Skeletal muscle adaptation in response to mechanical stress in p130cas$^{-/-}$ mice

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Skeletal muscle is constantly exposed to mechanical forces throughout its life. Many lines of investigation have shown that mechanical forces regulate muscle function and that a moderate amount of mechanical loading is essential for muscle homeostasis. The signals generated by mechanical forces are converted into molecular events that, in turn, regulate multiple anabolic and catabolic processes, including cell proliferation, organogenesis, repair, regeneration, adaptation, fatigue, and muscle pathologies (16, 30). For example, skeletal muscle hypertrophy in response to physical exercise, muscle atrophy with immobilization, and satellite cell proliferation following the mobilization of inflamed muscle all point to the mechanosensing by and mechanoresponsiveness of muscle cells (10, 19, 27). However, the mechanisms of action of the biomechanical signals that regulate gene expression during skeletal muscle adaptation in response to mechanical stress are not yet well understood.

The ability of cells to respond to mechanical cues is governed by mechanosensitive receptors or structures, called “mechanosensors,” that sense and convert mechanical signals into biochemical signaling events. Mechanical force can activate several putative mechanosensitive structures, including cytoskeletal proteins, stretch-activated ion channels, and G protein-coupled receptors. There is evidence that the unfolding of protein domains under tension mediates the responses to applied forces. The first-reported protein in the cytoplasmic region of integrin-mediated adhesions is the adaptor protein p130Cas (also known as Bcar1; Refs. 25, 26). Sawada et al. (24) demonstrated in an in vitro model that p130Cas acts as a mechanosensor, transducing mechanical extension into cellular signaling by priming the phosphorylation and activation of the p38 mitogen-activated protein kinase (MAPK) signaling pathway. However, the mechanosensor involved in this process has not been identified in skeletal muscle.

We have previously shown that enhanced mechanical stress, such as exercise, promotes the transcription of the peroxisome proliferator-activated receptor-γ coactivator-1α gene (Pgc-1α) and mitochondrial biogenesis in skeletal muscle through the activation of the p38 MAPK pathway (2). PGC-1α is a transcriptional coactivator, originally discovered through its interaction with peroxisome proliferator-activated receptor-γ in a yeast two-hybrid screen of a cDNA library from a differentiated brown fat cell line (21). To date, numerous studies have suggested that PGC-1α has a pivotal function in endurance exercise-induced skeletal muscle adaptation (5, 13, 18). Previous studies have also reported that PGC-1α possibly attenuates muscle atrophy (8, 23).

In this study, we used a loss-of-function molecular genetic approach with a physiological model of mechanical stress in mice to delineate the functional role of p130Cas in mechanical-stress-induced skeletal muscle adaptation. Because p130Cas is expressed in skeletal muscle and is activated after exercise, we hypothesized that the activation of p130Cas promotes mechanical-stress-induced skeletal muscle adaptation through its transcriptional control of the Pgc-1α gene via p38 MAPK.

MATERIALS AND METHODS

Animal experiments. All mice were housed in temperature-controlled quarters (21°C) on a 12:12-h light-dark cycle and were provided with water and food ad libitum. To determine the effects of increased mechanical stress on skeletal muscle, the mice were housed individually in cages (15 × 32 cm$^2$) equipped with running wheels (11 cm in diameter; Ref. 1). The running distance was monitored every 1 min with a homemade computer system (Melquest, Toyama, Japan). The running data were recorded and quantified with the Cif3Win Acquisition Software. The running wheels were locked after the last episode of running for a 24-h period before the mice were killed, and their muscles were harvested. To apply acute mechanical

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stress to the soleus and plantaris muscles, two distinct models were used; the whole hindlimb was immobilized with the ankle allowed full dorsal flexion and the knee joint angle at 90° for 30 min; the mice were exercised for 30 min on a two-lane enclosed treadmill with an adjustable belt speed (0–50 m/min), an adjustable incline (0–30°), and a shock plate (0–2 mA; Melquest). A writing brush was also used to encourage the mice to run. The treadmill exercise protocol was as previously described (15). The mice were familiarized with the equipment for two days by running on a motor-driven treadmill for 10-min periods at a speed of 15 m/min with no incline (0% grade). The exercise protocol consisted of total 30 min of running (25–30 m/min, 0% grade). The animals started at 5 m/min for 5 min, and then the incremental protocol was begun at 10 m/min for 5 min. The speed was increased by 5 m/min every 5 min to a maximum of 30 m/min.

The animals were killed and their muscles harvested in an appropriate buffer or embedded in OCT compound. The animal protocols were approved by the Animal Care and Use Committee of the University of Tokyo.

Generation of muscle-specific p130Cas−/− mice. Knockout mice lacking the muscle-specific p130Cas gene were generated by cross-breeding Ckmm-cre mice (Jackson Laboratory) and floxed-p130Cas mice (B6;129P2-p130Castrm2Homy). The genomic DNA from mouse tails was used for PCR-based genotyping with the following primers: Cre-F (forward), 5′-ATGTCCAAATTTACGTACC-3′; Cre-R (reverse), 5′-GGCCGCAATAACCACTGAAC-3′; Cas15 (forward), 5′-AAAGATTGACCCGTTTGGCTCC-3′; and Cas16 (reverse), 5′-CAGAATCTTGGGCTGCACG-3′. To confirm the excision of the loxP-flanked (floxed) sequence in p130Cas gene, the genomic DNA from mouse muscle and spleen were used for genomic PCR with the following primers: p130Cas-5arm-8860-F and extension (72°C for 30 s) were repeated 35 times for genotyping.

RNA analysis. Total RNA was extracted from animal tissues using ISOGEN (WAKO, Osaka, Japan) according to the manufacturer’s protocols, and 1 μg of RNA was reverse transcribed using SuperScript III reverse transcriptase (RT; Invitrogen). Oligo-dT primers were used to generate cDNA, and an aliquot of the RT reaction was used directly for PCR with Ex Taq HS (TaKaRa, Osaka, Japan) and gene-specific primers. Primer sequences were as follows: p130Cas, 5′-CAAAGGT-GTTGTTCCCTA CG-3′ and 5′-CCTCTGGCCCATCCTGTTA-3′; and GAPDH, 5′-GGGCGAAAGTGAGGAGATTGTTGCC-3′ and 5′-GATGATGACC CCTTGTGCTCC-3′. GAPDH was used as an internal standard.

Immunoblotting analysis. The mouse skeletal muscles were homogenized with glass homogenizers in complete protein-loading buffer containing 50 mM Tris-HCl (pH 6.8), 1% SDS, 10% glycerol, 20 mM dithiothreitol, 127 mM 2-mercaptoethanol, and 0.01% bromophenol blue, supplemented with protease inhibitors (Roche) and phosphatase inhibitors (Sigma-Aldrich). The muscle homogenates were transferred to microfuge tubes, heated for 5 min at 100°C, and centrifuged in a microfuge for 5 min at 12,000 g at room temperature. The protein concentrations were determined (Bio-Rad), and 10–30 μg of total protein were loaded onto 7.5–15% gels (depending on the molecular weight of the protein) for SDS-PAGE, transferred to a nitrocellulose membrane, and immunodetected with an enhanced chemiluminescence kit (Amersham) using a LAS-3000 imaging system (Fuji Film, Japan). The antibodies directed against the following proteins were used for the immunoblotting analysis of the p130Cas−/− mice: cytochrome c oxidase IV (Cox IV; ab14744; Abcam Novus Biologicals); cytochrome c, phospho-p130Cas (Tyr410), phospho-MKK3/6, and phospho-p38 MAPK (Cell Signaling Technology); PGC-1α (Chemicon); GAPDH (Abcam); Cre recombinase (Novagen, San Diego, CA); and γ-tubulin (Sigma-Aldrich). The secondary antibodies used were sheep anti-mouse IgG-horseradish peroxidase (NA931; Amersham) and goat anti-rabbit IgG-horseradish peroxidase (Amersham). Protein quantification was performed with the ImageJ software (National Institutes of Health, Bethesda, MD).

Immunohistochemistry. Monoclonal antibodies directed against myosin heavy chain (MyHC) I (BA.F8), MyHC IIA (SC.71), and MyHC IIB (BF.F3) were used as the primary antibodies to immunostain the OCT-embedded cross sections of mouse skeletal muscle (29). Mouse anti-dystrophin antibody (D8043; Sigma) with a Alexa-488-conjugated secondary antibody was used to determine the muscle

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Fig. 1. Mechanical-stress-induced phosphorylation of p130Cas, mitogen-activated protein kinase kinase (MKK)3/6, and p38 MAPK. The ankles of C57BL6 mice retained dorsal flexion while their knees were maintained in the flexed position for 30 min. The soleus muscles were harvested for phospho-specific immunoblotting analysis. A: representative images of phospho-p130Cas, phospho-MKK3/6, and phospho-p38 MAPK proteins in skeletal muscle after passive stretching for 30 min (St). B: representative images of phospho-p130Cas, phospho-MKK3/6, and phospho-p38 MAPK proteins in skeletal muscle after treadmill running for 30 min (Run). Quantification of the relative abundances of the phospho-proteins after the passive stretching (C) and the treadmill running (D; n = 5–6). *P < 0.05, **P < 0.01 vs. control (Con).
fiber shapes. The cross sections were fixed in PBS with 4% paraformaldehyde, permeabilized in PBS with 0.3% Triton X-100, and incubated overnight with the primary antibody. The secondary antibody was either Alexa-405-conjugated anti-mouse IgG2a, Alexa-488-conjugated anti-mouse IgG1, or Alexa-549-conjugated anti-mouse IgM (for MyHC I, MyHC IIa, and MyHC IIb, respectively; Jackson ImmunoResearch Laboratories, West Grove, PA). Finally, the coverslips were mounted with VectaShield (Vector Laboratories, Burlingame, CA). The cross-sectional area was measured with the ImageJ software.

**Statistics.**
Data are presented as means ± SE. Statistical significance (P < 0.05) was determined with Student’s t-test or a two-way ANOVA followed by Newman-Keuls test for multiple comparisons (genotype × treatment).

**RESULTS**

Mechanical-stress-induced phosphorylation of p130Cas in skeletal muscle. We first investigated whether mechanical stress induces the phosphorylation of p130Cas in skeletal muscle, so we performed a phosphoprotein-specific immunoblotting analysis of soleus muscle that had been recruited during passive stretching and running (n = 5–6, each). The phosphorylation of p130Cas was induced by the passive stretching of the skeletal muscle for 30 min (Fig. 1, A and C).

Fig. 2. Generation of muscle-specific p130Cas knockout mice. The muscle-specific p130Cas−/− mice were obtained by crossbreeding genetically modified mice, in which the p130Cas allele was flanked by loxP sites, with Ckmm-cre transgenic mice. A: PCR of the genomic DNA with the appropriate primers (see MATERIALS AND METHODS) to detect the loxP-flanked p130Cas allele in wild-type (+/+; WT), heterozygous (+/−), and homozygous (−/−) mice. Only the homozygous mice with the Cre transgene (cre/+) were considered to be muscle-specific-p130Cas knockout (p130Cas−/−) mice. B: confirmation of the excision of the floxed sequence in p130Cas gene. Only muscle tissue from the homozygous mice with the Cre transgene were detected the excision of the p130Cas genome. The p130Cas mRNA was also measured. C: immunoblotting analysis of the p130Cas and cre proteins in the skeletal muscle and nonmuscle tissue (spleen) of p130Cas−/− mice compared with their WT littermates. γ-tubulin was used as the loading control. D: immunoblotting analysis of muscle proteins in three distinct muscles of the p130Cas−/− mice compared with those in their WT littermates. γ-tubulin was used as the loading control. MyHC, myosin heavy chain; PGC-1α, peroxisome proliferator-activated receptor γ-coactivator 1α; COX IV, cytochrome c oxidase IV.

**Table 1. Physical characteristics of muscle-specific p130Cas−/− mice and their wild-type littermates**

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<thead>
<tr>
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<th>WT (n = 5)</th>
<th>p130Cas−/− (n = 5)</th>
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<tr>
<td>Body weight, g</td>
<td>19.16 ± 0.54</td>
<td>21.57 ± 0.81</td>
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<tr>
<td>Muscle weight, mg/g body wt</td>
<td>0.33 ± 0.01</td>
<td>0.30 ± 0.01</td>
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<tr>
<td>Soleus</td>
<td>0.53 ± 0.02</td>
<td>0.59 ± 0.02</td>
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<tr>
<td>Plantaris</td>
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Values are means ± SE. p130Cas−/−, muscle-specific p130Cas−/− mice; WT, wild-type littermate mice.

**Table 2. Distances run by p130Cas−/− mice and their wild-type littermates**

<table>
<thead>
<tr>
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<th>Genotype</th>
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<tr>
<td>Distance, km/day</td>
<td>WT</td>
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<td></td>
<td>5.04 ± 0.69</td>
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Values are means ± SE.
increased in the wild-type (WT) mice in response to treadmill running for 30 min (2) (Fig. 1, B and D). We also observed that the phosphorylation of p130Cas was induced in the skeletal muscle by running for 30 min (Fig. 1, B and D). These results clearly indicate that the phosphorylation of p130Cas, MKK3/6, and p38 MAPK is induced by mechanical stress.

Muscle-specific deletion of p130Cas does not influence basic skeletal muscle properties. To determine whether p130Cas is required to maintain the properties of skeletal muscle, we crossed Ckmm-cre transgenic mice (7) with genetically modified mice in which the p130Cas alleles were flanked by loxP sites. Expression of the Cre gene under the control of the muscle creatine kinase promoter allowed us to delete the gene of interest specifically in the striated muscle. Following three rounds of crossbreeding, we obtained mice in which the p130Cas gene was muscle-specifically deleted (Fig. 2A). The muscle-specific p130Cas−/− mice were born in normal Mendelian ratios and were fertile (data not shown). The efficacy of the Cre/loxP genetic deletion system was confirmed by genomic PCR (Fig. 2B) and immunoblotting analysis (Fig. 2C). We detected the excision of the floxed sequence in p130Cas gene only in muscle tissue in the muscle-specific p130Cas−/− mice. A specific antibody that recognizes only p130Cas detected only trace amounts of p130Cas protein in the plantaris muscles of the muscle-specific p130Cas−/− mice, whereas p130Cas expression was as abundant in the p130Cas-floxed mice with no Cre expression (Fig. 2C). Postnatally, the muscle-specific p130Cas−/− mice showed a normal skeletal muscle mass, with body weights similar to those of their WT littermates (n = 5–7). *P < 0.05 and **P < 0.01 vs. control.

To characterize the skeletal muscle phenotype of the muscle-specific p130Cas−/− mice in a sedentary state, an immunoblotting analysis was performed to measure the expression of the MyHC proteins in three muscles with distinct fiber type compositions: the soleus (predominantly type I and IIa fibers), plantaris (predominantly type IIa and IIb fibers), and white vastus lateralis muscles (predominantly type IIb fibers). No striking differences were observed in these muscles, although
the expression of the MyHC proteins was slightly lower in the type IIb fibers (Fig. 2D). These biochemical characteristics of the muscles were not associated with any significant difference in the muscle masses of the muscle-specific p130Cas mice and their WT littermates (Table 1).

Mechanical-stress-induced muscle adaptation in muscle-specific p130Cas mice. To ascertain the importance of p130Cas in mechanical-stress-induced skeletal muscle adaptation, we used an in vivo model of mechanical-stress-induced adaptation. When the muscle-specific p130Cas mice were subjected to voluntary running for 2 wk, they ran similar daily running distances as their WT littermates (Table 2). An immunofluorescence-based fiber-type analysis of the soleus and plantaris muscles after voluntary running for 2 wk suggested that the deletion of the p130Cas gene had no significant impact on exercise-induced fiber-type transformation in the plantaris muscle, as shown by increases in the percentage of type IIA myofibers (Fig. 3, D and E). This finding was further confirmed by an immunoblotting analysis of the expression of the MyHC proteins, which showed similar degrees of skeletal muscle adaptation in the IIb-to-IIa fiber type transformation (enhanced MyHC IIa expression) and mitochondrial biogenesis (enhanced Cox IV and cytochrome c expression) to those in the control mice (Fig. 4, A–G), consistent with the notion that p130Cas is not required for exercise-induced skeletal muscle adaptation.

Muscle-specific deletion of the p130Cas gene has no effect on mechanical-stress-mediated p38 MAPK signaling. Orchestrated signaling/transcription events that are elicited by mechanical stimuli play essential roles in skeletal muscle adaptation. To examine mechanical-stress-induced p38 MAPK signaling, we performed a phospho-protein-specific immunoblotting analysis of the soleus and plantaris muscles, which is recruited during stretching and running. The phospho-p38 MAPK was induced by 30 min of planter flexion (stretching; Fig. 5A). Consistent with a previous finding that phospho-p38 MAPK

Fig. 4. Mechanical-stress-induced expression of muscle proteins in p130Cas mice was similar to that in their WT littermates. A: immunoblotting analysis of MyHC types I, IIa, and IIb, myoglobin (Mb), PGC-1α, and Cox IV proteins in the plantaris muscle of p130Cas mice (−/−) and their WT littermates. γ-tubulin was used as the loading control and to confirm the quality of the protein samples. B–G: quantification of the relative abundances of the proteins after normalization to the abundance of γ-tubulin. (n = 5). *P < 0.05, **P < 0.01 vs. control.

Fig. 5. Muscle-specific deletion of the p130Cas gene does not affect mechanical stretch-induced p38 MAPK signals. The muscle-specific p130Cas mice (−/−) and their WT were subjected to a 30-min passive mechanical stretching, and their soleus muscles were harvested for immunoblotting analysis (n = 5–6). Representative images (A) and quantitative data (B) for phospho-p38 MAPK protein. Appreciable increases in phospho-p38 MAPK were noted after stretching in both groups. C: representative images for phospho-p38 MAPK proteins. Representative images (D) and quantitative data (E) for phospho-MKK3/6 proteins. **P < 0.01 vs. control.
increased in their WT littermates after running for 30 min (2), the deletion of the p130Cas gene surprisingly had no significant impact on mechanical stress-induced phospho-p38 MAPK expression in the plantaris muscle (Fig. 6A). We then determined whether upstream signals of p38 MAPK were activated in the muscle-specific p130Cas−/− mice by 30-min stretching and a bout of acute running. We first confirmed that the phosphorylation of p130Cas was blunted by the deletion of the p130Cas gene (Figs. 5C and 6C). We also confirmed that the phosphorylation of MKK3/6 was induced by stretching and a single bout of running, as shown in Figs. 5, D and E, and 6, D and E.

DISCUSSION

We have demonstrated in this study that adult skeletal muscle fibers have a normal phenotype in p130Cas flox/flox; Ckmm-cre+/- mice. Furthermore, the mechanical-stress-induced alteration of the skeletal muscle phenotype was not affected by the deletion of p130Cas.

p130Cas has a possible functional role in sensing mechanical stress in the skeletal muscle to facilitate the activation of signaling pathways that maintain and adapt the muscle functions, including the muscle mass, muscle fiber type, and mitochondrial biogenesis, in response to mechanical loading. According to this hypothesis, we would expect that the targeted mutation of the p130Cas gene would disrupt skeletal muscle adaptation, such as the muscle fiber type switching induced by enhanced mechanical loading and the expression of proteins essential for mitochondrial biogenesis. Contrary to our original hypothesis, we provide biochemical evidence that muscle-specific p130Cas−/− mice undergo skeletal muscle adaptation like their WT littermates, including fast-to-slow fiber type switching in response to increased contractile activity. Furthermore, the expression of PGC-1α and other mitochondrial proteins in the p130Cas−/− mice was induced by increased contractile activity in a manner similar to that observed in their WT littermates. The induced expression of other exercise-responsive genes, such as that encoding myoglobin, was also unaffected by the deletion of the p130Cas gene in the skeletal muscle. These findings indicate that p130Cas is not essential for exercise-induced mitochondrial biogenesis or type IIb-to-IIa fiber type switching in skeletal muscle.

Similarly, the muscle-specific deletion of p130Cas had no significant impact on the mechanical stress-induced MKK-p38 MAPK signaling. The functional role of p38 MAPK in mechanical-stress-induced skeletal muscle adaptation is supported by several studies in which muscle contractile activity activated both the p38 MAPK pathway and PGC-1α expression (2, 3, 4, 9, 12, 20, 22, 29). The tyrosine phosphorylation of p130Cas creates a docking site for the Sh2 adaptor, Crk, which in turn recruits a Rap guanine nucleotide exchange factor, Rapgef1, to activate Rap1 GTPase and p38 MAPK signaling (11, 17, 24, 25).

Although p130Cas has been implicated in the stretch-induced activation of p38 MAPK signaling in vitro, the elimination of p130Cas by targeted gene disruption did not disturb mechanical stress-induced p38 MAPK phosphorylation in adult skeletal muscle. This is not surprising because a previous study showed that Rap1 activity was not completely eliminated in p30Cas-deficient cells exposed to cyclic stretch (25). Therefore, although p130Cas is not required for mechanical-stress-induced muscle adaptation, it remains possible that the mechanical-stress-induced changes in the skeletal muscle phenotype are dependent on p38 MAPK activation. This is consistent with our finding that p38 MAPK signaling was stimulated by both passive mechanical stretching and a single bout of exercise in the p130Cas−/− mice. Thus it appears that both p130Cas-dependent and -independent activation of Rap1 occur in response to mechanical stimulation.

The only change we observed in the muscle-specific p130Cas−/− mice was a reduced proportion of type IIB fibers in the plantaris and white vastus lateralis muscles. This unexpected finding may imply that the targeted mutation of the p130Cas gene results in a reduction in fast fibers. The exact mechanism underlying this phenotypic change is currently unknown. Nevertheless, the finding that fast fibers are reduced in mice lacking p130Cas has important implications.

It should be noted that although we used muscle-specific p130Cas−/− mice to minimize the possibility of developmental and organ effects caused by the global disruption of the gene, we cannot rule out the possibility that other regulatory factors functioned in a compensatory manner. The facts that exercise-induced fiber type transformation and mitochondrial biogenesis are normal in the muscle-specific p130Cas−/− mice suggest
that the essential function of p130Cas in these fundamental adaptive processes might be compensated adequately by other mechanotransduction pathways. Another possibility is that the mild phenotype of the p130Cas−/− mice arises from insufficient Ckmm-cre expression, required to generate the muscle-specific p130Cas deletion.

The observation that the muscle-specific deletion of p130Cas had no effect on mechanical-stress-induced skeletal muscle adaptation does not imply that p130Cas is not required for the mechanical-stress-induced changes in myofiber specialization. The mechanical-stress-induced phosphorylation of p38 MAPK was not impaired in the muscle-specific p130Cas−/− mice. These findings suggest that future research should focus on the potential functional roles of mechanosensors and other protein kinases that act on substrates they share with p130Cas in the mediation of skeletal muscle plasticity.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


