BL/6J male mice. Skeletal muscles were removed 3 days later and analyzed for the regulation of critical determinants of muscle mass homeostasis (Akt/mammalian target of rapamycin pathway, myostatin-Smad2/3 and bone morphogenetic protein-Smad1/5/8 signaling pathways, ubiquitin-proteasome and autophagy-lysosome proteolytic pathways).

Results—Cerebral ischemia induced severe sensorimotor deficits associated with muscle mass loss of the paretic limbs. Mechanistically, cerebral ischemia repressed Akt/mammalian target of rapamycin pathway and increased expression of key players of ubiquitin-proteasome pathway (MuRF1 [muscle RING finger-1], MAFbx [muscle atrophy F-box], Musa1 [muscle ubiquitin ligase of SCF complex in atrophy-1]), together with a marked increase in myostatin expression, in both paretic and nonparetic skeletal muscles. The Smad1/5/8 pathway was also activated.

Conclusions—Our data fit with a model in which a repression of Akt/mammalian target of rapamycin pathway and an increase in the expression of key players of ubiquitin-proteasome pathway are critically involved in skeletal muscle atrophy after cerebral ischemia. Cerebral ischemia also caused an activation of bone morphogenetic protein-Smad1/5/8 signaling pathway, suggesting that compensatory mechanisms are also concomitantly activated to limit the extent of skeletal muscle atrophy. (Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.114.008574)

Key Words: atrogenes ▪ bone morphogenetic protein ▪ myostatin ▪ proteolysis ▪ Smad proteins ▪ stroke

Loss of skeletal muscle mass and function is a severe complication in patients with stroke. Skeletal muscle atrophy occurs primarily in the paretic limb, but also to a lesser extent in the nonparetic limb.1 Accordingly, cross-sectional area of type II muscle fibers2,3 or both type I and type II muscle fibers4,5 is reduced in patients with stroke. One major consequence of skeletal muscle atrophy is a decrease in muscle strength and a critical deterioration of muscle function.6–8 All these factors promote physical inactivity and disability after stroke, leading to prolonged hospitalization, prolonged weakness, and less efficient rehabilitation.

Evidence from clinical trials indicate that loss of skeletal muscle mass after stroke is because of the brain lesion and the subsequent alterations in nerve impulse from upper to lower motor neurons.9,10 However, muscle atrophy is also attributable to nutritional deficits, commonly observed in patients with stroke,11 and disuse atrophy because of extended phase of bed rest.12 Moreover, a systemic inflammatory response is observed after stroke,13 as well as the production of cytokines (tumor necrosis factor-α and myostatin) by skeletal muscle,14,15 indicating that both systemic and local inflammatory responses could also contribute to altering muscle homeostasis and promoting disability after stroke.

The harmful consequences of skeletal muscle loss after cerebral ischemia in patients with stroke underline the necessity to identify the intracellular mechanisms triggered by cerebral ischemia in skeletal muscle. We therefore determined the molecular mechanisms involved in the regulation of skeletal muscle mass in paretic and nonparetic limbs of mice.

Materials and Methods

Animals and Middle Cerebral Artery Occlusion

The protocol has been approved by the regional committee on animal ethics (CENOMEXA) according to the guidelines of the European Community Council for the Ethical Treatment of Animals (86/609/ EEC). Animals were housed at the Central Animal Care Facility of...
Caen University (France). Under isoflurane (1.5% in N2O/O2) anesthesia, transient focal cerebral ischemia (60 minutes) was induced by intraluminal right middle cerebral artery occlusion (MCAO), in 13-week-old C57BL/6J male mice (n=8; Janvier, Le Genest Saint-Isle, France). Sham C57BL/6J male mice (n=8) were subjected to the same procedure, but the occlusive monofilament was immediately withdrawn. Shortly after surgery and daily, the animals received 1 mL of physiological saline to avoid dehydration. All experiments were performed randomly and analyzed in a blind manner.

Magnetic Resonance Imaging Analyses and Behavioral Tests
T2-weighted brain magnetic resonance images were acquired (echo time/repetition time, 46 ms/5000 ms; number of excitation, 6; field of view, 20 mm²; matrix, 256x192; 15 slices; thickness, 0.75 mm) 2 days after MCAO using a 7-T Pharmscan (Bruker, Ettlingen, Germany). The delineated lesion areas were summed and multiplied by the slice thickness to determine the infarct volume.

Hind-paw footprints were analyzed before and 2 days after surgery. Coordination was analyzed (Rotarod test) before, 2 and 3 days after MCAO. The maximal peak force of both forelimb and hindlimb was assessed (grip test) before and 3 days after surgery.

Immunohistomorphometry
Tibialis anterior muscles were cut (12 μm) in a cryostat (Leica CM 1950). Transverse sections were fixed in 4% paraformaldehyde and incubated with antialaminin (1:200; Sigma-Aldrich, Saint-Quentin Fallavier, France). Fluorescent muscle fibers were visualized with a Leica TCS-SP2 confocal scanning laser inverted microscope (Leica-Microsystem, Heidelberg, Germany). Nuclei were counterstained with 4',6-diamidino-2-phenylindole. Approximately 200 muscle fibers per mouse were randomly selected and used for the quantification of cross-sectional area (Image J software, http://rsb.info.nih.gov/ij/).

RNA Isolation, cDNA Synthesis, and Real-Time Polymerase Chain Reaction
Total RNA extraction, synthesis of cDNA, and real-time quantitative polymerase chain reaction was performed as previously described. The selected forward and reverse primer sequences are listed in Table I in the online-only Data Supplement. Peptidylpropyl isomerase A and hypoxanthine-guanine phosphoribosyltransferase were used as reference genes.

Protein Extraction and Immunoblotting
Protein extraction from quadriceps muscles and immunoblot analysis were performed as previously described. Primary antibodies are listed in Table II in the online-only Data Supplement. α-Tubulin immunoblots were used to check for equal protein loading between samples.

Statistical Analysis
All values are expressed as mean±SEM. Statistical analyses were performed using GraphPad PRISM 5.0 (GraphPad Software). Data were tested for normal distribution using Shapiro–Wilks test. Data with normal distribution were analyzed with ANOVA followed by Tukey test. Data without normal distribution were analyzed by Kruskal–Wallis and Dunn tests. Behavioral tests were analyzed by repeated measures ANOVA. All remaining variables were compared using Mann–Whitney and Wilcoxon tests. The α-level of significance was set at 0.05.

Results
Cerebral Ischemia Induces Atrophy of Paretic Skeletal Muscles
MCAO induced a brain lesion that affected the lateral striatum and parietal cortex (Figure 1A). The whole brain lesion averaged 111±27 mm³ 3 days after cerebral ischemia. Mice subjected to MCAO could hardly move (Figure 1B). Accordingly, neurobehavioral scores (Figure I in the online-only Data Supplement), motor coordination (Figure 1C), and muscle strength (Figure 1D) were significantly impaired.

Surgery led to a decrease in body weight of Sham and MCAO animals (Figure 2A). Cerebral ischemia induced atrophy of quadriceps, soleus, and tibialis anterior muscles of the paretic side (Figure 2B; Figure IIA in the online-only Data Supplement), whereas weight of nonparetic muscles remained unchanged. Accordingly, tibialis anterior muscle fiber cross-sectional area was only significantly decreased in paretic muscles (Figure 2C and 2D; Figure IIB in the online-only Data Supplement).

Cerebral Ischemia Inhibits Akt/Mammalian Target of Rapamycin Pathway and Increases Expression of Critical Players of Ubiquitin-Proteasome Pathways in Paretic and Nonparetic Muscles
Muscle atrophy may originate from a decrease in protein synthesis and increase in protein degradation. As shown in Figure 3, cerebral ischemia downregulated the Akt/mammalian target of rapamycin pathway, a crucial regulator of skeletal muscle hypertrophy, as evidenced by the decrease in phosphorylation level of Akt, rpS6, and 4E-BP1 (Figure 2C and 2D), in both paretic and nonparetic muscles. Total protein level of Akt, rpS6, and 4E-BP1 remained unchanged.

Expression of muscle-specific E3 ubiquitin ligases, muscle RING finger-1 (MuRF) and Atrogin1/muscle atrophy F-box (MAFbx), TRAF6 (tumor necrosis factor receptor-associ- related factor 6), and muscle ubiquitin ligase of SCF complex in atrophy-1 (Musa1) were all significantly increased in paretic and nonparetic muscles of MCAO mice (Figure 3C). Histone deacetylase 4 (HDAC4) is a critical determinant of neurogenic atrophy that upregulates the expression of MuRF1 and MAFbx/atrogin-1 through an increased expression and transcriptional activity of myogenin. Histone deacetylase 4 protein level remained unchanged, whereas myogenin mRNA level was dramatically decreased in both paretic and nonparetic muscles (Figure 3D).

Autophagy plays a critical role for myofiber maintenance. Phosphorylation of Ulk1, the target residue of mammalian target of rapamycin that prevents Ulk1 activation, was markedly decreased after cerebral ischemia (Figure 4A). The increase in the mRNA level of autophagy-related genes, Ulk1, LC3, and cathepsin L, in paretic and nonparetic limbs, did not match a corresponding increase in protein content (Figure 4B and 4C; Figure III in the online-only Data Supplement). Atg5, Atg4b, and cathepsin B mRNA level, as well as the protein content of Atg13 and Atg5–Atg12 protein complex, remained unchanged (Figure 4B and 4C and Figure IIIA in the online-only Data Supplement).
Expression of Neuromuscular Junction Proteins After Cerebral Ischemia

To further delineate the mechanisms potentially involved in the atrophy of paretic muscles, we hypothesized that cerebral ischemia could alter the expression of neuromuscular junction proteins. However, mRNA levels of α- (Chrna1) and δ-subunits (Chrnd) of acetylcholine receptor remained unchanged after cerebral ischemia (Figure 5A). Similarly, MusK mRNA level and rapsyn protein content, required for the maintenance of synaptic structure and aggregation of postsynaptic acetylcholine receptors, respectively, remained unchanged (Figure 5A and 5B).

Figure 1. A, Infarction of the right hemisphere 48 hours after middle cerebral artery occlusion (MCAO) and quantification of cerebral infarct volume. B, Foot print analysis. C, Evolution of performances in the Rotarod test. D, Specific muscle strength. Data are mean±SE (n=7–8 per group).  *P<0.01: significantly different from MCAO D-1. aP<0.01 and bP<0.001: significantly different from Sham at the same time point.

Figure 2. A, Body weight in response to cerebral ischemia. B, Quadriceps and Tibialis anterior muscle weight. C, Representative sections immunostained with laminin and muscle fiber cross-sectional area (D) of Sham, nonparetic (middle cerebral artery [MCAO] NP) and paretic (MCAO P) tibialis anterior muscles 3 days after surgery. Scale bar, 100 μm. Data are mean±SE (n=8 per group).  *P<0.001: significantly different from corresponding group at D-1. *P<0.05: significantly different from NP muscle.
Cerebral Ischemia Increases Myostatin mRNA Level and Upregulates BMP Signaling Pathway

A local inflammatory response could promote atrophy of paretic skeletal muscle. The analysis of interleukin-1β, interleukin-6, interferon-γ, and tumor necrosis factor-α protein content in skeletal muscle did not show any significant change after cerebral ischemia (Figure IV A in the online-only Data Supplement). However, we cannot exclude the possibility that the inherent variance of such measurements may have masked some minor changes. Serum tumor necrosis factor-α concentration also remained unchanged (Figure IVB in the online-only Data Supplement).

Myostatin is a negative regulator of skeletal muscle mass that signals through the activin type 2 receptors (Acvr2a and Acvr2b) and activin type 1 receptors (Acvr1b/Alk4 and Tgfr1/Alk5) to phosphorylate Smad2 and Smad3 proteins. Although transcript levels of Acvr2b and Alk5 remained unchanged, myostatin and Alk4 mRNA levels were markedly increased in both paretic and nonparetic muscles (Figure 6A). Surprisingly, Smad2/3 phosphorylation was decreased in skeletal muscles of MCAO mice (Figure 6B).

BMP-Smad1/5/8 signaling pathway is an important positive regulator of skeletal muscle mass that counters the myostatin-Smad2/3 pathway. BMPs bind to dedicated BMP receptors (Alk3, Alk6) that in turn phosphorylate Smad1/5/8 proteins. Although cerebral ischemia did not alter the expression of BMP1 and BMP receptors (Bmpr2 and Bmpr1a;
Figure 6C). Smad1/5/8 phosphorylation was markedly upregulated in muscles of MCAO mice (Figure 6D). Protein content of Smad4, the regulatory Smad, that forms a transcriptionally active complex with Smad2/3 or Smad1/5/8, remained unchanged (Figure V in the online-only Data Supplement). Skeletal muscle atrophy was observed 3 days after cerebral ischemia in paretic limb, whereas muscle mass remained unchanged in nonparetic limb. Previous studies indicate that muscle atrophy is consistently observed both in paretic and nonparetic limbs later after cerebral ischemia (7 days). Therefore, atrophy of paretic skeletal muscles could be an early event that will be followed by the atrophy of nonparetic muscles. Importantly, muscle mass is restored within 21 days in mouse models of stroke, which indicates that muscle atrophy is also a transient event that is rapidly followed by the stimulation of recovery processes. This markedly contrasts with reports in human studies where muscle mass loss is observed several months after the onset of stroke (>6 months).1

Mechanistically, skeletal muscle atrophy involved a downregulation of Akt/mammalian target of rapamycin pathway and an increase in the expression of E3-ubiquitin ligases MuRF1, MAFbx/Atrogin1, and Musa1. Importantly, these molecular adaptations were also observed in the nonparetic muscle. Whether these molecular events in nonparetic muscles will trigger atrophy later is currently unknown, but this is strongly suggested by the observation that muscle atrophy of both limbs is consistently found 7 days after cerebral ischemia. Therefore, atrophy of paretic skeletal muscles could be an early event that will be followed by the atrophy of nonparetic muscles. Importantly, muscle mass is restored within 21 days in mouse models of stroke, which indicates that muscle atrophy is also a transient event that is rapidly followed by the stimulation of recovery processes. This markedly contrasts with reports in human studies where muscle mass loss is observed several months after the onset of stroke (>6 months).1

Mechanistically, skeletal muscle atrophy involved a downregulation of Akt/mammalian target of rapamycin pathway and an increase in the expression of E3-ubiquitin ligases MuRF1, MAFbx/Atrogin1, and Musa1. Importantly, these molecular adaptations were also observed in the nonparetic muscle. Whether these molecular events in nonparetic muscles will trigger atrophy later is currently unknown, but this is strongly suggested by the observation that muscle atrophy of both limbs is consistently found 7 days after cerebral ischemia. Therefore, atrophy of paretic skeletal muscles could be an early event that will be followed by the atrophy of nonparetic muscles. Importantly, muscle mass is restored within 21 days in mouse models of stroke, which indicates that muscle atrophy is also a transient event that is rapidly followed by the stimulation of recovery processes. This markedly contrasts with reports in human studies where muscle mass loss is observed several months after the onset of stroke (>6 months).1

Autophagy plays a critical role for myofiber maintenance and its activation is necessary to avoid the accumulation of dysfunctional organelles and toxic proteins that would lead to muscle atrophy and weakness. von Walden et al recently reported that cerebral ischemia was associated with a decreased expression of several autophagy-related genes in human, suggesting that a defect in autophagy could impair muscle fiber maintenance. Although we cannot preclude that...
the reported increase in mRNA level will be sustained later by an increase in protein level, our data do not provide any conclusive evidence of an alteration in autophagy-lysosome pathway 3 days after cerebral ischemia.

The observation that muscle mass loss is only found in paretic muscles strongly suggests the existence of specific mechanisms that may accelerate the onset of atrophy in paretic muscles. Atrophy of paretic skeletal muscles could thus be attributed to the alteration in nerve impulse from upper to lower motor neurons.2,39 Furthermore, muscle denervation is well known to induce skeletal muscle atrophy40,41 and to alter the expression of neuromuscular junction proteins.42 However, our data showed no change in the expression of neuromuscular junction proteins.42 Similarly, expression of histone deacetylase 4 and myogenin, critical determinants of neurogenic atrophy,24,43 remained unchanged (histone deacetylase 4) or even decreased (myogenin) after cerebral ischemia. Further studies are essential to determine the molecular mechanisms involved in the alteration of nerve impulse transmission in the acute phase of stroke.

Other factors may also contribute to trigger molecular adaptations in skeletal muscle including disuse, malnutrition, and inflammation. Springer et al14 recently assayed locomotor activity in response to cerebral ischemia and reported a significant increase in locomotor movement 3 days after MCAO, suggesting that disuse may not be a major factor involved in skeletal muscle atrophy. In the same study, a transient decrease in food intake was also reported, whereas Sham animals did not experience malnutrition. Therefore, stroke-associated malnutrition could contribute to muscle loss. However, Choe et al35 reported that MCAO induced a greater muscle mass loss compared with Sham pair-fed rats, indicating that cerebral ischemia exerts hypophagia-independent effect on muscle mass loss.

Myostatin is a master negative regulator of skeletal muscle mass.44 In agreement with studies in mouse34 and human,15 mRNA level of myostatin and Acvr1b (Alk4) were increased by ≈2-fold in skeletal muscles of both paretic and nonparetic limbs, clearly identifying myostatin as a critical regulator of skeletal muscle mass after stroke and a potential target for a therapeutic intervention aimed at limiting muscle mass loss after stroke. Surprisingly, Smad2/3 phosphorylation was also markedly decreased in both paretic and nonparetic muscles. However, extensive activation of myostatin signaling has been shown to trigger the expression of Smad7,45 which in turn represses Smad2/3 phosphorylation.46 Such a mechanism could thus limit the extent of skeletal muscle atrophy and increase the recovery process after cerebral ischemia.
Activation of BMP signaling prevents excessive muscle mass loss in response to denervation and fasting. Accordingly, this pathway was activated in response to cerebral ischemia. The activation of BMP signaling pathway as a compensatory mechanism to limit the extent of muscle mass loss is also in agreement with the observation that complete recovery of skeletal muscle mass is observed 21 days after loss is also in agreement with the observation that complete compensatory mechanism to limit the extent of muscle mass atrophy after stroke through targeted therapeutic interventions is an important outcome to restore physical capacity and mobility, thus ultimately improving poststroke recovery and patients’ life quality.

In conclusion, our study identified for the first time that Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat Cell Biol. 2001;3:1014–1019. doi: 10.1038/ncb101-1014.

References


Molecular Mechanisms of Skeletal Muscle Atrophy in a Mouse Model of Cerebral Ischemia

Marine Maud Desgeorges, Xavier Devillard, Jérome Toutain, Didier Divoux, Josiane Castells, Myriam Bernaudin, Omar Touzani and Damien Gilles Freyssenet

Stroke. published online May 7, 2015;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/early/2015/05/07/STROKEAHA.114.008574

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2015/05/08/STROKEAHA.114.008574.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Supplemental methods

Behavioral tests
Irwin screen. The evaluation of neurobehavioral and physiological state of mice (consciousness, alertness, muscle tone, posture and gait, motor control and coordination, seeking behavior, ptosis and stereotypies including head movements, chewing, and sniffing) was assessed according to the Irwin screen\(^1\) before surgery and every day after surgery.

Enzyme assay
Cathepsin B+L activities were fluorometrically measured (\(\lambda_{\text{exc}}=380\) nm and \(\lambda_{\text{em}}=460\) nm) by following the hydrolysis of specific fluorogenic substrates. The assay was started by the addition of 10 mM Z-Phe-Arg-AMC (Bachem, I-1160).

Luminex analysis
Interleukin (IL)-1\(\beta\), IL-6, Interferon-\(\gamma\) and TNF-\(\alpha\) were assessed on serum samples and skeletal muscle using a multiplex-assay (Luminex IS 100 system) as described by the manufacturer (Bio-Rad, Marnes-la-Coquette, France).
**Supplemental Table I**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Reference</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>4E-BP1&lt;sup&gt;Thr37/46&lt;/sup&gt;</td>
<td>Cell signaling, CS9459</td>
<td>1:500</td>
</tr>
<tr>
<td>4E-BP1</td>
<td>Cell signaling, CS9452</td>
<td>1:500</td>
</tr>
<tr>
<td>Akt&lt;sup&gt;Ser473&lt;/sup&gt;</td>
<td>Cell signaling, CS9271</td>
<td>1:500</td>
</tr>
<tr>
<td>Akt</td>
<td>Cell signaling, CS9272</td>
<td>1:500</td>
</tr>
<tr>
<td>AMPK&lt;sup&gt;Thr172&lt;/sup&gt;</td>
<td>Cell signaling, CS2531</td>
<td>1:500</td>
</tr>
<tr>
<td>AMPKα</td>
<td>Cell signaling, CS2532</td>
<td>1:500</td>
</tr>
<tr>
<td>Atg5-Atg12 conjugate</td>
<td>Sigma-Aldrich, A0856</td>
<td>1:800</td>
</tr>
<tr>
<td>Atg13</td>
<td>Sigma-Aldrich, SAB4200100</td>
<td>1:800</td>
</tr>
<tr>
<td>Foxo3&lt;sup&gt;Thr32&lt;/sup&gt;</td>
<td>Upstate, 07-695</td>
<td>1:500</td>
</tr>
<tr>
<td>LC3b</td>
<td>Sigma-Aldrich, L7543</td>
<td>1:800</td>
</tr>
<tr>
<td>rpS6</td>
<td>Cell Signaling, CS2217</td>
<td>1:1000</td>
</tr>
<tr>
<td>rpS6&lt;sup&gt;Ser235/236&lt;/sup&gt;</td>
<td>Cell Signaling, CS4856</td>
<td>1:1000</td>
</tr>
<tr>
<td>α-Tubulin</td>
<td>Sigma-Aldrich, T5168</td>
<td>1:1500</td>
</tr>
<tr>
<td>Ulk1&lt;sup&gt;Ser757&lt;/sup&gt;</td>
<td>Cell signaling, CS6888</td>
<td>1:500</td>
</tr>
<tr>
<td>Ulk1</td>
<td>Santa Cruz, SC33182</td>
<td>1:200</td>
</tr>
</tbody>
</table>

**Supplemental Table I.** Antibody used in the study.

Abbreviations: AMPK, AMP-activated protein kinase; Atg, Autophagy related gene; Foxo3, Forkhead box O3; LC3b/Map1lc3b, Microtubule-associated protein 1 light chain 3 beta; rpS6, Ribosomal protein s6; Ulk1, Unc-51 like kinase 1.
Supplemental Table II. Primers were designed using Primer 3 software from gene sequences obtained from Genebank. Primer specificity was determined using a BLAST search.

<table>
<thead>
<tr>
<th>gene</th>
<th>Forward primer sequence (5’-3’)</th>
<th>Reverse primer sequence (5’-3’)</th>
<th>Gene Bank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acvr1b</td>
<td>TGCTGAGCTTCCTGTGCGAT</td>
<td>GAGAAGCAACACGCACTCGA</td>
<td>NM_007395.3</td>
</tr>
<tr>
<td>Acvr2b</td>
<td>ATCAAGGAGTCATGGCTGAAC</td>
<td>GACTCTTTAGGGAGCAGGTGTC</td>
<td>NM_007397.2</td>
</tr>
<tr>
<td>Atg4b</td>
<td>ACAGATGATCCCTGGCCAGG</td>
<td>TAGACTGGCTCTGCGCCA</td>
<td>NM_174874.3</td>
</tr>
<tr>
<td>Atg5</td>
<td>TGAAAGATGTGCTTCTCTCTG</td>
<td>GCTCCACCTGAAACTGGTCG</td>
<td>NM_053069</td>
</tr>
<tr>
<td>Bmp1</td>
<td>GGACTCTGGGGAAGAAGACCTAT</td>
<td>ACCCTACATGTTGGGAACATG</td>
<td>NM_009755.3</td>
</tr>
<tr>
<td>Bmpr1a</td>
<td>GAAAGACCTGGATGACCCATGCC</td>
<td>CCCATACATCTTCTCCATACG</td>
<td>NM_009758.4</td>
</tr>
<tr>
<td>Bmpr2</td>
<td>TGGACATCAGCTAGGTGGAAGGT</td>
<td>TGAGAAAGAACAGCTGGCAATCC</td>
<td>NM_007561.4</td>
</tr>
<tr>
<td>Cat B</td>
<td>GAAGAAGCTGTTGTTGGCACTG</td>
<td>GTGTCGTGAGAAATGGGTCTC</td>
<td>NM_007798.3</td>
</tr>
<tr>
<td>Cat L</td>
<td>GTGACTGTCTCTACGCAGCAGA</td>
<td>TCCGCTCTTCGCTTGCAAGG</td>
<td>NM_009984.3</td>
</tr>
<tr>
<td>Chrna1</td>
<td>ACCTGCGACCTTTGAGGGCTCT</td>
<td>AGTACTGAGTGGGCTGCTGG</td>
<td>NM_007389.5</td>
</tr>
<tr>
<td>Chrmd</td>
<td>CTCGAGTTGATCTATCATGGAT</td>
<td>CCGCGGATGTAAGTGGAA</td>
<td>NM_021600.3</td>
</tr>
<tr>
<td>Foxo3</td>
<td>AGGATAAGGGCGAGCAGCA</td>
<td>CATTCTGAGGGCGCAGTAA</td>
<td>NM_019740.2</td>
</tr>
<tr>
<td>HPRT</td>
<td>CAGGCCGACCTTTGTTGGAT</td>
<td>TTGGCCTCTCATTTAAGGCTT</td>
<td>NM_013556.2</td>
</tr>
<tr>
<td>Lc3b</td>
<td>CACTGCTCTGTCTGTGTAGGTG</td>
<td>TCTGGTGCGGCCTTTTGATAGGCTG</td>
<td>NM_026160.4</td>
</tr>
<tr>
<td>Mafb</td>
<td>GTTTTCAGAGGCGCCAAGAAG</td>
<td>TTGGCCTCTCATTTAAGGCTT</td>
<td>NM_026346.2</td>
</tr>
<tr>
<td>Maff1</td>
<td>ACCCTGCTGCTGGAAGACAA</td>
<td>AGGAGAAGTACGATGGGCACTG</td>
<td>NM_00013904.2</td>
</tr>
<tr>
<td>Maff2</td>
<td>TCCGAGTTGATCTATCATGGAT</td>
<td>CCGCGGATGTAAGTGGAA</td>
<td>NM_00116829.3</td>
</tr>
<tr>
<td>Musk</td>
<td>CTTCAACGGGGCCACTGAAACCA</td>
<td>TGGTCTGAGGGCGCAGTAA</td>
<td>NM_010944</td>
</tr>
<tr>
<td>Myogenin</td>
<td>TGATGAGGATCTATCATCCCAAGG</td>
<td>CAGACATATGCTGCTGGCGG</td>
<td>NM_031189.2</td>
</tr>
<tr>
<td>Myostatin</td>
<td>AGCTGCGAGGAGGACGACAGG</td>
<td>ACATTTGCGCTCGCATCGC</td>
<td>NM_010834.2</td>
</tr>
<tr>
<td>Ppia</td>
<td>AGCATAACGAGCCGCTGCACTG</td>
<td>TCTCGCTGCTGCTGCACTG</td>
<td>NM_008097.1</td>
</tr>
<tr>
<td>Tgfb1</td>
<td>AGCTCCTGCTATCGGTGTGTG</td>
<td>GAGAGGTGGACGAGAAACCTGTA</td>
<td>NM_009370.2</td>
</tr>
<tr>
<td>Trarf6</td>
<td>TGGTGGAGCCACATACACA</td>
<td>CACATCGCTGTCAGCAGTGA</td>
<td>NM_009424.2</td>
</tr>
<tr>
<td>Ulk1</td>
<td>TCTGGTGCTATCTGGCTGCTG</td>
<td>TGAACAGAGCCGTGCAAA</td>
<td>NM_009469.3</td>
</tr>
</tbody>
</table>

Abbreviations: Acvr1b, activin A receptor, type 1B; Acvr2b, activin receptor 2B; Atg4b, autophagy related 4B cysteine peptidase; Atg5, Autophagy related 5; Bmp1, Bone morphogenetic protein 1; Bmpr1a, Bone morphogenetic protein receptor, type 1A; Bmpr2, Bone morphogenetic protein receptor, type II (serine/threonine kinase); Ctsb, Cathespin B; Ctsl Cathepsin L; Chrna1, Cholinergic receptor nicotinic alpha polypeptide 1; Chrmd, Cholinergic receptor nicotinic delta polypeptide; Fbxo30/Musa1, F-box protein 30; Fbxo32/MAFbx, F-box protein 32; Foxo3, Forkhead box O3; HPRT, Hypoxanthine guanine phosphoribosyl transferase; Map1lc3b/Lc3b, Microtubule-associated protein 1 light chain 3 beta; MusK, Muscle skeletal receptor tyrosine kinase; Ppia, Peptidylprolyl isomerase A; Tgfb1, Transforming growth factor beta receptor I; TRAF6, TNF receptor-associated factor 6; Trim63/Murf1, Tripartite motif-containing 63; Ulk1, Unc-51 like kinase 1.
Supplemental Figure I

Supplemental Figure I. Neurologic deficits were measured with the Irwin modified score. A normal neurological score = 8. Data are means ± SE (n=7-8/group). $^a$p<0.01: significantly different from MCAO at D-1. $^b$p<0.01 and $^c$p<0.001 significantly different from sham at the same time point.
Supplemental Figure II

**A**

Soleus muscle weight.

**B**

Frequency histograms of muscle fiber cross-sectional area. All analyses were performed in sham, nonparetic (MCAO NP) and paretic (MCAO P) soleus muscles 3 days after surgery. Data are means ± SE (n=8/group).

*P < 0.05 : significantly different from non paretic limb.
Supplemental Figure III

Supplemental Figure III. A, Transcript level of cathepsin B and cathepsin L. B, Cathepsin B+L enzyme activity. All analyses were performed in sham, nonparetic (MCAO NP) and paretic (MCAO P) quadriiceps muscles 3 days after surgery. Data are means ± SE (n=6-8/group). *P<0.05: significantly different from nonparetic limb.
Supplemental Figure VI

Supplemental Figure IV. A, IL-1-β, IL-6, IFN-γ and TNF-α concentration in sham, paretic (MCAO P) and nonparetic (MCAO NP) protein extracts. B, Serum TNF-α concentration in sham and MCAO animals. Data are means ± SE (n=4-8/group).
Supplemental Figure V

Supplemental Figure V. Representative immunoblot and quantification of Smad4 protein level. All analyses were performed in sham, nonparetic (MCAO NP) and paretic (MCAO P) quadriceps muscles 3 days after surgery. Data are means ± SE (n=6-8/group).
Supplemental references