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A novel voluntary weightlifting model in mice promotes muscle adaptation and insulin sensitivity with simultaneous enhancement of autophagy and mTOR pathway

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Abstract

Our understanding of the molecular mechanisms underlying adaptations to resistance exercise remains elusive despite the significant biological and clinical relevance. We developed a novel voluntary mouse weightlifting model, which elicits squat-like activities against adjustable load during feeding, to investigate the resistance exercise-induced contractile and metabolic adaptations. RNAseq analysis revealed that a single bout of weightlifting induced significant

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Authors Contributions.

D. Cui and Z. Yan designed research; D. Cui, O. L. Sabik, C. R. Farber, and Z. Yan analyzed data; D. Cui, J. C. Drake, R. J. Wilson, B. Lewellen, M. Zhang, H. Zhao, O. L. Sabik, and S. Onengut performed research; D. Cui, S. S. Berr, S. S. Rich, and Z. Yan wrote the paper.

transcriptome responses of genes that function in post-translational modification, metabolism and muscle differentiation in recruited skeletal muscles, which were confirmed by increased expression of fibroblast growth factor-inducible 14 (*Fn14*), Down syndrome critical region 1 (*Dscr1*) and Nuclear receptor subfamily 4, group A, member 3 (*Nr4a3*) genes. Long-term (8 weeks) voluntary weightlifting training resulted in significantly increases of muscle mass, protein synthesis (puromycin incorporation in SUNSET assay) and mTOR pathway protein expression (raptor, 4e-bp-1 and p70S6K proteins) along with enhanced muscle power (specific torque and contraction speed), but not endurance capacity, mitochondrial biogenesis and fiber type transformation. Importantly, weightlifting training profound improved whole-body glucose clearance and skeletal muscle insulin sensitivity along with enhanced autophagy (increased LC3 and LC3-II/I ratio, and decreased p62/Sqstm1). These data suggest that resistance training in mice promotes muscle adaptation and insulin sensitivity with simultaneous enhancement of autophagy and mTOR pathway.

Keywords

Resistance exercise; hypertrophy; muscle power; contractile adaptation; metabolic adaptation

Introduction

Skeletal muscle makes up approximately 40% of body weight and plays an essential role daily life, metabolism and other physiological functions (1). It is well known that aging and physical inactivity cause loss of muscle mass and function, compromising mobility and life quality. Importantly, skeletal muscle abnormalities increase the propensity of co-morbidities, contributing significantly to the overall mortality under various diseased conditions. On the contrary, regular exercise improves muscle and whole-body function with many health benefits (2, 3). In particular, resistance exercise, a type of exercise that employs resistive load during muscle contraction, is the most effective in building up muscle mass and strength as well as preventing muscle atrophy and frailty in humans (4). Furthermore, resistance exercise promotes metabolic function across all age groups under various conditions (5–7). It is for this reason that muscle-strengthening activities that work all major muscle groups in addition to aerobic exercise are highly recommended for the general population (5). However, we still have a poor understanding of the molecular mechanism underlying resistance exercise.

Much of our knowledge about the mechanisms of resistance exercise has come from studies using animal models, including surgical ablation of synergistic muscles (8, 9), ladder climbing (10), passive stretching (11), and electric stimulation against load or with a stimulation frequency that mimics resistance exercise (12, 13). The invasive nature of these models causes inevitable injury and inflammation (14). A model of voluntary weightlifting was developed in rats in 1998 by Klitgaard et al., showing significant increases of plantar flexor muscle weight and strength after a very long duration of training (36 weeks) (15). Their model requires human handling during each session of the whole training period and, as a result, has not been employed widely by the research community. Thus, the pre-existing animal models of resistance training in animals either involve invasive procedures, which

will inevitably confound the physiological relevance of the findings due to injury and inflammation, or require constant human handling during the intervention, which preclude large-scale, in depth mechanistic studies. There is an urgent need of developing a physiological animal model of resistance exercise.

Current research findings in human and animals support a potent anabolic effect of resistance exercise. Mechanistic target of rapamycin (mTOR) complex 1 (mTORC1), a highly conserved, serine/threonine kinase of the phosphatidylinositol kinase-related kinase family, is a central node in this process (16). mTORC1 and its upstream regulators and downstream effectors as well as protein synthesis can all be activated by an acute bout of isometric contractions induced by high-frequency (100 Hz) electrical stimulation that mimics resistance exercise in rat skeletal muscle (13). Electrical stimulation-induced muscle contractions also stimulate glucose transporter Glut4 translocation to plasma membrane (17), which promotes glucose uptake. Pharmacological and genetic interventions revealed that mTORC1 is not only required for the maintenance of insulin sensitivity and intramyocellular lipid content (18) but also for increased protein synthesis and muscle hypertrophy induced by high-frequency electrical stimulation (19). Whether mTORC1 signaling plays a central role in the anabolic and metabolic adaptations in physiological model of resistance exercise has yet to be resolved.

Recent evidence show that resistance training also promotes autophagy, a highly conserved cellular catabolic process for aggregate proteins and dysfunctional organelles. Resistance exercise in humans led to increased autophagosome content (20)(21). Findings of resistance exercise in animal models is inconclusive, due to multiple confounding factors. For example, ladder-climbing in rats was reported to have increased autophagy along with increased expression of autophagy related proteins in one study (22), but decrease autophagy in another (23). Since autophagy/mitophagy is required for metabolic adaptation to endurance exercise training (24–26), and resistance training improves insulin sensitivity and glucose metabolism in skeletal muscle (26), it is conceivable that resistance training may promote insulin sensitivity and glucose metabolism in skeletal muscle by promoting autophagy/mitophagy.

We developed a novel model of voluntary weightlifting in mice that does not involve human handling during training. This model employs a cage top on a regular mouse cage where an individually housed mouse performs squat-like activity against controlled load during feeding, recapitulating many of the known responses in humans to a single bout, short-term and long-term resistance exercise training. Voluntary weightlifting had effects on transcriptional, biochemical, cellular, contractile and metabolic adaptations. In addition, orchestrated, concurrent regulation of autophagy and mTOR signaling pathway was associated with physiological contractile and metabolic adaptations. These findings provide a research platform to provide interventions to combat muscle atrophy, frailty and other muscle related disease conditions.

Materials and Methods

Animals

Male C57BL/6J mice (male, 26~28 g, 10~14 weeks old) were obtained from Jackson Laboratory and housed in the animal facility at 22–24°C with a 12 h/12 h light/dark cycle and with access to food and water *ad libitum*. The Institutional Animal Care and Use Committee at the University of Virginia approved all animal procedures. All samples were harvested after mice were euthanized under anesthesia following an overnight fasting at least 48 hours after the last bout of weightlifting.

Voluntary weightlifting exercise

For acute weightlifting exercise and long-term weightlifting training, we replaced the regular cage with the newly designed weightlifting cage top at the beginning of the dark cycle (18:00) and switched to regular cage top next morning (9:00). This was to minimize the potential impact of limited food intake during the dark cycle. We confirmed that neither acute weightlifting nor long-term weightlifting training resulted in significant loss of body weight comparing with mice housed in regular cages. Following two days of acclimatization with elevated lever plate with elevated ramp (mice can reach food without additional load to the hindlimbs), mice in the acute weightlifting group were allowed to push against 150%-body weight load for one night. Mice in the long-term (8 weeks) weightlifting group were allowed to push against resistance 100% body weight load on the first day with incremental increase of load by 20% body weight each day until reaching 240% of body weight load. The training includes 5 sessions/week with 1-day rest after 3 days of training and another one after another 2 days of training.

Magnetic resonance imaging (MRI)

MRI was performed under anesthesia using 1.25% isoflurane in oxygen as previously describe (27). Imaging was performed on a 7.0T MR system (Clinscan, Bruker Biospin, Ettlingen, Germany) using a 35mm volume coil and an MR-compatible physiological monitoring and gating system for mice (SA Instruments, Inc., Stony Brook, NY). Maximum gradient strength of the system was 600 mT/m and the peak slew rate achievable was 6000 mT/m/ms.

Glucose tolerance test

This test was performed at least 24 hours after the last bout of weightlifting training to avoid acute effects of exercise as described previously (26).

In vivo muscle function

Muscle contractile function of hindlimb plantar flexion muscles was measured *in vivo* under anesthesia by using the Aurora Dual-Mode Lever System (Model 300C-LR; Aurora Scientific, Aurora, Canada) at least 24 hours after the last bout of weightlifting activities as described previously (28).

Treadmill running test

We performed this procedure, which allows measurement of endurance capacity in mice, as described previously (26).

Echocardiography

Echo was performed with measurements of LV end-diastolic and end-systolic diameters, end-diastolic and end-systolic wall thicknesses of interventricular septum, end-diastolic and end-systolic LV posterior wall thickness. Fractional shortening and ejection fraction were calculated as described (29).

Insulin sensitivity in skeletal muscle

This analysis of insulin stimulated Akt phosphorylation in skeletal muscle provides direct evidence of insulin sensitivity in living mice. Briefly, following an overnight fasting, a muscle biopsy (gastrocnemius muscle) was taken from a hindlimb of the mouse under anesthesia induced by isoflurane. Another biopsy was taken from gastrocnemius muscle of the other hindlimb 10 min after a single bolus injection of insulin (5 U/kg body weight). Western blot analysis was then performed for phosphorylated (S473) and total Akt, and increased ratio of p-Akt (S473) to total Akt was calculated as insulin sensitivity in skeletal muscle. A pilot study with a range between 1 and 20 U/kg body weight showed that 5 U/kg body weight elicited an optimal stimulation of Akt phosphorylation in skeletal muscle.

Surface sensing of translation assay (SUnSET)

SUnSET assay allows estimation of global protein synthesis in cultured mammalian cells and in tissues of living animals (30). We performed this assay according to previously described method (30). Briefly, we injected mice under anesthesia with puromycin (AG Scientific, San Diego, CA) at 0.04 $\mu\text{mol/g}$ body weight (i.p.) 30 min before sample harvesting. Western blot was then performed with a mouse anti-puromycin antibody (Kerafast, Boston, MA) and FITC-conjugated anti-mouse secondary antibody. Puromycin incorporation was measured by using LI-COR system with both ponceau S staining and Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) quantity as loading control.

RNAseq and semi-quantitative PCR

RNA was isolated by using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNAseq library construction and analysis were performed as previously described (31). Semi-quantitative PCR was performed as described previously (32). Additional primers were used including: *Tweak* forward primer AGTGAGTGGCTGGGAAGAGA, reverse primer CAGCCTTAAGATGAGCCCAG; *Cytb* forward primer ATATACACGCAAACGGAGCC, reverse primer CTGTTTCGTGGAGGAAGAGG; *18S rRNA* forward primer AAACGGCTACCACATCCAAG, reverse primer CCCTCTTAATCATGGCCTCA.

Western blot analysis

Muscle sample preparation and western blot analysis were performed as described previously (26). The following primary antibodies were used: LC3 (Novus Biological

NB100–2220), p62/SQSTM1 (sigma P0067), Atg6 (Novus Biological NB500–249), Atg7 (R&D systems MAB6608), Puromycin (Kerafast EQ001), Akt (Cell signaling CST9272), p-Akt (S473) (Cell signaling CST9271), Raptor (Cell signaling CST2280), p-p70S6k (T389) (Cell signaling CST9205), p70S6k (Abcam AB37699), p-4e-bp-1 (S65) (Cell signaling CST9451), 4e-bp-1 (Cell signaling CST9452), Cox4 (Abcam AB33985), OXPHOS cocktail (Thermo PA5–28220), and Gapdh (Cell signaling CST2118).

Statistical analysis

All data were presented as mean \pm standard error (SEM). Unpaired t-test or Two-Way ANOVA was used for comparisons between groups or among groups using GraphPad Prism 7.0 with Sidak's multiple comparisons post hoc analysis. A statistically significant difference was achieved when $p < 0.05$.

Results

Establishment of voluntary weightlifting model in mice

Back squat is a common, compound movement in human resistance exercise that engages a large group of lower extremity muscles, including quadriceps, gastrocnemius, plantaris and soleus muscle (33). This exercise mode leads to functional improvements, such as balance, strength, mobility, and glucose metabolism. An animal model that recapitulates this resistance exercise would permit understanding the underlying cellular and molecular adaptations. We developed a mouse cage environment that is conducive to voluntary hindlimb plantar flexion movement against shoulder-loaded resistance during feeding. A mouse in the exercise group wore a neck collar (Fig. S1B) and extended hips, knees and raised heels from a height-adjustable metal ramp to push a weight-loaded lever plate in order to access food in a container through a hole in the lever plate (Fig. 1A). The regular cage top was replaced with a weightlifting cage top during the dark cycle, so the weightlifting activities were voluntary without the need of human handling. A magnet was attached to the lever plate, and weightlifting activities were recorded by a magnetic sensor connecting to a computer recording system (DSI). Each cage top was calibrated by measurement of the force generated at the hole of the lever plate when different weights were added on the extended arm of the lever plate (Fig. S1A).

Based on pilot studies, the height of the metal ramp was adjusted such that the distance from the top of the ramp to the food in the food container is 0.7 cm shorter than the distance from the nose to the heels of the mouse (please see the Methods section). This setting ensured food accessibility as well as full hindlimb extension during weightlifting verified by video recording (Fig. 1B). The sedentary control mice were housed in the same cage environment except that the lever plate was fixed at the lifted position, and the metal ramp was adjusted at an elevated position such that the mouse did not have to lift weight to access food. This weightlifting model mimics the heel raised, squat-like task to recruit multiple muscle groups by flexing and extending at the hip, knee, and ankle joints in a single maneuver, recapitulating the ascent phase of the human heel raised squat with loading.

Validation of weightlifting activity was made by subjecting mice to an overnight acute bout of weightlifting against 150%-body weight load followed in assessment of mRNA expression. Our pilot study results instructed us to use this workload for untrained mice to have reliable weightlifting activity. Mice in the weightlifting group lifted the plates 419 ± 82 times (Fig. 1C) with no aberrant effect on body weight (Supplemental Fig. S1C). At the end of the dark cycle, all mice were euthanized, and the hindlimb muscles, including quadriceps, gastrocnemius and tibialis anterior muscles, as well as the heart and liver were harvested. Three genes were chosen to validate the transcriptional responses by semi-quantitative RT-PCR: Down syndrome critical region gene 1 (*Dscr1* or *Rcan1*), fibroblast growth factor-inducible 14 (*Fn14* or *Tnfrsf12a*) and Nuclear receptor subfamily 4, group A, member 3 (*Nr4a3* or *Nor-1*) mRNA. These three genes have been shown to be responsive to acute exercise (32, 34). mRNA was measured for tumor necrosis factor superfamily, member 12 (*Tweak*) that encodes the ligand for Fn14 and mitochondrial cytochrome b (*Cytb*) that encodes a mitochondrial respiratory protein. Significantly increased mRNA for *Dscr1* (1.5-fold; $p < 0.01$), *Fn14* (2.3-fold; $p < 0.01$) and *Nr4a3* (3.0-fold; $p < 0.01$) was observed in gastrocnemius muscle of weightlifting mice compared with the sedentary control mice, but not for *Tweak* and *Fn14* mRNA (Fig. 1D), consistent with the transcriptional responses being gene-specific. We measured *Fn14* and *Dscr1* mRNA in quadriceps and tibialis anterior muscles, heart and liver. Significantly induced expression was observed only in recruited quadriceps, but not in antagonistic tibialis anterior muscle. A subtle, but statistically significant, increase in *Fn14* mRNA was observed in the heart, but none of these changes were observed in the liver (Fig 1E). Collectively, these findings demonstrate that acute weightlifting activity induces specific transcriptional responses in recruited skeletal muscles.

Transcriptome responses to a single bout of weightlifting activity

Culmination of transcriptome responses to single bouts of exercise underscores skeletal muscle adaptations induced by training. RNA sequencing of recruited gastrocnemius muscle showed significantly increased (341 genes) and suppressed (149 genes) mRNA expression compared with sedentary mice (Fig. 2A). The 30 most highly responsive genes from differential expression (DE) analysis include *Dscr1* and *Fn14* and *Nr4a3* (Fig. 2B), indicating a high degree of muscle plasticity to resistance exercise at the transcriptome level. PANTHER gene ontology was used to analyze and identify the highly regulated biological processes. Based upon gene ontology (PANTHER) analysis, 490 genes are classified into 34 biological processes (top 20 shown in Fig. 2C). The biological processes with highest enrichment were protein phosphorylation, modification, metabolism process, and skeletal muscle cell differentiation (top 20 up-regulated and top 20 down-regulated genes, Table 1 and 2). These findings are consistent with the importance of transcriptional control of protein turnover, muscle differentiation and extracellular matrix remodeling in the process of adaptations to resistance exercise in mice. It is important to note that our analysis following an acute bout of weightlifting may elicit many stress responses; not all the regulated genes are directly involved in the muscle adaptations.

Long-term weightlifting training induces skeletal muscle hypertrophy with enhanced expression mTOR pathway

To ascertain the impact of weightlifting training on muscle mass, mice were subjected to voluntary weightlifting for 8 weeks (5 days/week). The training protocol began with no addition of weight on the plate lever (a workload equivalent to ~100% body weight) and gradually increased (20% increment on each training day) to 240% body weight in 10 days (referred to as staging phase). As the workload increased, there was a progressive decline of weightlifting activity (repetition per night) (Fig. 3A). The workload was kept at 240% of body weight for the remainder of the training period (referred to as steady phase). The weightlifting activity per night reached and remained at about 200 repeats during this phase (Fig. 3A). After 8 weeks of training, the cross-sectional area (CSA) of the lower hindlimb was assessed using magnetic resonance imaging (MRI). A moderate but significant increase of CSA by 14% ($p < 0.05$) was observed in weightlifting trained mice compared with sedentary control mice (Fig 3B). Hypertrophy of recruited skeletal muscle was further confirmed by increased wet weight of soleus, plantaris, and gastrocnemius muscles, but not that of tibialis anterior muscle (Fig. 3C and Supplemental Fig. S2A). There was no change of heart weight and any of the cardiac functional parameters assessed by electrocardiogram and echocardiogram (Supplemental Fig. S2A and Supplemental Table S1 and S2). Long-term voluntary weightlifting resulted in increased anteroposterior (AP) femur width, but not medial-lateral (ML) femur width, tibia and femur lengths and bone mineral density assessed by dual-energy X-ray absorptiometry (DEXA) (Fig. 3D and Supplemental Fig. S2B). These findings revealed adaptations to the musculoskeletal system of the weightbearing hindlimbs.

Skeletal muscle hypertrophy is thought to be caused by increased protein synthesis rather than decreased protein degradation, where protein synthesis is primarily controlled by mTORC1 (13). Surface sensing of translation (SUnSET) assay (35) was employed, in which a structural analogue of the tyrosyl-tRNA (antibiotic puromycin) is injected *in vivo* to label nascent peptide chains for a fixed duration before sample harvesting. A significant increase of puromycin incorporation in gastrocnemius, plantaris and quadriceps muscles was detected, as well as a moderate increase in the heart in weightlifting trained mice, but not in the liver (Fig. 3E and supplemental Fig. S2C). Similar findings were observed after 4 weeks of training in an independent series of mice (Supplemental Fig. S2D). To investigate the role of mTORC1 in resistance training, phosphorylation and content of key signaling molecules were measured in this pathway. A moderate, but significant, increase of Akt (1.27-fold; $p = 0.05$) and p-Akt (3.87-fold; $p < 0.001$) was observed, as well as a significant increase of regulatory-associated protein of mTOR (raptor) (1.67-fold; $p < 0.05$) (Fig. 3F). Importantly, mTORC1 targets ribosomal protein S6 kinase 1 (p70S6K) (12.0-fold; $p < 0.001$) and eukaryotic translation initiation factor 4E binding protein 1 (4e-Bp1) (2.40-fold; $p < 0.001$) showed profound increases with no significant increases of phosphorylation state (ratio of phosphorylated protein to total protein) at T389 and S65, respectively (Fig. 3F). For the first time, these data reveal profoundly enhanced protein expression of mTORC1 pathway as a possible mechanism for driving protein synthesis following weightlifting training.

Long-term voluntary weightlifting training promotes muscle contractile function

Resistance training is not only effective in building up muscle mass, but also in promoting muscle power (36, 37). We performed non-invasive muscle contractile function assay for plantar flexor muscles in the hindlimb *in vivo* via sciatic nerve stimulation. Weightlifting trained mice showed significantly increased specific twitch (1.28-fold; $p < 0.05$) and tetanic torque (1.13-fold; $p < 0.01$) in comparison with sedentary control mice (Fig. 4A and 4B). Weightlifting trained muscles had significantly greater contraction (max dx/dt) (1.33-fold; $p < 0.01$) and relaxation (min dx/dt) speed (1.34-fold; $p < 0.01$) during twitch contraction with similar trends in tetanic contraction (Fig. 4A and 4B). When the motor nerve was stimulated with electrical pulses of increasing frequencies (force-frequency curve), a significant change in the force frequency curve was not observed, but there was an upshift of the curve in weightlifting trained mice (Fig. 4C). Repeated tetanic contraction induced by pulses at physiological frequency (60 Hz) (fatigability test) showed no significant change in time to 50% maximal contraction but a significantly increased integrated work (1.37-fold; $p < 0.001$) in weightlifting trained mice compared with sedentary control mice (Fig. 4D) (Table 3). Similar but slightly variable findings were observed following 4 weeks of training (Supplemental Fig. S3A–D, Supplemental Table S3). It is well known that voluntary running in mice improves endurance capacity primarily through adaptations, including mitochondrial biogenesis, angiogenesis and fiber type transformation (25). There were no differences in running distance and in induction of blood lactic acid level in exhaustive treadmill running test between weightlifting trained and sedentary control mice (Fig. 4E). Consistent with these findings, there were no significant changes in mitochondrial electron transport chain protein Cox4, proteins in the electron transport chain protein complex I, II, III, IV and V, or fiber type composition, based on the expression of myosin heavy chain proteins Mhy7 (type I), Myh2 (type IIa) and Myh4 (type IIb) (Fig. 4F and Supplemental Fig. S3E). Together, these findings demonstrate that voluntary weightlifting training in mice specifically leads to musculoskeletal adaptations with improved strength as well as contraction and relaxation speed in recruited muscles.

Long-term voluntary weightlifting training improves metabolic function and insulin signaling in skeletal muscle along with enhanced autophagy

Endurance exercise training is one of the most efficient interventions for improving metabolic function along with reduced adiposity in mice and humans (38). Weightlifting did not lead a significant change in body weight. In contrast, sedentary mice showed a slight decline of body weight in the first week after being housed individually (Fig. 5A). These results suggest a negative impact of social separation on body weight, which could be mitigated by weightlifting. Consistent with increased energy expenditure with weightlifting activity, mice in the weightlifting group had greater daily food consumption (~16%) than the sedentary mice when they reached the workload of 240% body weight resistance (Supplemental Fig. S4).

MRI-based body composition analysis showed no significant changes in total fat mass, lean body mass, free water content and total water content between the two groups (Fig. 5B). Weightlifting training led to a 19.7% ($p < 0.05$) reduction of epididymal fat mass (Fig. 5C). These findings showed a moderate impact of resistance training on visceral fat with no

major impact on the body composition in mice on normal diet. In contrast, there was a profound improvement of whole-body glucose clearance by glucose tolerance test (GTT) with a 38% decrease ($p < 0.01$) in area under curve (AUC) (Fig 5D). To determine whether the improved glucose clearance in resistance exercise-trained mice was due to increased insulin sensitivity in skeletal muscle, Akt phosphorylation was measured in gastrocnemius muscle pre- and 10 min post-insulin injection (5 unit/kg, i.p.). An elevated baseline (2.84-fold; $p < 0.05$) and a more robust responsiveness of Akt phosphorylation at S473 was detected in weightlifting-trained mice (13.1-fold) compared with sedentary control mice (5.89-fold; $p < 0.01$) (Fig. 5E). Glut4 is a key glucose transporter responsible for insulin- and exercise-stimulated glucose transport in skeletal muscle (39), and increased Glut4 expression has been reported in endurance exercise training (39). No increased Glut4 protein expression was observed in gastrocnemius muscle (Fig. 5F), a result different from the findings in endurance exercise models.

It has been shown that an acute bout of resistance exercise in humans reduces microtubule-associated proteins 1A/1B light chain 3B (LC3)-II/I ratio, an index of autophagy activation (21). Autophagy is important for endurance exercise-induced adaptations in different tissues/organs (40, 41), but its role in resistance training is not clear. Significantly increased LC3 content (sum of LC3-I and LC3-II) and decreased LC3-II/I ratio were observed in weightlifting trained muscle along with reduced level of p62/Sequestosome 1 (p62/sqstm1) (Fig. 5F), a cargo protein for autophagy that is degraded upon incorporation into the autolysosome (23). There was no evidence of increased autophagy-related 6 (Atg6) and autophagy-related (Atg7), both previously shown to be induced by endurance exercise (25). These findings suggest that resistance exercise training leads to increases of autophagy in skeletal muscle. Therefore, long-term weightlifting training improves whole-body glucose clearance along with improved insulin responses in skeletal muscle in the absence of enhanced mitochondrial biogenesis and glucose transporter expression; but it increases autophagy, which may underlie improved metabolic functions.

Discussion

Skeletal muscle is a very malleable organ that is responsive to a variety of cues, including physical activity, nutrients, aging, and diseases. Resistance training promotes skeletal muscle hypertrophy and improves cardiovascular and metabolic functions (5). However, the underlying molecular mechanism(s) for the myriad benefits of resistance training remains elusive. Several existing experimental animal models mimic human resistance training (8, 12, 15, 42); however, the invasive and/or the labor-intensive features of these models have limited them being widely used for mechanistic studies (43). Here, we developed a novel resistance exercise model in mice that involves voluntary recruitment of a large group hindlimb muscles against adjustable load during feeding. This human labor-free, non-invasive model makes large scale mechanistic studies a reality.

A single bout of weightlifting activity in this model led to increased mRNA of *Dscr1*, *Fn14* and *Nr4a3*, but not that of *Tweak* and *Cytb*, in recruited gastrocnemius muscle (Fig. 1D), demonstrate gene specificity of impact of exercise. Further, highly induced *Dscr1* and *Fn14* mRNA in recruited quadriceps muscle, but not in antagonist tibialis anterior muscle, heart

and liver, reveals the tissue specificity of the transcriptional responses (Fig. 1E), consistent with the electromyography (EMG) findings in human that ascent squat movement results in robust EMG activity in gastrocnemius and quadriceps muscles with little in antagonist tibialis anterior muscle (44). The lack of gene responses in tibialis anterior muscle was further corroborated by the lack of significant hypertrophy following long-term training (Fig. 3C).

RNAseq analysis revealed 341 up-regulated and 149 down-regulated genes in gastrocnemius muscle following a single bout overnight weightlifting. These are mainly functional genes in protein phosphorylation, protein modification, skeletal muscle cell differentiation, cellular protein metabolism and other cellular responses to extracellular stimuli, supporting the importance of these processes in early remodeling induced by resistance exercise. Increased *Dscr1* (or *Rcan1*) mRNA is consistent with previous findings in skeletal muscle following eccentric contractions for promoting hypertrophy (45). Increased *Fnl14* mRNA is also in line with findings in human resistance exercise (34). Interestingly, *Nr4a3* (also known as *Nor1*) gene was the most robustly (17-fold) induced in this study (Fig. 2D). *Nr4a3* gene is highly responsive to endurance exercise (46) and has been shown to promote oxidative phenotype, autophagy and hypertrophy (47). *Ankrd1* (also known as *Carp*) mRNA showed a 11-fold increase following overnight weightlifting, which has previously been shown to be induced by eccentric contractions in mice (48) and by exhaustive jumping exercise in humans (49). Finally, a robust upregulation of *Atf3* mRNA (8-fold) was observed following weightlifting (Fig. 2D). Upregulation of *Atf3* gene, which was induced by eccentric contractions (50), has anti-inflammatory roles by inhibiting chemokine and cytokine expression (51). These findings support that acute weightlifting elicits transcriptome responses as an early step in skeletal muscle adaptation to resistance exercise, laying a foundation for future dissection of molecular mechanisms underlying structural and functional adaptations to resistance training.

Resistance exercise promotes muscle hypertrophy and strength by enhancing anabolic processes in humans (52). Our long-term weightlifting training (8 weeks) resulted in moderate, but significant, hypertrophy in recruited muscles as indicated by increased CSA of plantar flexor muscles measured by MRI (17%) and muscle wet mass (14%) with no significant changes in tibialis anterior muscle and heart weight. The negative findings in antagonist tibialis anterior muscle and heart suggest that the increased muscle mass in recruited muscles (gastrocnemius, soleus and plantaris muscles) in weightlifting trained mice are not due to a general effect of moderately reduced body weight in the sedentary control mice. We did not observe changes of any of the parameters of cardiac function assessed by electrocardiogram and echocardiogram (Supplemental Fig. S2A and Supplemental Table S1 and S2). These findings are consistent with findings in human high-volume resistance exercise training (53). The degree of muscle hypertrophy in this study is highly consistent with that (8–12%) observed in humans (54). This is clearly different from the dramatic hypertrophy observed following surgical ablation of synergistic muscle, which reaches ~50% hypertrophy within a few days (8). The relatively moderate degree and slow process of muscle hypertrophy in our model suggest a very different underlying regulatory mechanisms compared with invasive muscle hypertrophy model, such as synergic muscle ablation.

Organ/tissue mass is determined by a balance between protein synthesis and degradation (i.e. protein turnover), and resistance training promotes anabolism in skeletal muscle, favoring protein synthesis over degradation (52). As a nodal sensor and integrator of environmental cues for organismal growth and homeostasis, the mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) is particularly relevant since an acute muscle contraction increases phosphorylation of mTOR at Ser-2448, downstream p70S6 kinase-1 (S6K1) at Thr-389 and rpS6 at Ser-240/244 (13) along with increased protein synthesis (13). Importantly, mTORC1, but not mTORC2, is critical for maintaining muscle mass and metabolic function (55), and activation of mTORC1 contributes significantly to increased protein synthesis and hypertrophy induced by resistance exercise (19, 56). We speculated that resistance training induces both catabolic and metabolic adaptations in skeletal muscle through mTORC1 pathway for the following reasons: 1) Both mTOR-dependent and -independent mechanisms contribute to increased protein synthesis and muscle hypertrophy induced by resistance exercise-mimicking contractions (19); 2) mTORC1 in skeletal muscle is required for normal insulin sensitivity and intramyocellular lipid content (18), and 3) Isometric contractions increase Akt phosphorylation and Glut4 translocation to plasma membrane (17). Here, we showed significant increases of protein synthesis as measured by puromycin incorporation in hypertrophied muscle after 8 weeks of weightlifting training (Fig. 3E and Supplemental Fig. S2C) and at early time (Supplemental Fig. S2D) prior to detectable changes in muscle mass, consistent with the notion the increased protein synthesis is primarily responsible for skeletal muscle hypertrophy. To investigate the signaling pathway that is potentially involved in hypertrophic adaptation, we measured mTORC1 protein phosphorylation and expression in recruited skeletal muscle and observed enhanced protein expression of this pathway in the absence of profound changes in phosphorylation state (Fig. 3F), suggesting that enhanced protein expression as a novel mechanism of enhanced mTORC1 signaling capacity in promoting hypertrophy. Since the samples were harvested after overnight fast, this enhanced expression of mTORC1 proteins along with increased insulin response (Fig. 5E) may lead to a more dramatic increase of protein synthesis. This enhanced anabolic response may play a very important role in promoting skeletal muscle hypertrophy in these mice. To our knowledge, this is the first study that showed clear evidence of enhanced protein expression of mTORC1 pathway in promoting muscle hypertrophy following resistance exercise training.

Resistance training in humans increases the force and velocity of muscle contraction, collectively called muscle power (work/time). In this study, 8 weeks of weightlifting training resulted in significant increases of maximal force of twitch and tetanic contractions (Fig. 4A and 4B) similar to what was observed in humans (36, 37). Weightlifting training in mice also resulted in moderate increases of velocity of both twitch and tetanic contractions (Fig. 4A and 4B) as additional evidence of increased muscle power. Our measurements of maximal twitch and tetanic contractions were normalized by muscle mass, demonstrating improved contractile function beyond hypertrophy. This is highly consistent with findings in humans where improved maximal force by resistance training exceeded the increases of muscle cross-sectional area (36, 37). Since the measurements of muscle contractions were performed *in vivo* by motor nerve stimulation for isometric contractions with the same preload and maximal stimuli (currency and frequency) for all groups of mice, the differences

in magnitude of torque generation are unlikely due to muscle mechanics, morphological factors and neural factors. We measured myosin heavy chain (MHC) proteins in the recruited gastrocnemius muscles and found no evidence of fiber type transformation (Fig. 4F and Supplemental Fig. S3E). The fact that the overall increases of muscle tetanic contraction without major impacts on the shape of force-frequency curve (Fig. 4C and Supplemental Fig. S3C) suggests that the improved muscle power is not due to alterations of calcium handling, i.e. release of Ca^{2+} from SR and/or restoration of Ca^{2+} level in the cytosol. While a previous study has shown progressive resistance exercise training increased cross-sectional area and peak Ca^{2+} -activated force of all three types of isolated fiber *ex vivo* without affecting specific force (57), more recently studies have shown increased *in vivo* muscle specific force and myofibrillar ATPase activity following resistance training (58). The mechanisms underlying the improved muscle power, particularly specific force and velocity of muscle contraction, remain to be elucidated.

Autophagy is an important cellular process that facilitates unnecessary or toxic cargos removal in muscle quality control (59), and both insufficient and excessive autophagy contribute to muscle atrophy (60). Aerobic exercise promotes autophagy to clear toxic components, balance redox state and maintain cellular homeostasis (25, 26), whereas the effect of resistance exercise is unclear. It was reported that progressive resistance exercise-induced muscle hypertrophy was associated with reduced autophagy as indicated by decreased in LC3-II/I ratio and an increase in p62 level (23). In this study, we observed increased autophagy capacity (increased total LC3) and flux (decreased LC3-II/I ratio and p62 level) with no changes in Atg7 and Atg6 proteins (Fig. 5F), which is consistent with findings in humans (20). Autophagy is a catabolic process, which is unlikely to be directly related to hypertrophy. However, its impact on metabolic adaptation in endurance exercise is well known (24–26). In this study, weightlifting training led to enhanced autophagy concurrent with improved clearance of blood glucose in GTT (Fig. 5D) and increased Akt phosphorylation after insulin injection (i.p.) (Fig. 5E), recapitulating the findings in humans (61). To the best of our knowledge, this is the first study showing a profound positive impact of resistance exercise on whole body glucose metabolism with direct evidence of improved insulin sensitivity in skeletal muscle in an animal model. It is of note that the moderate muscle hypertrophy in recruited muscles alone does not appear to be responsible for the profound improvement of glucose metabolism. Future studies should focus on the signaling mechanism(s) by which resistance training promotes insulin sensitivity in skeletal muscle.

mTOR activates anabolic process under the condition of great availability of nutrient supply, including promoting protein synthesis and inhibition of catabolic autophagy through activating phosphorylation of p70S6K at threonine 389 (T389) and inhibitory phosphorylation of Ulk1 at serine 757 (S757) (62). On the contrary, energetic stress, such as exercise, stimulates catabolic processes of autophagy/mitophagy through activation of AMP-activated protein kinase (AMPK) and consequent activating phosphorylation of Unc-51 like autophagy activating kinase 1 (Ulk1) at serine 555 (S555) (24, 26). These two processes are typically considered antagonizing signaling events. However, these signaling pathways can be activated simultaneously as long as the activation mechanisms do not overlap. For example, system biology studies have shown that amino acid can acutely activate AMPK and Ulk1 through Ca^{2+} /calmodulin-dependent protein kinase kinase ($\text{CaMKK}\beta$)

concurrently with activation of mTOR (63). We observed that weightlifting training stimulates autophagy flux and concurrently promotes protein expression in mTOR pathway along with enhanced responsiveness to insulin-stimulated Akt. These findings demonstrate that these two pathways can be enhanced through different mechanisms in physiological model of resistance exercise in mice.

In summary, we have developed a novel voluntary weightlifting model in mice, mimicking physiological adaptation similar to back squat exercise in humans. Using this model, we observed significant increased mRNA of genes that function in post-translational modification, metabolic control and muscle differentiation following a single bout exercise. We also observed that improved skeletal muscle power along with increased protein synthesis, muscle mass, and expression of proteins in the mTOR pathway following long-term (8 weeks) training. Weightlifting training resulted in profound improvement of whole-body glucose metabolism and skeletal muscle insulin sensitivity along with enhanced autophagy in recruited skeletal muscles. We conclude that resistance training promotes skeletal muscle adaptation and insulin sensitivity with simultaneous enhancement of autophagy and mTOR pathway. We believe that this study has paved the way for future studies to mechanistically dissect the underlying mechanisms of the physiological adaptations and their impact on physiology, health and disease prevention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations

4e-bp-1	Eukaryotic translation initiation factor 4E-binding protein 1
AMPK	AMP-activated protein kinase
Atg6	Autophagy-related 6
Atg7	Autophagy-related 7
CSA	cross-sectional area
DEXA	dual-energy X-ray absorptiometry
Dscr1	Down syndrome critical region 1
EMG	electromyography
Fn14	Fibroblast growth factor-inducible 14
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase

GTT	Glucose tolerance test
LC3	Microtubule-associated proteins 1A/1B light chain 3B
MRI	<i>Magnetic resonance imaging</i>
mTOR	Mechanistic target of rapamycin
Nr4a3	Nuclear receptor subfamily 4, group A, member 3
p62/Sqstm1	p62/Sequestosome 1
p70S6K	p70S6 kinase
Raptor	Regulatory-associated protein of mTOR
SUnSET	<i>Surface sensing of translation</i>
Ulk1	Unc-51 like autophagy activating kinase 1

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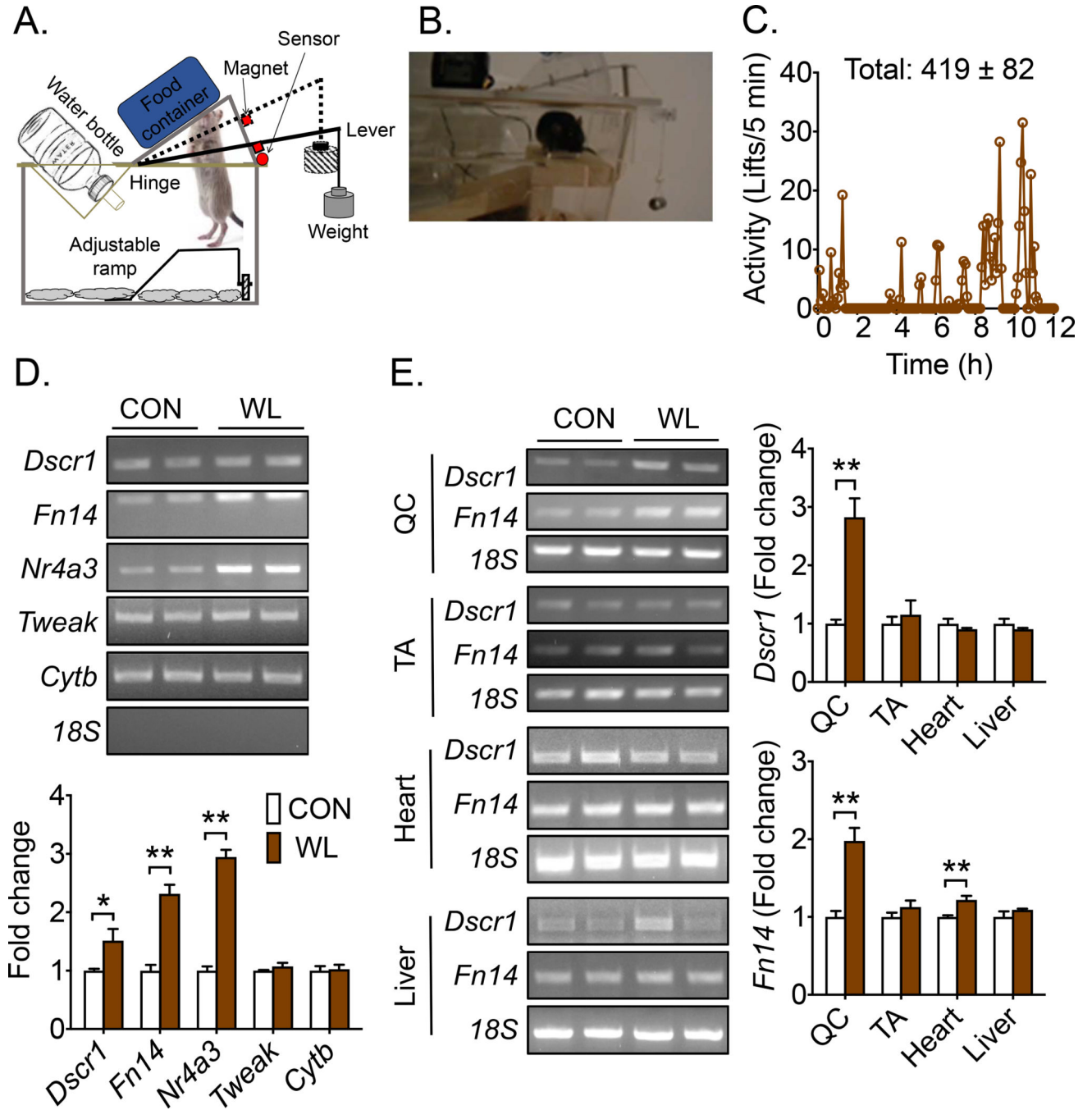


Fig. 1. A single bout of voluntary weightlifting activates recruited muscles.

Mice were subjected a single bout of overnight voluntary weightlifting (WL) in cages equipped with newly invented weightlifting cage tops with mice subjected to sedentary cage activity as control (CON) followed by semi-quantitative RT-PCR analysis of mRNA expression in recruited muscles and other tissues. (A) A cartoon illustrating the design of the weightlifting cage top, depicting a mouse performing squat-like movement against a weighted lever to access food; (B) A video recording of weightlifting activity of a mouse; (C) A representative record of weightlifting activity with quantification for 9 mice over the

12-hour dark cycle (419 ± 82); (D) Semi-quantitative RT-PCR analysis of *Dscr1* ($n = 5$), *Fn14* ($n = 5$), *Nr4a3* ($n = 5$), *Tweak* ($n = 3-4$), and *Cytb* ($n = 3-4$) mRNA in gastrocnemius muscle using *18S RNA* as reference control; and (E) Semi-quantitative RT-PCR analysis of *Dscr1*, *Fn14* mRNA in quadriceps (QC; $n = 5$), tibial anterior muscle (TA; $n = 5$), heart ($n = 3-4$), and liver ($n = 3-4$). Bars represent mean \pm SE. * and ** denote $p < 0.05$ and $p < 0.01$, respectively.

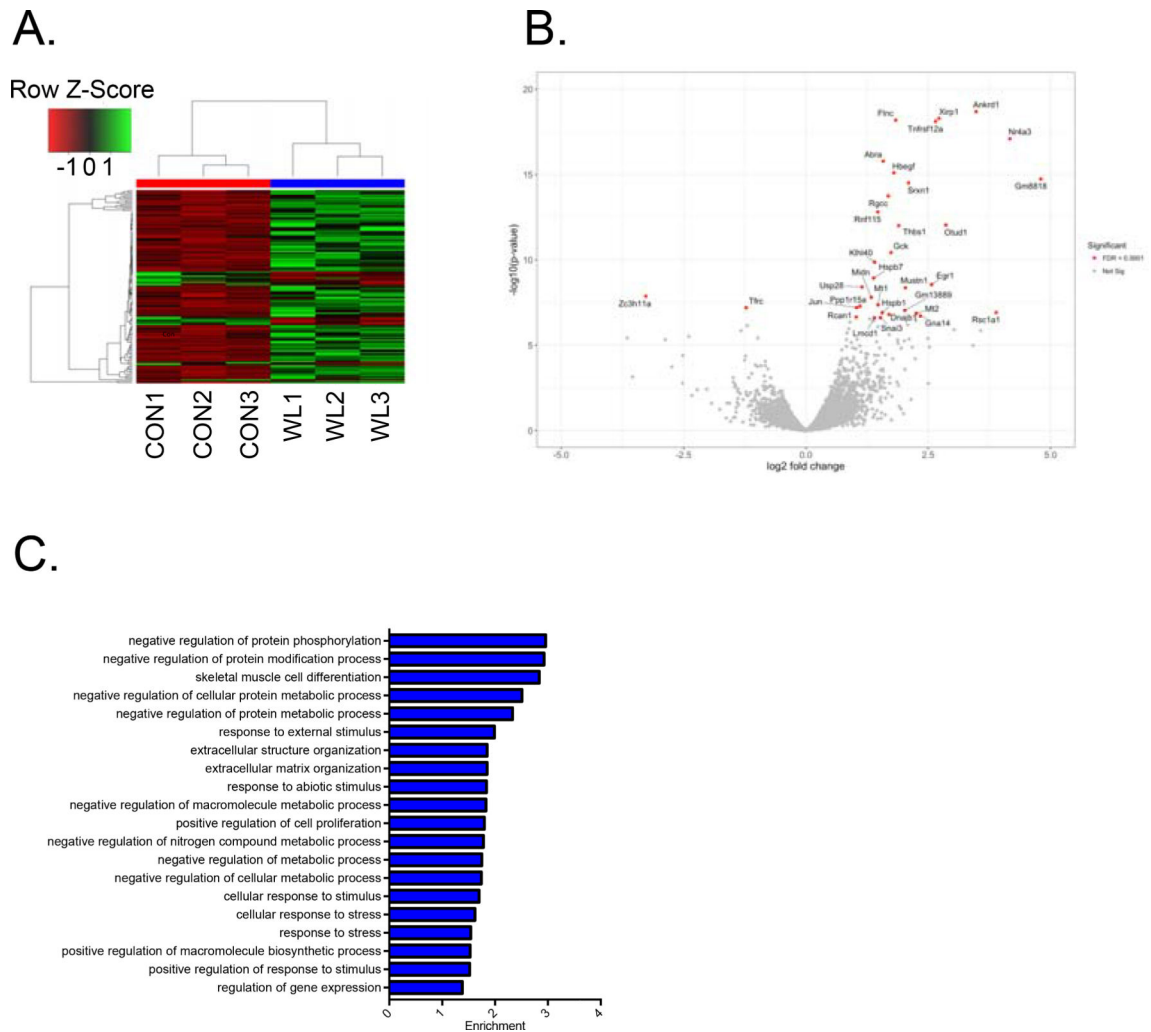


Fig. 2. A single bout of weightlifting activity induces transcriptome regulation.

Total RNA was isolated from gastrocnemius muscles in sedentary control mice (CON) and acutely (overnight) weightlifting exercised mice (WL) and used for RNAseq analysis. (A) A transcriptome heatmap of RNA sequencing data of significantly changed genes in gastrocnemius muscles of CON and WL mice (n = 3 for each group); (B) A volcano plot of all the genes highlighting the 30 top responsive genes (red spots); (C) Top 20 biological processes of gene ontology analysis; and (D) A list of top 20 upregulated and downregulated genes.

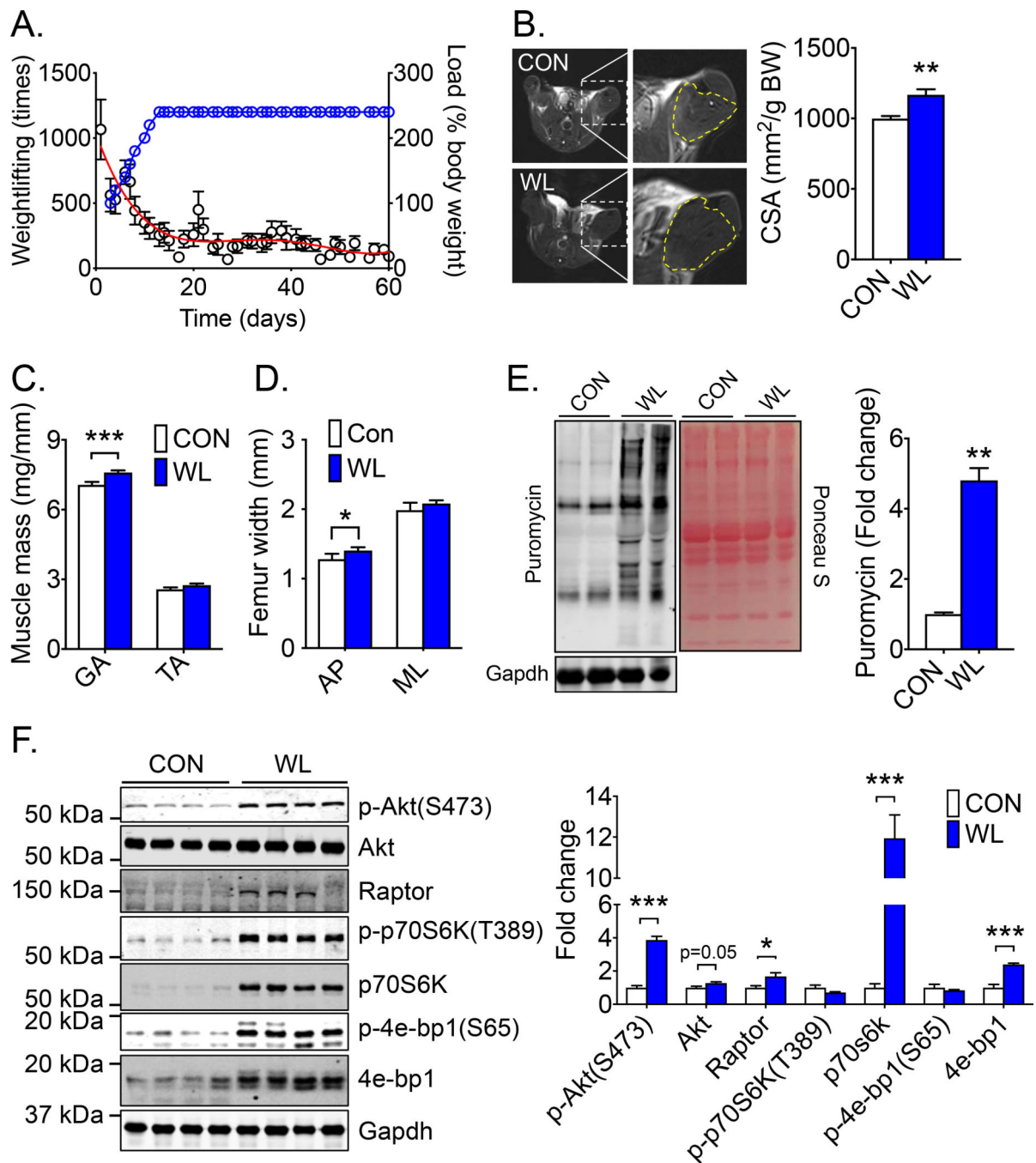


Fig. 3. Long-term weightlifting training induces skeletal muscle hypertrophy and skeletal adaptations along with increased protein synthesis and enhanced Akt-mTOR pathway in skeletal muscle.

Mice were subjected to 8 weeks of voluntary weightlifting (WL) with sedentary mice as control (CON) followed MRI imaging, SUnSET and western blot analyses of the recruited muscles. (A) Weightlifting training activity in WL mice, displaying the weightlifting repeats (dots in black) (left Y axis) with a red trending curve for % body weight load (dot in blue) (right Y axis, n = 9); (B) Representative MRI and quantification of hindlimb muscle cross-sectional area normalized by body weight (n = 4–5); (C) Muscle wet weight of

gastrocnemius and tibialis anterior muscles (n = 12 for each group); (D) Anteroposterior (AP) and medial-lateral (ML) femur width (n = 5 for each group); (E) Representative SUnSET image with *ponceau S* staining and *Gapdh* as loading control (n = 4–5); and (F) Representative western blot images and quantification of Akt-mTOR pathway proteins (p-Akt (S473), Akt, Raptor, p-p70S6k (T389), p70S6k, p-4e-Bp-1 (S65), and 4e-Bp-1) in gastrocnemius muscle with *Gapdh* served loading control (n = 4). Bars represent mean \pm SE. *, ** and *** denote $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

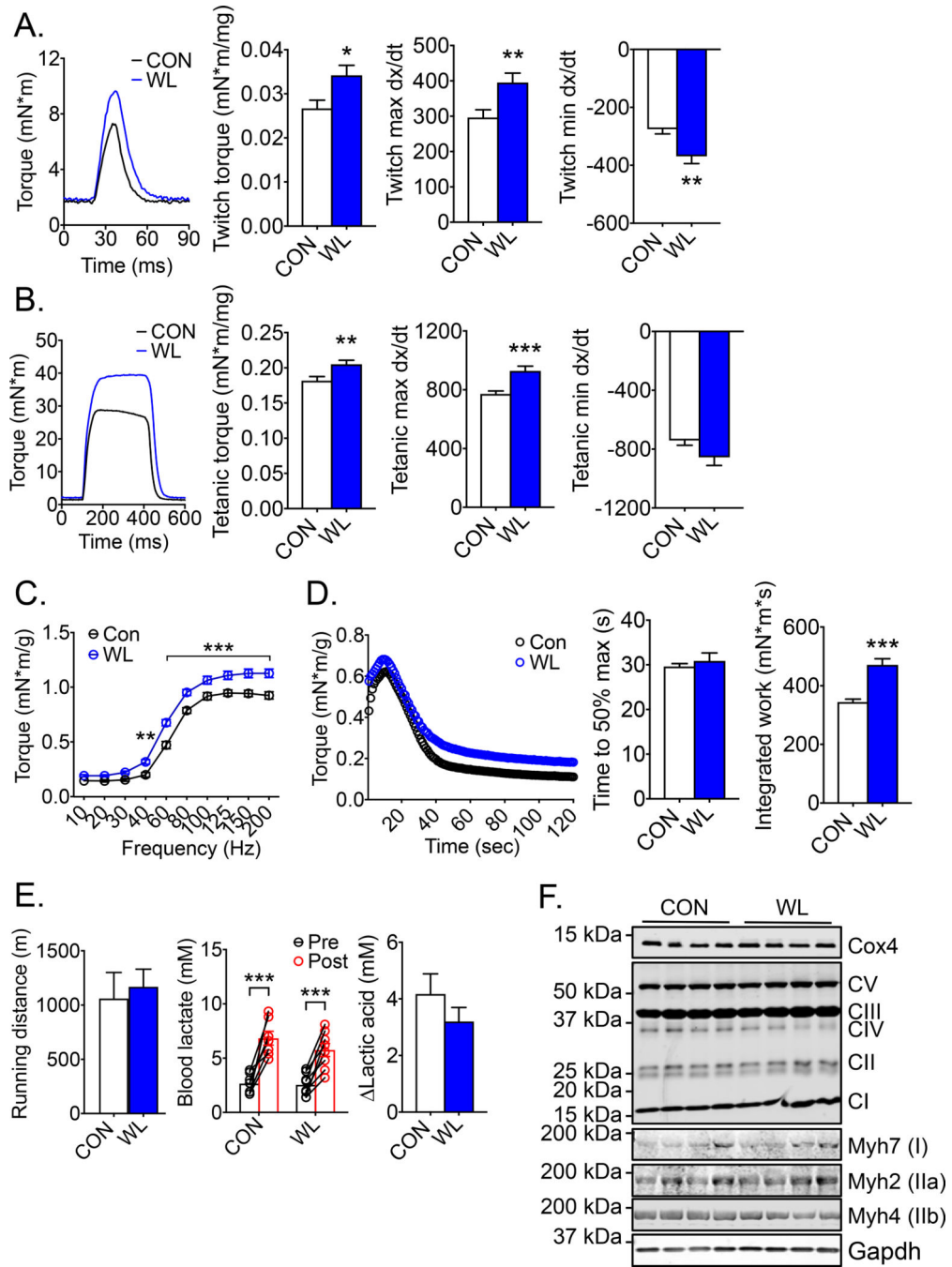


Fig. 4. Long-term weightlifting training enhances skeletal muscle power.

Mice were subjected to 8 weeks of voluntary weightlifting (WL) with sedentary mice as control (CON) followed by measurements of contractile function and endurance exercise capacity in vivo as well as measurements of mitochondrial and contractile proteins in skeletal muscle. (A) Representative twitch force of planta flexor muscles of the hindlimbs with quantification for twitch torque normalized by muscle mass, twitch max dx/dt and min dx/dt (n = 12 for each group); (B) Representative tetanic force of planta flexor muscles of the hindlimbs with quantification for tetanic torque normalized by muscle mass, tetanic max

dx/dt and min dx/dt (n = 12 for each group); (C) Force-frequency curve, displaying torque normalized by body weight (n = 6–7); (D) Fatigability curve, display torque normalized by body weight with quantification of time to 50% maximal force and integrated work performed during the test (n = 6–7); (E) Running distance and blood lactic acid level pre- and post-test during treadmill running test (n = 7–8); and (F) Representative images and quantification of mitochondrial (Cox4 and Complex I-V) and contractile proteins (MHC I, IIa and IIb) in gastrocnemius muscles with Gapdh as loading control (n = 4–6); Bars and dots represent mean \pm SE. *, ** and *** denote $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

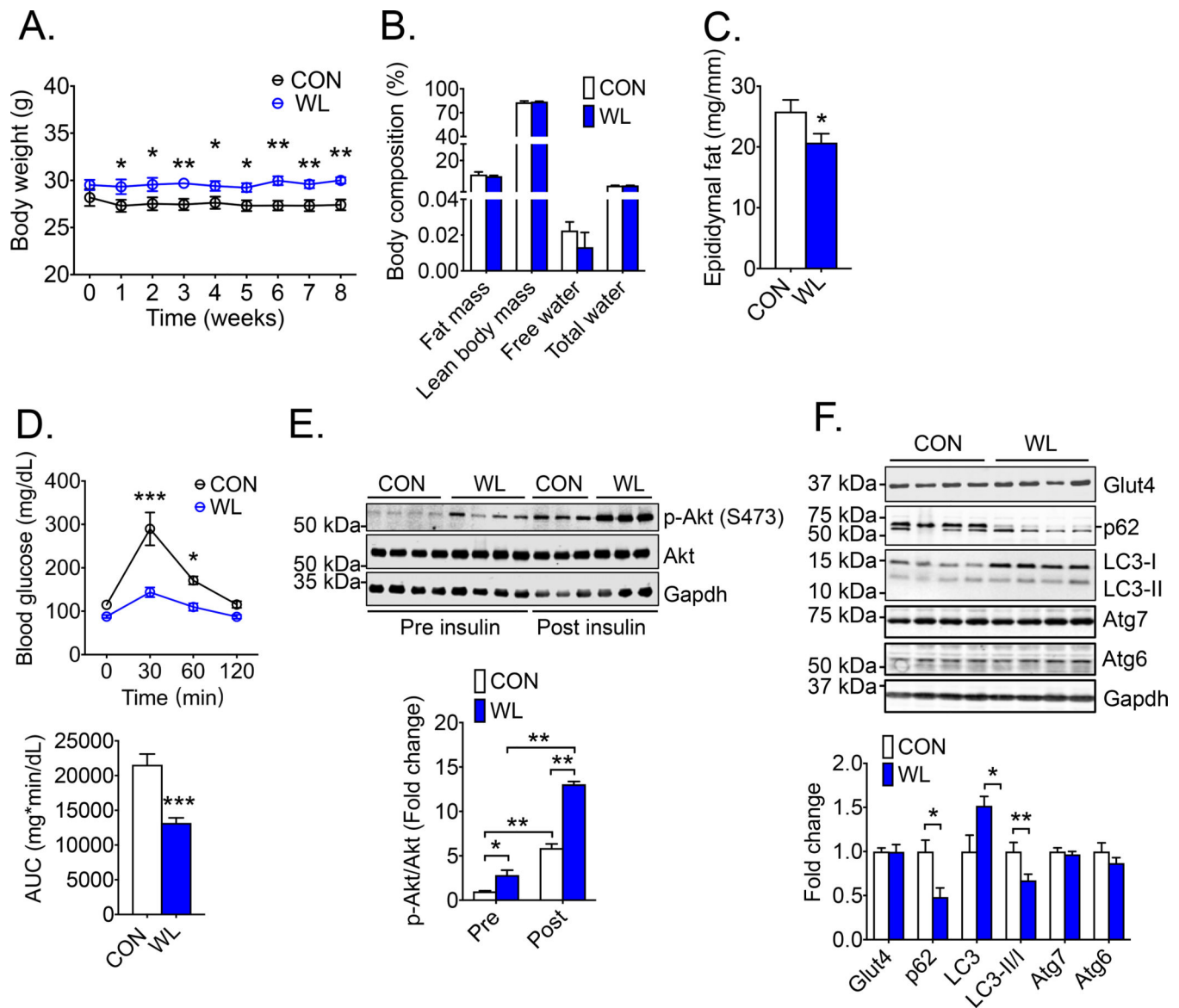


Fig. 5. Long-term weightlifting training enhances metabolic function with increased insulin sensitivity in skeletal muscle along with increased autophagy.

Mice were subjected to 8 weeks of voluntary weightlifting (WL) with sedentary mice as control (CON) followed by measurements of body composition, epididymal fat mass, whole-body glucose tolerance, insulin stimulated Akt phosphorylation as well as autophagy in gastrocnemius muscles. (A) Body weight during 8 weeks of weightlifting training; (B) MRI-based measurements of body composition (n = 4 for each group); (C) Epididymal fat mass normalized by tibia length (n = 12); (D) Blood glucose detected by tail vein blood during glucose tolerance test before and at 30, 60, and 120 min after intraperitoneal injection of glucose with quantification of the area under curve (AUC) (n = 12); (E) Representative image and quantification of p-Akt (S473)/Akt in gastrocnemius muscle pre- and 10 min post-intraperitoneal injection of insulin with Gapdh as loading control (n = 3–4); and (F) Representative images and quantification of glucose transporter (Glut4) (n = 4) and

autophagy proteins (p62, LC3, Atg7 and Atg6) (n = 6–11) in gastrocnemius muscles with Gapdh as loading control; Bars and dots represent mean \pm SE. *, ** and *** denote $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Table 1.

Top 20 up-regulated genes by an acute bout of weightlifting

Gene ID	Gene Name	Log2 (Fold change)
XLOC_014928	Nr4a3	4.14
XLOC_011380	Ankrd1	3.48
XLOC_001742	Unknown	3.11
XLOC_001384	Atf3	3.04
XLOC_011579	Otud1	2.76
XLOC_023770	Xirp1	2.65
XLOC_021891	Unknown	2.63
XLOC_021639	Mt2	2.45
XLOC_002378	Unknown	2.40
XLOC_009593	Tnfrsf12a	2.30
XLOC_016477	Uchl1	2.30
XLOC_017518	Serpine1	2.28
XLOC_010181	Egr1	2.18
XLOC_012311	Srxn1	2.15
XLOC_004693	Fos	2.14
XLOC_023769	Csrp1	2.11
XLOC_017298	Unknown	2.08
XLOC_009776	Hspa1b	2.04
XLOC_022720	Sln	1.99
XLOC_009777	Hspa1a	1.96

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Table 2.

Top 20 down-regulated genes by an acute bout of weightlifting

Gene ID	Gene Name	Log2 (Fold change)
XLOC_004993	Unknown	-2.33
XLOC_013177	Actc1	-2.04
XLOC_012015	Unknown	-1.73
XLOC_000988	Unknown	-1.61
XLOC_010450	Aqp4	-1.56
XLOC_007275	Unknown	-1.50
XLOC_015924	Foxo6	-1.42
XLOC_009700	Mdga1	-1.35
XLOC_015188	Cited4	-1.35
XLOC_010486	Nrep	-1.32
XLOC_001313	Grem2	-1.31
XLOC_011439	Pdzd7	-1.30
XLOC_015378	Lrrc38	-1.29
XLOC_008241	Tfrc	-1.24
XLOC_001389	Unknown	-1.21
XLOC_009154	Col11a2	-1.20
XLOC_008805	Tiam1	-1.19
XLOC_019299	Dpf1	-1.17
XLOC_022995	6430571L13Rik	-1.09
XLOC_008102	Rmi2	-1.08

Table 3.*In vivo* muscle function analysis at 8 weeks of weightlifting training

	CON (n = 12)	WL (n = 12)
Twitch torque (mN*m/g)	0.14 ± 0.01	0.19 ± 0.01 *
Twitch max dx/dt	296 ± 22	395 ± 26 **
Twitch min dx/dt	-275 ± 17	-369 ± 25 **
1/2RT twitch (s)	0.015 ± 0.000	0.014 ± 0.000
Tetanic torque (mN*m/g)	0.96 ± 0.02	1.15 ± 0.03 ***
Tetanic max dx/dt	772 ± 19	928 ± 33 ***
Tetanic min dx/dt	-741 ± 33	-856 ± 55
1/2RT tetanic (s)	0.219 ± 0.006	0.174 ± 0.014 **
Time to 50% max (s)	29.6 ± 0.6	30.9 ± 1.8
Integrated work (mN*m*s)	344 ± 10	471 ± 21 ***

Values are presented as mean ± SE. *, ** and *** denote $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, in comparison with CON mice.