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Effect of flywheel-based resistance exercise on processes contributing to muscle atrophy during unloading in adult rats

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Dupont-Versteegden, Esther E., James D. Fluckey, Micheal Knox, Dana Gaddy, and Charlotte A. Peterson. Effect of flywheel-based resistance exercise on processes contributing to muscle atrophy during unloading in adult rats. *J Appl Physiol* 101: 202–212, 2006. First published April 6, 2006; doi:10.1152/jappphysiol.01540.2005.—Flywheel-based resistance exercise (RE) attenuates muscle atrophy during hindlimb suspension. We have previously shown that protein synthesis is elevated in response to RE, but the effect on protein degradation, cell proliferation, or apoptosis was not investigated. We hypothesized that, in addition to affecting protein synthesis, RE inhibits processes that actively contribute to muscle atrophy during hindlimb suspension. Male rats were housed in regular cages (control), tail suspended for 2 wk (HS), or HS with RE every other day for 2 wk (HSRE). Although RE attenuated soleus muscle atrophy during HS, the observed fivefold elevation in apoptosis and the 53% decrease in cell proliferation observed with HS were unaffected by RE. Expression of genes encoding components of the ubiquitin-proteasome pathway of protein degradation were elevated with HS, including ubiquitin, MAFbx, Murf-1, Nedd4, and XIAP, and proteasome subunits C2 and C9. Total ubiquitinated protein was increased with HS, but proteasome activity was not different from control. RE selectively altered the expression of different components of this pathway: MAFbx, Murf-1, and ubiquitin mRNA abundance were downregulated, whereas C2 and C9 subunits remained elevated. Similarly, Nedd4 and XIAP continued to be upregulated, potentially accounting for the observed augmentation in total ubiquitinated protein with RE. Thus a different constellation of proteins is likely ubiquitinated with RE due to altered ubiquitin ligase composition. In summary, the flywheel-based resistance exercise paradigm used in this study is associated with the inhibition of some mechanisms associated with muscle atrophy, such as the increase in MAFbx and Murf-1, but not with others, such as proteasome subunit remodeling, apoptosis, and decreased proliferation, potentially accounting for the inability to completely restore muscle mass. Identifying specific exercise parameters that affect these latter processes may be useful in designing effective exercise strategies in the elderly or during spaceflight.

apoptosis; MAFbx; Murf-1; Nedd4; XIAP; satellite cells

SKELETAL MUSCLE FIBERS ARE very adaptable to changes in activity. Muscle atrophy occurs with disuse, whereas myofibers hypertrophy with increased use, such as during resistance exercise. The final outcome of the adaptive response of muscles to changes in activity depends on the balance between protein synthesis and degradation. Hindlimb suspension in rodents has been used as a model of disuse to mimic responses

of muscles occurring during spaceflight and bed rest (32, 57). Hindlimb suspension is associated with a decrease in muscle mass mainly of postural muscles, such as the soleus, which is at least partly due to a reduction in muscle protein synthesis (16, 56, 57). We have previously shown that in adult rats resistance exercise using flywheel technology during the suspension period attenuates the atrophy in soleus muscle and maintains rates of protein synthesis to nonsuspension levels at 4 wk as well as 4 days after the onset of hindlimb suspension (15, 16). This form of exercise is unique because it is completely gravity independent and is therefore the preferred model for testing exercise as a countermeasure for unloading-induced muscle loss.

Other changes that occur during disuse atrophy that play an important role in the loss of muscle mass include 1) an increase in protein degradation, 2) an elevation of apoptosis, and 3) a decrease in cell proliferation (1, 10, 13, 19, 29). Protein degradation has been shown to be elevated during hindlimb suspension, and the ubiquitin-proteasome system is the main proteolytic system responsible for the elimination of the bulk of myofibrillar protein (64; for review, see Refs. 29, 46). Many components of the ubiquitin-proteasome protein degradation pathway are elevated during disuse muscle atrophy (for review, see Ref. 46). Additional evidence for the important role of the ubiquitin-proteasome pathway in muscle protein degradation during disuse atrophy came from the findings that two E3 ubiquitin ligases [muscle atrophy F-box (MAFbx) or atrogen-1, and muscle RING (really interesting novel gene) finger (Murf)-1] were specifically elevated in multiple models of diminished muscle use (3, 22) and have been suggested as markers of muscle atrophy (21). The effect of resistance exercise on muscles under atrophy-inducing conditions has only recently been investigated. Haddad et al. (24) reported that the hindlimb suspension-induced elevation of the ubiquitin ligases MAFbx and Murf-1 reversed to control with resistance exercise (24), even though muscle weight under these conditions was only minimally affected. However, under normal weight bearing, the effects of resistance exercise on the ubiquitin-proteasome pathway are equivocal with studies showing increased (43–45, 49, 52, 58, 63), as well as decreased (33, 61, 62), activity of the pathway.

Coincident with a loss of protein, muscles undergoing disuse-induced atrophy exhibit a decrease in myonuclear number due to the loss of myofiber nuclei through apoptotic-like

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processes (1, 13, 36; for review, see Ref. 10). Apoptosis in skeletal muscle occurs in a segmental fashion, but the underlying molecular mechanisms by which apoptosis during disuse atrophy ensues are unclear. Whether apoptosis has a causal relationship to atrophy or is merely a consequence of the loss of muscle mass is unknown, but it has been shown that exercise, which decreases muscle loss due to hindlimb suspension or spinal cord injury, also attenuates the extent of apoptosis (1, 13).

Finally, cell proliferation and the total number of supporting cells decrease in muscles during disuse atrophy (19, 41). This decrease in proliferation will likely impact the maintenance of muscle mass, because cell proliferation is necessary to recover from a period of disuse (40) and for hypertrophy induced by resistance exercise (47). Therefore, the ability of an intervention to restore cell proliferation in muscles may partially determine the extent to which muscle mass maintenance can occur.

Because muscle mass is only partially maintained with resistance exercise during hindlimb suspension (16, 17, 24), it is possible that not all of the above-mentioned processes are attenuated by resistance exercise, and, thus, the extent to which exercise can maintain muscle mass under atrophy-inducing conditions may be limited. Therefore, the goal of this study was to investigate the effects of gravity-independent flywheel-based resistance exercise, known to maintain protein synthesis during atrophy (15, 16), on apoptosis, on cell proliferation, and on components of the ubiquitin-proteasome degradation pathway. We hypothesized that flywheel-based resistance exercise attenuates muscle loss by inhibiting processes that actively contribute to muscle atrophy during hindlimb suspension.

METHODS

Animals

The research project was approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences (UAMS). All procedures on animals were performed in accordance with institutional guidelines for the care and use of laboratory animals. Male Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN) at 6 mo of age, allowed to acclimate for 1 wk in the animal facilities of UAMS, fed standard laboratory rat chow, and maintained on a 12:12-h light-dark cycle before operant conditioning was started. All animals underwent operant conditioning, regardless of the group to which they were assigned. Because different assays required different manipulation of the muscle tissues, we performed two different experiments (*experiment 1* and *experiment 2*); the details of these experiments are described below.

Operant Conditioning

Before the start of the experiment, operant conditioning of the rats to engage in resistance exercise training was performed as described previously (16, 18). Briefly, all rats were taught to press an illuminated lever in a specially designed cage to avoid a brief foot shock stimulus (<1 mA, 60 Hz, 1–5 V). The movement facilitated by the entrainment process required full extension and flexion of the hindlimbs. All animals were entrained to conduct this movement in an upright or standing position. The movement facilitated by the entrainment is similar to a squatting movement and is the same whether performed in vertical or horizontal (with hindlimb suspension) position. Once appropriately conditioned, the animals engaged in the flywheel training protocol (as described below) with very little or no shock. Conditioning to reach this point required approximately four

distinct sessions lasting 30–45 min, with sessions separated by 48–72 h. All operant conditioning and resistance exercise sessions were conducted at the onset of the dark cycle.

Hindlimb Suspension

For *experiment 1*, after operant conditioning, rats were randomly assigned to control ($n = 9$), hindlimb suspended (HS) ($n = 10$), or hindlimb suspended with resistance exercise using flywheel technology (HSRE) ($n = 6$) groups. Animals in the HS and HSRE groups were hindlimb suspended for a 2-wk period by a method previously described (34). Briefly, rats were anesthetized with ketamine and xylazine (60 and 10 mg/kg, respectively), and a steel needle was inserted into the tail of the animals through which a stainless steel cable was threaded. During the anesthesia, a continuous release pellet containing 5'-bromo-2'-deoxyuridine (BrdU) (Innovative Research America, Sarasota, FL), constructed to give a dose of $0.022 \text{ mg BrdU} \cdot \text{g body wt}^{-1} \cdot \text{day}^{-1}$, was implanted subcutaneously in the subscapular region as described previously (5, 19). After the animals regained consciousness, the tail device was connected to a pulley sliding on a vertically adjustable stainless steel bar running longitudinally above a high-sided cage. The system was designed in such a way that the rats cannot rest their hindlimbs against any side of the cage but are still capable of reaching their food and water easily.

Resistance Exercise

Flywheel technology was used to perform resistance training on the rats as previously described (15, 16). The advantage of this type of resistance training is that it can be conducted while the animal is hindlimb suspended and is therefore independent of gravity. Briefly, a nylon cord was attached to the stainless steel thread which was secured to the rat's tail, as described above. The nylon cord was then spooled around an inertia wheel located on the outside of the resistance exercise apparatus. The rat was allowed to place its feet on the shock grid, which was suspended at the top of the cage, and the illumination bar was located in the apparatus opposite of the shock grid (for details about the design, see Ref. 16). Upon illumination of the bar, the rat extended its hindlimbs and pulled against the tether using enough force to overcome the inertia of the wheel. Once the movement was completed and the animal was back in the starting position, the bar was illuminated again. The movement performed by the animal requires force production of muscles involved in movements of the hip, knee, and ankle joints. When needed, shock was applied briefly to facilitate the movement. The resistance protocol consisted of six exercise sessions over a 2-wk period, with two sets of a maximum of 25 repetitions for each session. This protocol has been shown to be effective in elevating rates of muscle protein synthesis and attenuating atrophy of soleus muscle over a 4-wk period (15, 16). Force generation during the exercise sessions was recorded using a load cell (Entran Devices, Fairfield, NJ) attached to the flywheel apparatus, which was integrated to a personal computer.

For *experiment 2*, to obtain muscles for the proteasome activity measurements, a second set of rats was trained. As in *experiment 1*, animals were randomly assigned to control ($n = 8$), HS ($n = 9$), or HSRE ($n = 8$) groups, after operant conditioning. Rats in the HS and HSRE groups were hindlimb suspended for a 2-wk period by a method previously described (19, 36). Briefly, a tail device containing a hook was attached with gauze and cyanoacrylate glue while the animals were anesthetized with ketamine and xylazine (60 and 10 mg/kg, respectively). The tail device was connected via a thin cable to a pulley sliding on a vertically adjustable stainless steel bar running longitudinally above a high-sided cage as above. For the resistance training, the rats were tethered via a leather and Velcro vest attached to a nylon cord that was spooled to the inertia wheel (16), and training was performed as described above. Rats received seven exercise sessions, but the outcome of the training was similar to *experiment 1* as judged by the muscle weights as reported in Table 2.

Tissue Collection

Approximately 16 h after the last exercise session, animals were anesthetized with pentobarbital sodium (60 mg/kg), and soleus and gastrocnemius muscles were dissected and weighed. Soleus muscles were used for the analysis, because of all hindlimb muscles soleus is affected to the greatest extent by HS, and this form of resistance exercise has been shown to induce an anabolic response in soleus muscle (16, 18). Gastrocnemius muscle also responds to this form of resistance exercise, and the weight of the gastrocnemius muscles was used as an indicator of the effectiveness of the training, such that *experiments 1* and *2* could be compared. Soleus muscles, to be used for immunohistochemistry and determination of apoptotic nuclei, were embedded in freezing medium at resting length and frozen in liquid nitrogen cooled isopentane. Muscles to be used for all other analyses were frozen in liquid nitrogen, and all muscles were stored at -80°C until further analysis.

Apoptosis Determination

Detection of apoptotic nuclei. Nuclei exhibiting apoptotic changes were identified by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL), as described (13, 36) and according to the manufacturer's recommendations (Roche Molecular Biochemicals, Pleasanton, CA). Briefly, soleus muscle cross sections were cut on a cryostat (6 μm) and fixed in 4% paraformaldehyde at room temperature, blocked in 3% H_2O_2 in 100% methanol at room temperature, and permeabilized in 0.1% Triton-X and 0.1% sodium citrate at 4°C . TUNEL reaction mix was added in a 1:7.5 dilution and incubated at 37°C for 1 h. Sections were reacted with fluorescein antibody for 30 min at 37°C , followed by substrate addition for color development. The number of positive nuclei was expressed per whole muscle section.

Cytosolic mono- and oligonucleosomes. Apoptotic DNA fragmentation was quantified by measuring the amount of cytosolic mono- and oligonucleosomes in the muscle using a Cell Death ELISA kit (Roche Molecular Biochemicals), as previously described (36). Soleus muscles were homogenized by using a polytron in isolation buffer (220 mM D-mannitol, 75 mM sucrose, 0.1% fatty acid-free bovine serum albumin, 0.5 mM EGTA, 2 mM HEPES, pH 7.4) (1:10 wt/vol). The homogenate was centrifuged at 700 g at 4°C for 10 min, and the supernatant was centrifuged again at 8,000 g at 4°C for 10 min. The supernatant was carefully collected, and protein concentration was determined according to the Bradford method (4). Mono- and oligonucleosomes were measured in the supernatant according to the manufacturer's recommendations. Because total protein content decreases with atrophy, results are reported as arbitrary optical density units normalized to milligrams of protein.

Measurement of Cell Proliferation

Total number of BrdU-positive nuclei was measured as an index of cell proliferation. BrdU-positive nuclei were measured and counted as described previously (13, 19). Briefly, soleus muscle cross sections were cut (8 μm) and incubated in 0.25% hydrogen peroxide in PBS to block endogenous peroxidase activity. Muscle sections were reacted with 0.25% hydrogen and fixed in methanol. Sections were then incubated in 2 N HCl for 60 min at 37°C to denature the DNA followed by neutralization in 0.1 M borate buffer at pH 8.5. Muscle sections were then incubated in PBS containing 1.0% Igepal (Sigma, St. Louis, MO) to permeabilize the tissue, and all further washes contained 0.1% Igepal. BrdU antibody (Boehringer Mannheim, Indianapolis, IN) was applied at a concentration of 6–8 ng/ μl and incubated for 1 h at room temperature. After washing, a secondary rat anti-mouse IgG1 biotin-conjugated antibody (Zymed, San Francisco, CA) was applied at 1:100 dilution for 1 h at room temperature. Streptavidin-peroxidase was applied followed by 3,3'-diaminobenzi-

dine (DAB) peroxidase substrate (Vector, Burlingame, CA). The number of positive nuclei was expressed per whole muscle section.

Real-Time RT-PCR

RNA isolation and detection were performed as described previously (11, 14). Briefly, total RNA was isolated from soleus muscles by the guanidinium thiocyanate-phenol-chloroform extraction method as described by Chomczynski and Sacchi (6). Total RNA was treated with DNase (Ambion, Austin, TX) before measurement of mRNA abundance by real-time RT-PCR. Quantitative real-time RT-PCR was performed by using the protocols, chemistries, and amplification and detection systems of Applied Biosystems (Applied Biosystems, Foster City, CA). For each sample, cDNA was synthesized from 2 μg of total RNA by using components from the Taqman reverse transcription reagents (Applied Biosystems). The reaction contained $1 \times$ RT buffer, 5.5 mM MgCl_2 , 0.5 mM dNTPs, 2.5 mM random hexamers, 40 units of RNase inhibitor, and 375 units of Multi-Scribe reverse transcriptase. The primers were allowed to anneal for 10 min at 25°C before the reaction proceeded for 1 h at 37°C followed by 5 min at 95°C . The resulting cDNA samples were aliquoted and stored at -80°C .

Primer sequences were selected from the accession numbers in the National Center for Biotechnology Information database using the Taqman probe and primer design function of the Primer Express v1.5 software (Applied Biosystems) and are listed in Table 1. PCR reactions were assembled using the SYBRgreen PCR master mix that required only the addition of cDNA template and primers. Control reactions were run lacking cDNA template to check for reagent contamination and to determine the melting temperature of any primer dimer. To optimize assay efficiency, PCR standard curves were produced by use of a pool containing each sample cDNA. Data points were generated by fourfold serial dilutions of cDNA. Gene expression was compared in individual samples by using 16 ng (1 ng for 18s) RNA equivalents of cDNA and the standard curve method described in Applied Biosystems User Bulletin No. 2. The reactions were performed by using the ABI PrismTM 7700 sequence detection system (Applied Biosystems) and the instrument's universal cycling conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, and then 60°C for 1 min. An additional cycle was added in which the ramp time to 95°C was increased to 19 min and 59 s, during which time data were collected for melting curve analysis. RNA abundance for each gene of interest is expressed as a ratio normalized to RNA abundance of 18S in the same sample.

Isolation of Protein and Western Analysis

Western analysis of proteins was performed as described previously with minor modifications (12). Briefly, soleus muscles were homogenized in a buffer containing 10 mM MgCl_2 , 10 mM KH_2PO_4 , 1 mM EDTA, 5 mM EGTA, 1% Igepal, 50 mM β -glycerophosphate (βGPO_4), 1 mM PMSF, 1 mM Na_3VO_4 , 1 $\mu\text{g}/\text{ml}$ leupeptin, 2 $\mu\text{g}/\text{ml}$ antipain, 10 $\mu\text{g}/\text{ml}$ benzamide, 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ chymostatin, and 1 $\mu\text{g}/\text{ml}$ pepstatin. After homogenization, samples were centrifuged for 10 min at 1,000 g at 4°C . Protein concentration of the supernatants was determined according to Bradford (4) by using the Bio-Rad (Hercules, CA) protein assay reagent. For determination of total ubiquitinated protein content, 40 $\mu\text{g}/\text{lane}$ total protein were loaded and separated on a 4–15% polyacrylamide gradient gel (Bio-Rad). For detection of BRUCE (BIR repeat-containing ubiquitin-conjugating enzyme) protein abundance, 100 μg total protein was separated on a 5% polyacrylamide gel (Bio-Rad). After electrophoretic separation, proteins were transferred to nitrocellulose membranes (Bio-Rad) for Western analysis. Subsequently, the membranes were incubated in Ponceau S solution (Sigma) for 5 min for visualization of the protein and assurance of equal loading in all the lanes and pictures were saved for normalization of total protein after Western analysis. Membranes were incubated in blocking solution

Table 1. Real-time RT-PCR primer sequences

Gene	Accession Number		Primer Sequence
18S	X01117	forward	TTCGGACGTCTGCCCTATCAA
		reverse	ATGGTAGGCAGGGCGACTA
MAFbx	AY059628	forward	GACCTGCATGTGCTCAGTGAAG
		reverse	GGATCTGCCGCTCTGAGAAGT
Murf-1	AY059627	forward	TGCCCTGCCAGCACAAAC
		reverse	GGATTGGCAGCCTGGAAGAT
Ubiquitin	NM_017413	forward	CGTACCTTTCTCACCACAGTATCTAGA
		reverse	GAAAACCTAAGACACCTCCCATCA
C2 subunit	M29859	forward	GGTGTGGGCTGCTCATTG
		reverse	CAGATGGGCAGGTTTGGAAA
C9 subunit	X53304	forward	GGCCATCCGGGTTGGT
		reverse	GGAAGACAGTGAGGTGCAGAT
Nedd4	U50842	forward	GATCACCTCTCATACTTCAAGTTCATTG
		reverse	CATCCAACAGCTTGCCATGATA
XIAP	AF183429	forward	TGAAAACGGTGCCACACAGT
		reverse	TGTTTCCCACAGTTTTCAGATT

MAFbx, muscle atrophy F-box; Murf-1, muscle really interesting novel gene Finger-1; Nedd4, neuronal precursor cell-expressed developmentally downregulated-4; XIAP, X-chromosome-linked inhibitor of apoptosis.

[3% blocking grade nonfat dry milk (Bio-Rad) in Tris-buffered saline + 0.1% Tween] for 1 h at room temperature followed by incubation with ubiquitin antibody (1:200, Sigma) or BRUCE (1:250, BD Transduction Laboratories, Franklin Lakes, NJ). Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies, respectively (Pierce, Rockford, IL). Antibody binding was detected by incubating membranes for 5 min in SuperSignal (Pierce) at room temperature. Membranes were exposed to X-ray film or ChemImager 5500 (AlphaInnotech, San Leandro, CA), and density of the bands was determined by ChemImager software (AlphaInnotech).

Proteasome Activity Measurement

Soleus muscles were homogenized by using a polytron in lysis buffer (50 mM HEPES, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100), and homogenate was centrifuged at 1,500 g for 15 min at 4°C. Chymotrypsin-, trypsin-, and peptidyl-glutamyl-peptide hydrolase (PGPH)-like activities of the proteasome were measured by using the fluorogenic peptides *N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-AMC), Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin (LSRT-AMC), and Z-Leu-Leu-Glu-7-amido-4-methylcoumarin (LLE-AMC), respectively, as described in Ref. 31. All fluorogenic peptides, as well as free AMC for the generation of the standard curve, were purchased from Sigma. Enzymatic activity was determined by incubating 25 µg protein homogenate with 25 mM Tris·HCl, pH 7 and either 25 µM Suc-LLVY-AMC, 40 µM LSTR-AMC, or 150 µM LLE-AMC in total volume of 150 µl. After 30 min of incubation at 37°C, the reaction was stopped by the addition of 150 µl of ice-cold 96% (vol/vol) ethanol, and fluorescence was monitored by the release of AMC with a temperature-controlled microplate spectrofluorometer Spectramax (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 380 nm and an emission wavelength of 440 nm. A standard curve was generated by using known amounts of free AMC. The proteasome inhibitor MG-132 (20 µM, Sigma) was used to ensure that measurements of specific proteasome activities and data were expressed as nanomolar per microgram protein per minute.

Analysis and Statistics

Differences of means of maximum force values across sessions (each session consisting of 50 repetitions) or force through a range of motion (area under the curve) among sessions were tested by ANOVA. To test for statistically significant differences between the groups, one-way ANOVA was used; in case of significant differences,

a Tukey's multiple comparisons test was applied. Statistical significance was assumed at $P < 0.05$.

RESULTS

Force production by the rats was measured during each repetition of the flywheel-based resistance exercise sessions, such that the training status of the animals could be evaluated. The force produced during each repetition of the six HSRE rats of *experiment 1* was averaged and plotted in Fig. 1A. Rats were able to progressively produce more force during the repetitions, because peak force in *session 6* was higher than in the other sessions and lowest in *session 1*. The total force was estimated by calculating the area under curve for the repetitions and was averaged for all six rats of *experiment 1* (Fig. 1B). Total force produced was increased in *sessions 5* and *6* compared with *sessions 1, 3, and 4*, and in *session 6* total force produced was higher than that in other sessions. The observation that force production increases in an incremental manner indicates that the exercise is progressive. This suggests that force per unit area was elevated in soleus and gastrocnemius muscles undergoing resistance exercise, because increased force was accomplished in the face of decreasing muscle mass and both muscles contribute to the exercise.

Soleus and gastrocnemius muscle weights were measured to ensure effectiveness of the exercise program. However, only soleus muscles were used for further analysis. Muscle atrophy was significantly attenuated by the exercise regimen in both soleus and gastrocnemius muscles in *experiment 1* and in *experiment 2* (Table 2). Averaged between the two experiments, soleus muscle weight-to-body weight ratio was 40% decreased in HS, but only 28% in HSRE rats. Similarly, gastrocnemius muscle weight-to-body weight ratio was decreased 20% in HS and only 11% in HSRE rats. The extent of atrophy in the present study is very similar to previously reported loss of muscle mass with hindlimb suspension (1, 2, 12, 23, 27, 60). Therefore, flywheel-based resistance exercise is able to decrease the loss of muscle mass during hindlimb suspension, as we have shown previously (16).

Apoptosis has been implicated as a factor contributing to the loss of muscle mass during disuse, and it has been shown

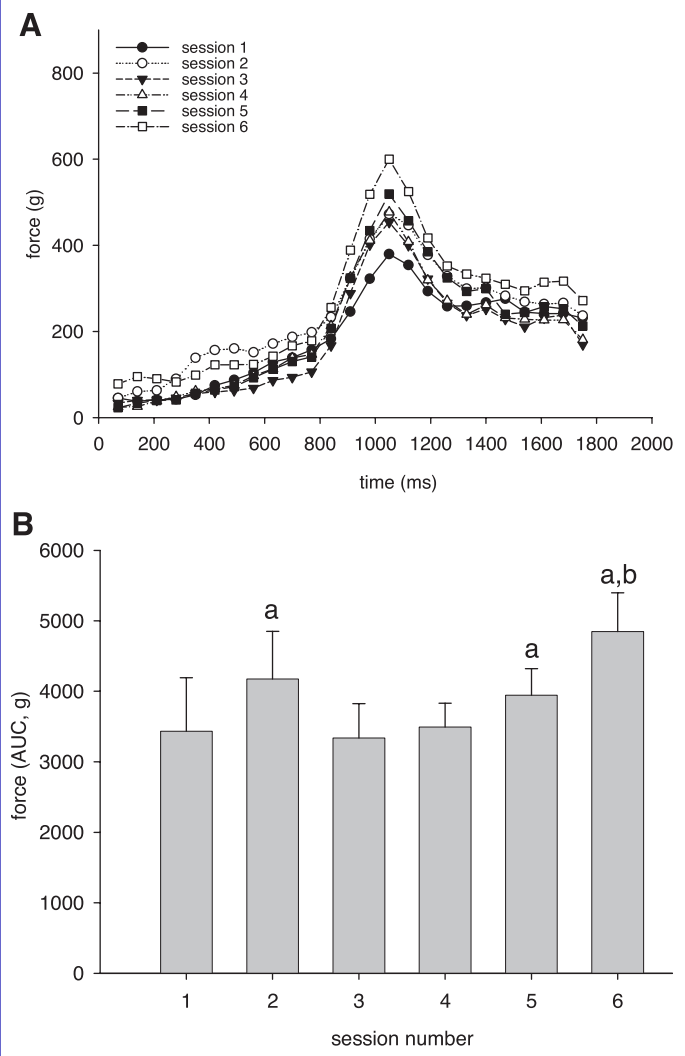


Fig. 1. Resistance exercise with flywheel-based technology progressively increases force production in hindlimb-suspended (HS) rats. *A*: average force of 6 HS rats undergoing flywheel-based resistance exercise (HSRE) produced overall repetitions for sessions 1 through 6. Each point represents the force generated for all repetitions per session (i.e., 25 repetitions per set per 2 sets) averaged for all rats ($n = 6$) at a certain point in time for each repetition. Peak force developed in session 6 was significantly higher and in session 1 was significantly lower than in the other sessions. *B*: total force produced for all repetitions per session average for all rats ($n = 6$) undergoing the flywheel-based resistance exercise. AUC, area under the curve; a, different from all other sessions; b, different from all other sessions. Values are means \pm SE; $P < 0.05$.

previously that exercise during atrophy-inducing conditions attenuated the extent of apoptosis, providing a possible mechanism for the attenuation of atrophy (1, 13). Therefore, we investigated whether apoptosis was involved in the attenuation of atrophy with flywheel-based resistance exercise. TUNEL-positive nuclei were elevated fivefold in soleus muscle of HS rats, and resistance exercise did not affect this increase (Fig. 2, A–D). Similarly, the apoptotic index, measured by quantifying mono- and oligonucleosomal content in soleus muscles, was increased by 3.2-fold and remained elevated with resistance exercise (Fig. 2E). These data indicate that apoptosis is not reduced by flywheel-based resistance exercise and likely does not play a role in the attenuation of atrophy under these conditions.

Satellite cell proliferation is required for hypertrophy (47) and recovery from atrophy (40), and therefore we hypothesized that resistance exercise would attenuate the decrease in proliferation that occurs with hindlimb suspension (19). To explore this possibility, slow-release BrdU pellets were implanted at the time of suspension. BrdU-positive nuclei in soleus muscle were decreased with HS (Fig. 3, A, B, and D). Flywheel-based resistance exercise was not associated with a change in BrdU labeling compared with hindlimb suspension (Fig. 3, C and D), indicating that the exercise did not overcome signals that induce a decrease in proliferation with disuse.

We have previously shown that rates of protein synthesis were decreased with hindlimb suspension and restored with flywheel-based resistance exercise after 4 days or 4 wk (15, 16). Whether a decrease in the major protein degradation pathway, the ubiquitin-proteasome pathway, was involved in the attenuation of atrophy with exercise during suspension was not investigated at that time. Here we investigated components of the ubiquitin-proteasome pathway that have been shown previously to be altered in the response to disuse atrophy. mRNA abundance of the ubiquitin ligases, MAFbx and MuRF-1, was elevated 3.7- and 2.9-fold, respectively, after 14 days of hindlimb suspension in soleus muscle, and resistance exercise was associated with a decrease of MAFbx and MuRF-1 by 50 and 40%, respectively, compared with HS (Fig. 4A). mRNA abundance of ubiquitin was increased by 2.2-fold with HS and restored to a level not different from control with resistance exercise (Fig. 4A). These data indicate that the relative abundance of components of the ubiquitin-proteasome pathway changed coordinately with changes in muscle size, as expected. A number of studies have shown an increase in the mRNA abundance for subunits of the proteasome under different atrophy-inducing conditions (26, 28, 38, 55), but the expression of these in combination with exercise has not been investigated. Therefore, we measured C2 and C9 proteasome subunit gene expression. C2 and C9 mRNAs increased with hindlimb suspension by 1.5- and 1.6-fold, respectively, and resistance exercise did not have an effect on this elevated expression (Fig. 4B). Therefore, the remodeling of the proteasome under atrophy-inducing conditions is not affected by exercise. Because the expression of ubiquitin ligases MAFbx and MuRF-1 was reduced with exercise, we expected that the total amount of ubiquitinated protein would also be decreased

Table 2. Flywheel-based resistance exercise attenuates muscle atrophy in soleus and gastrocnemius muscles

	Muscle Weight To Body Weight Ratio, mg/g	
	Soleus	Gastrocnemius
<i>Experiment 1</i>		
Control ($n = 9$)	0.414 \pm 0.015	5.113 \pm 0.057
HS ($n = 10$)	0.258 \pm 0.007*	4.176 \pm 0.068*
HSRE ($n = 6$)	0.317 \pm 0.010*†	4.764 \pm 0.089*†
<i>Experiment 2</i>		
Control ($n = 8$)	0.448 \pm 0.017	5.458 \pm 0.064
HS ($n = 9$)	0.265 \pm 0.006*	4.313 \pm 0.061*
HSRE ($n = 8$)	0.302 \pm 0.002*†	4.614 \pm 0.100*†

Values are means \pm SE. HS, hindlimb suspended; HSRE, hindlimb suspended and resistance exercised. *Significantly different from control in respective experiment. †Significantly different from HS in respective experiment: $P < 0.05$.

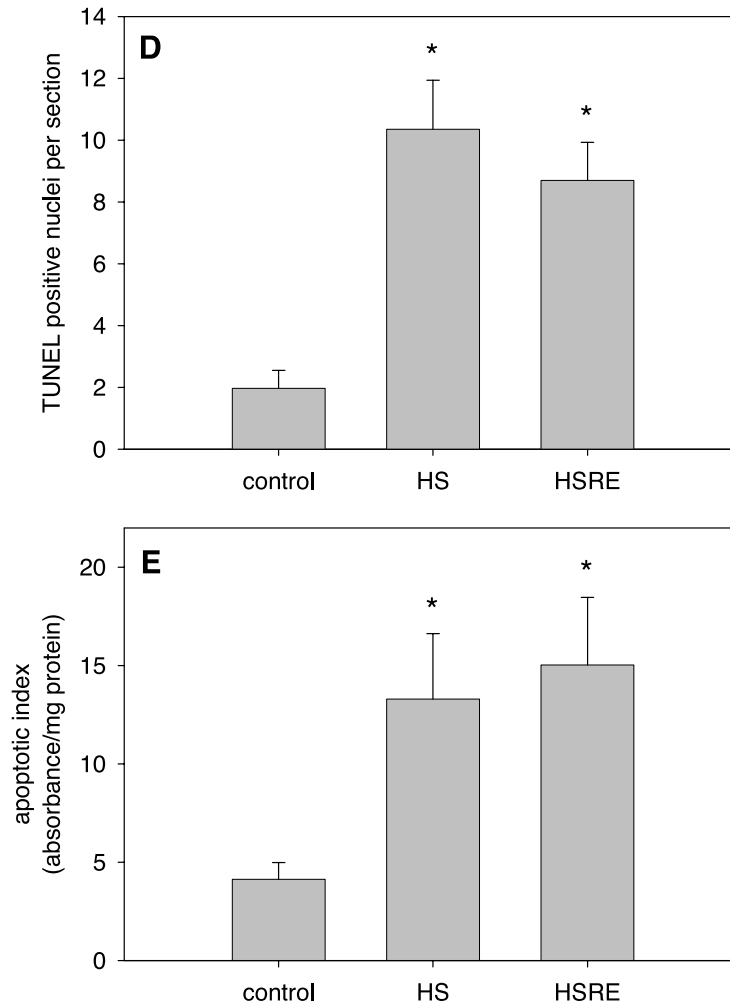
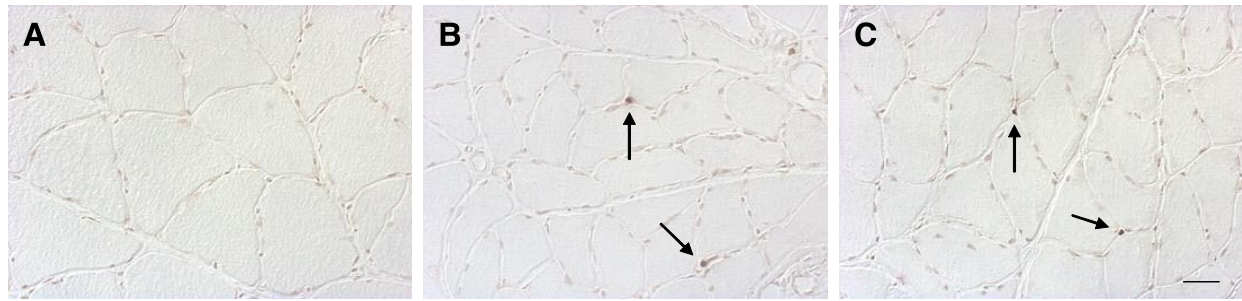


Fig. 2. Apoptosis is increased with HS and remains elevated with HSRE. Representative photographs of control (A), HS (B), and HSRE (C) soleus muscles reacted for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) activity (brown). Arrows indicate positive nuclei. For A, B, and C, bar in C is 25 μ m. D: TUNEL-positive nuclei per whole soleus muscle cross section were counted for control ($n = 9$), HS ($n = 10$), and HSRE ($n = 6$) rats. E: apoptosis quantified by measuring mono- and oligonucleosomal DNA fragmentation in soleus muscle homogenates from control ($n = 9$), HS ($n = 10$), and HSRE ($n = 6$) rats. Values are means \pm SE. *Significantly different from control, $P < 0.05$.

in response to exercise compared with the level in atrophied muscles. Surprisingly, we found that the level of ubiquitinated protein, which was elevated 1.6-fold in response to hindlimb suspension, was further increased an additional 1.7-fold in response to resistance exercise compared with HS (Fig. 5, A and B). This elevation could be explained if proteasome activity was decreased in response to resistance exercise, such that proteins that were ubiquitinated could not be processed through

the proteasome and would accumulate in the muscle cells. Therefore, the three major enzymatic activities of the proteasome, chymotrypsin-, trypsin- and PGPH-like activity, were measured by using specific substrates. No changes were observed in response to either hindlimb suspension or resistance exercise in soleus muscles for all three activities (Table 3). Thus we hypothesized that the ubiquitin ligases responsible for ubiquitin conjugation during atrophy may differ from those

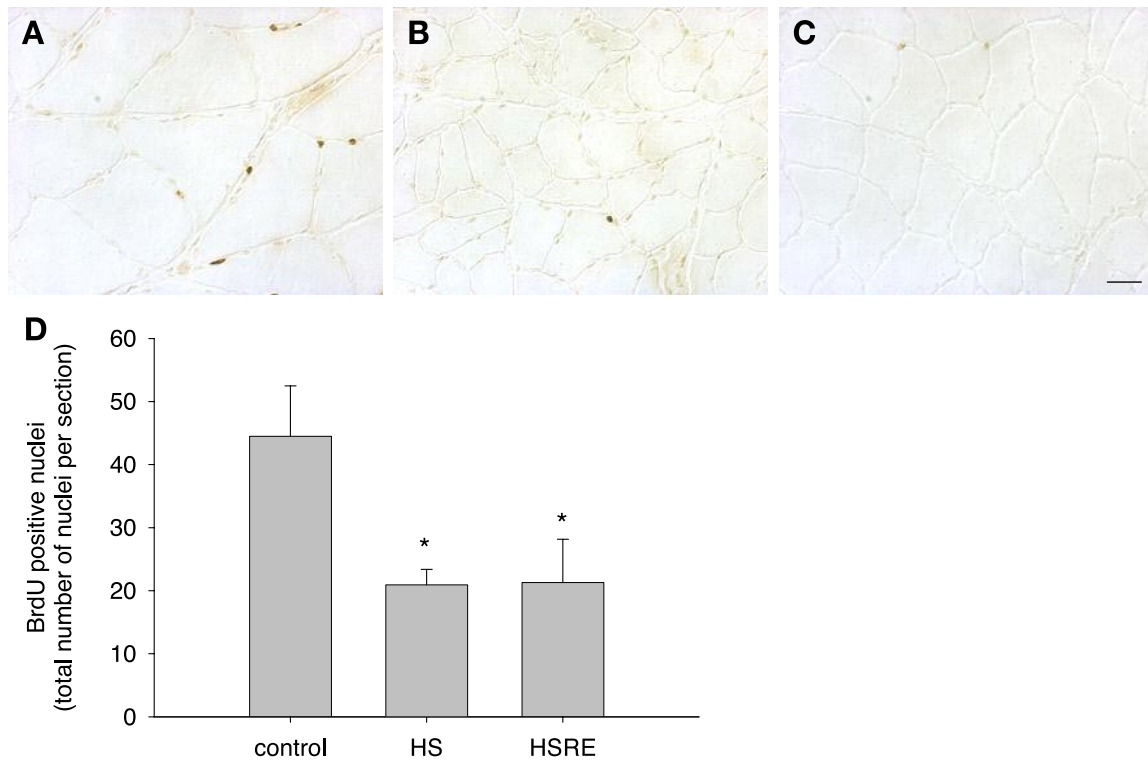


Fig. 3. Cellular proliferation decreases with HS and remains lower with HSRE. Representative photographs of control (A), HS (B), and HSRE (C) soleus muscles immunostained with 5'-bromo-2'-deoxyuridine (BrdU) antibody (brown). BrdU-positive nuclei were counted for whole soleus muscle cross sections from control ($n = 9$), HS ($n = 10$), and HSRE ($n = 6$) rats (D). Values are means \pm SE. *Significantly different from control, $P < 0.05$.

active during exercise, potentially affecting atrophy or other aspects of muscle function. In fact, it has been suggested that there may be as many as several hundred E3 ubiquitin ligases present in cells that remain to be identified (30). Therefore, we investigated the abundance of gene products encoding E3 ligases Nedd4 (neuronal precursor cell-expressed developmentally downregulated-4) and XIAP (X-chromosome-linked inhibitor of apoptosis), and an E2 conjugating enzyme with ligase activity Apollon/BRUCE, compared with MAFbx and MuRF-1. Nedd4 and XIAP gene expression were elevated by 2.5- and 1.5-fold, respectively, with hindlimb suspension and remained elevated with resistance exercise (Fig. 6), in contrast to MAFbx and MuRF-1, which decreased with exercise (Fig. 4). The protein abundance of BRUCE was not changed in HS or HSRE soleus muscle (Fig. 5C). These results indicate that the expression of different ubiquitin ligases is regulated independently in muscles under different loading conditions.

DISCUSSION

Resistance exercise induces hypertrophy of skeletal muscle and has also been shown to decrease the loss of muscle mass under atrophy-inducing conditions, such as hindlimb suspension (2, 27). Because previous models of resistance exercise have involved gravity-dependent models, we developed a flywheel-based exercise methodology for rats, which was modeled after a method that can be used for humans in spaceflight (16). We showed previously that muscle protein synthesis was restored with this form of exercise even though muscle mass was not returned to control (15, 16), suggesting that some processes that promote muscle atrophy may not be responsive

to this form of resistance exercise. It should be noted that, to our knowledge, no exercise intervention applied under atrophy-inducing conditions such as hindlimb suspension or spinal cord injury has been sufficient to restore muscle mass back to control, even though these interventions are known to induce hypertrophy in nondisused muscles (14, 24, 27). In this study, we investigated components of the proteasome pathway of protein degradation, apoptosis, and cell proliferation in response to flywheel-based resistance exercise during hindlimb suspension, and we found that some, but not all, processes associated with atrophy are attenuated with flywheel-based resistance exercise.

Apoptosis was elevated with hindlimb suspension, consistent with previous reports (1, 36), but did not change in response to exercise at the time point studied here. We showed previously that apoptosis was attenuated with cycling exercise after spinal cord injury, and Allen et al. (1) demonstrated that apoptosis decreased with reloading and growth hormone treatment in hindlimb-suspended rats (1, 13). Differences in modes and frequency of exercise and in combination with hormones may explain the observed difference in outcome. The extent of apoptosis as measured by TUNEL in this study was similar to that in previous studies in which atrophy was induced (1, 13, 36). The duration of the apoptotic process is only ~ 2 h (20), and therefore the increase reported in this study constitutes a substantial loss of nuclei, which has been shown to correlate well with the observed loss of myonuclear number (1). Thus, in the present model, apoptosis is a contributor to muscle atrophy, as previously suggested (48), and failure to downregulate

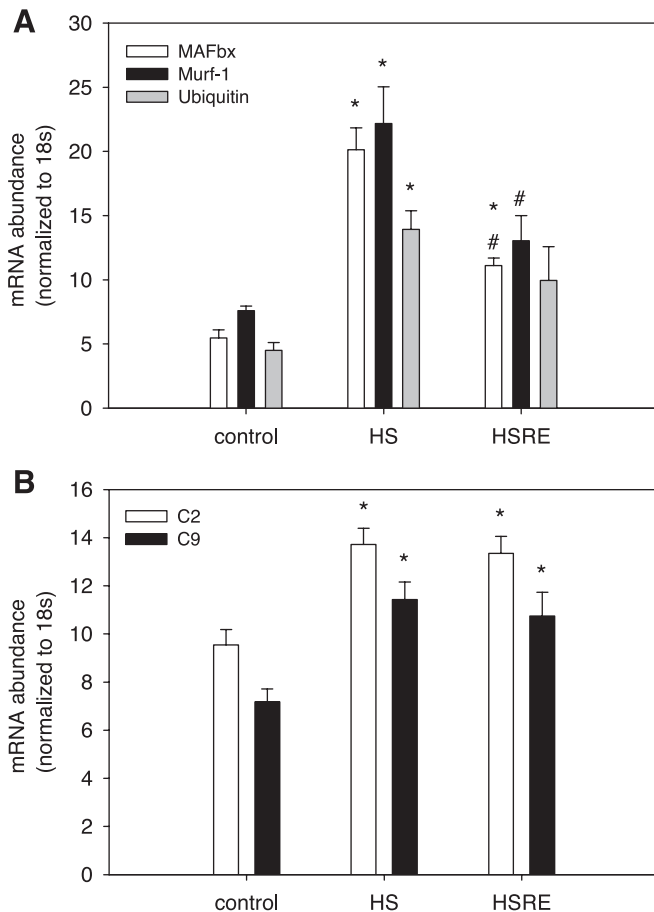


Fig. 4. Distinct changes in gene expression for components of the ubiquitin-proteasome pathway with flywheel-based resistance exercise. *A*: mRNA abundance of muscle atrophy F-box (MAFbx), muscle really interesting novel gene Finger 1 (Murf-1), and ubiquitin in soleus muscle from control ($n = 8$), HS ($n = 9$), and HSRE ($n = 8$) rats. *B*: mRNA abundance of C2 and C9 proteasome subunits in soleus muscle from control, HS, and HSRE rats. Values are means \pm SE. *Significantly different from control; #significantly different from HS, $P < 0.05$.

apoptosis with resistance exercise may contribute to the limited extent of muscle maintenance in this model.

Cell proliferation is required for hypertrophy and for complete regrowth of atrophied muscle after hindlimb suspension (40, 47). Satellite cells are thought to be the source of new nuclei for those lost during atrophy owing to apoptotic nuclear loss. In response to growth stimuli, such as occurs under hypertrophy-inducing conditions, satellite cells become activated and undergo cell division, after which they fuse to existing fibers. Therefore, we assumed that resistance exercise would be associated with maintenance or an increase in cell proliferation in the hindlimb-suspended rats. However, we observed that cell proliferation, which was decreased with unloading, did not change with exercise, indicating that signals that suppress cell proliferation during suspension were not alleviated by flywheel-based resistance exercise. This failure to restore cell proliferation may also contribute to the failure to fully restore muscle size with exercise under atrophy-inducing conditions.

We further found that components of the ubiquitin-proteasome pathway were regulated in a distinct manner under these

experimental conditions. The two ubiquitin ligases, MAFbx and Murf-1, which have been suggested as atrophy markers (21), indeed were associated with the changes in muscle mass. The extent of the changes was similar to those observed with hindlimb suspension previously (3, 24), and these molecules are therefore likely involved in regulating muscle mass in response to hindlimb unloading and exercise. The abundance of mRNA encoding these proteins was measured because this has been shown to be changed coordinately with muscle atrophy. It is therefore assumed that the protein abundance changes concurrently, even though this was not measured here. Our results are also consistent with previous reports that gene expression of ubiquitin is associated with changes in muscle mass (46). However, subunits of the proteasome (C2 and C9),

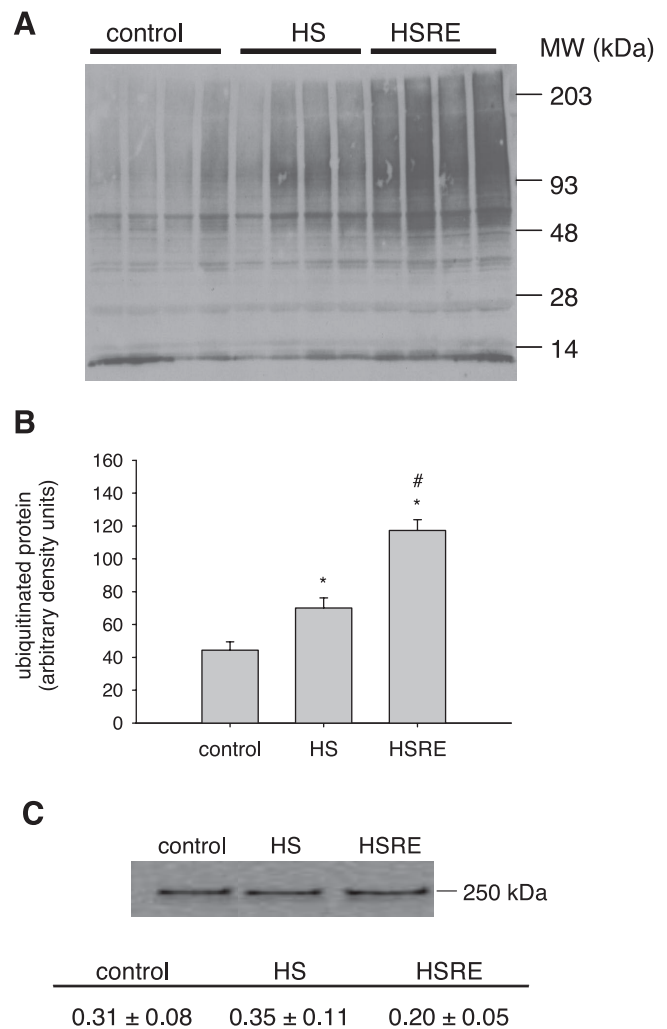


Fig. 5. Ubiquitinated protein accumulation is augmented by flywheel-based resistance exercise, but no change in BIR repeat-containing ubiquitin-conjugating enzyme (BRUCE). *A*: representative total ubiquitin-conjugated protein samples from control, HS, and HSRE soleus muscles. *B*: ubiquitinated protein quantified from Western blots of soleus muscle protein as shown in *A* from control ($n = 9$), HS ($n = 10$), and HSRE ($n = 6$) rats. *C*: representative lanes immunoblotted with BRUCE antibody from control ($n = 9$), HS ($n = 10$), and HSRE ($n = 6$) soleus muscles. Quantified values are depicted in table as arbitrary density units. For all blots protein levels were normalized to the intensity of Ponceau S staining. Values are means \pm SE. MW, molecular weight. *Significantly different from control, #significantly different from HS, $P < 0.05$.

Table 3. No changes in proteasome activity were observed with HS with or without HSRE

	Proteasome Activity, nM·mg protein ⁻¹ ·min ⁻¹		
	LLVY	LSRT	LLE
Control	0.415±0.063	0.423±0.031	0.474±0.077
HS	0.278±0.034	0.426±0.039	0.345±0.038
HSRE	0.344±0.050	0.450±0.031	0.286±0.056

Values are means ± SE for control ($n = 8$), HS ($n = 9$), and HSRE ($n = 8$). LLVY, chymotrypsin-like activity; LSRT, trypsin-like activity; LLE, peptidyl glutamyl peptide hydrolase (PGPH)-like activity.

which previously have been used as markers for atrophy (26, 28, 55), did not exhibit changes in gene expression, as would be predicted by the changes in muscle mass. The atrophic signals that induced the gene expression of these subunits during hindlimb suspension were not altered by exercise, such that the subunit composition of the proteasome in exercised animals remained different from control. Total ubiquitin-conjugated protein, which has also been used as an indicator of muscle protein breakdown and atrophy (46), showed the expected increase with hindlimb suspension but was further elevated by exercise. Indeed, previous studies have shown that ubiquitin-conjugated protein content is increased by exercise and implied that this is due to extensive remodeling of muscles after exercise (52, 58). A similar situation was observed in rats and mice that had been reloaded after hindlimb suspension (35, 54), in which accumulation of ubiquitin-conjugated proteins remained elevated, even though mRNA or protein levels for other proteolytic components were back to control levels and muscle size was increased compared with hindlimb suspended. The increased ubiquitin-conjugated protein could be explained if there was a decrease in proteasome activity with exercise, which would prevent the progression of ubiquitinated proteins through the proteasome. Proteasome activity has been reported to be unchanged in both atrophied gastrocnemius and soleus muscles with atrophy (28, 39, 42, 64), and conflicting results have shown either an increase (45) or a decrease (33) in proteasome activity after exercise. Our results indicate that proteasome activity was not different in soleus muscle 14 days after hindlimb suspension with or without exercise. The fact that proteasome activity was measured in whole muscle homogenates, instead of in isolated 20S proteasome, could be a factor in a lack of response to atrophy. Proteasome activity in isolated 20S proteasome is about threefold higher than in whole muscle homogenates (26, 39). However, proteasome activity was not decreased with muscle atrophy, similar to our findings, in a study in which isolated 20S proteasome was used (39). Also, our reported values are comparable to those in other studies using whole muscle homogenates in which changes were observed in muscles undergoing changes in size (7, 33) and were higher than those measured in the cytosolic fraction only (64). This indicates that the ability to measure changes in proteasome activity does not depend on the isolation technique. Collectively, these findings suggest that a change in proteasome activity does not explain the increase in ubiquitin-conjugated protein.

We investigated whether the expression of ubiquitin ligases was associated with changes during exercise. Nedd4 is an E3 ubiquitin ligase shown to be coregulated with

MAFbx, Murf-1, and ubiquitin during hindlimb suspension (51). Its regulation with exercise has not been investigated. XIAP and BRUCE (also named Apollon or BIRC6) are apoptosis inhibitors with ubiquitin ligase domains capable of the ubiquitination and targeting for proteasome degradation of a variety of proteins mainly involved in apoptosis (25, 53, 59). BRUCE protein expression was not changed in our experimental model, whereas Nedd4 and XIAP were upregulated with hindlimb suspension, regardless of exercise status. The functional significance of this regulation remains to be determined. Because Nedd4 is mainly involved in the degradation and ubiquitination of membrane proteins, it likely plays a role in the remodeling of muscle membrane properties in response to unloading. Interestingly, XIAP promotes the proteasomal degradation of caspase-3 (53), which in turn has been shown to be a likely protease involved in early protein degradation under muscle-wasting conditions (9). Therefore, the increase in XIAP with hindlimb suspension could be a compensatory mechanism to counteract the atrophic signals as has been shown in aged muscle where XIAP is also upregulated in the face of increased atrophy and apoptosis (8, 50). Because E3 ubiquitin ligases are thought to convey substrate specificity and are therefore instrumental in determining which proteins will be ubiquitinated and degraded, we suggest that the elevation of total ubiquitinated protein with resistance exercise may represent a distinct set of proteins from those ubiquitinated during hindlimb suspension only.

In summary, flywheel-based resistance exercise reverses some aspects of the atrophy process, such as the decrease in protein synthesis (15, 16), the increase in gene expression of muscle-specific and atrophy-associated ubiquitin ligases Mafbx and Murf-1, and ubiquitin. However, other atrophy-associated mechanisms, such as proteasomal subunit remodeling, the elevation in apoptosis, and the decrease in cellular proliferation, were unaffected by this form of exercise. Therefore, we suggest that muscle mass maintenance under atrophy-inducing conditions will require an exercise regi-

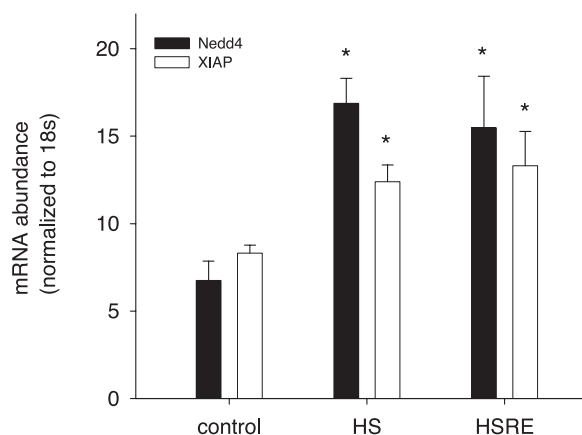


Fig. 6. Flywheel-based resistance exercise does not affect the HS-induced elevation of neuronal precursor cell-expressed developmentally downregulated-4 (Nedd4) and X-chromosome-linked inhibitor of apoptosis (XIAP) mRNA abundance, in contrast to MAFbx and Murf-1 (Fig. 3). mRNA abundance of Nedd4 and XIAP is from soleus muscles of control ($n = 8$), HS ($n = 9$), and HSRE ($n = 8$) rats. Values are means ± SE. *Significantly different from control, $P < 0.05$.

men that not only increases protein synthesis but also decreases apoptosis, restores cell proliferation, and normalizes components of the proteasome. Future research should be directed toward identifying exercise modalities and intensities that will counteract most if not all atrophy-inducing processes.

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GRANTS

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