Increased hypertrophic response with increased mechanical load in skeletal muscles receiving identical activity patterns

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It is often assumed that mechanical load is a primary trigger for muscle hypertrophy during strength training and under experimental overload conditions. There is, however, little direct evidence for an effect of mechanical factors per se, the problem being that, in most physiological and experimental models, force development is closely associated with changes in the pattern of action potentials evoked in the muscle. As reviewed previously (15, 56, 103), evoked electrical activity triggers massive influx and release of calcium from the sarcoplasmic reticulum, and this is known to have profound effects.

In the case of skeletal muscle, the amount of activity is higher when the muscle is immobilized by a cast in a lengthened position rather than a shortened position (14, 40, 43, 44, 47, 66, 81, 95, 105, 118), showing that atrophy can be partly counteracted when limbs are immobilized by a cast in a lengthened position rather than a shortened position (13). However, others have shown that hindlimb suspension changes the electrophysiological properties of the motor neurons (25) and that the integrated EMG levels are significantly reduced throughout the suspension period (13). The most influential evidence for a mechano-dependent mechanism comes from experiments over almost 100 years showing that atrophy can be partly counteracted when limbs are immobilized by a cast in a lengthened position rather than a shortened position (14, 40, 43, 44, 47, 66, 81, 95, 105, 118, 131–133, 147). However, integrated EMG measurements indicated that the amount of activity is higher when the muscle is immobilized in the lengthened position (43, 68), and the duration of the afterhyperpolarization of the motor neurons,
which influences firing frequency, can be altered by the length at which the muscles are immobilized (45).

To eliminate effects of nerve-evoked activity, a few studies have been performed on denervated muscles showing that immobilization in the stretched position (27, 87, 118, 130) or tenotomy of synergistic muscles (67, 123) partly counteracts atrophy even in absence of nerve-evoked activity. Similarly, in tissue culture, stretching of myotubes increases protein synthesis (137) and decreases proteolysis (138).

More recently, attempts have been made to train rat muscles by standardized electrical nerve stimulations to study muscle mechanosensation (2, 63, 144, 145). These studies have, however, failed to yield consistent results. In Wong and Booth (144), the training program failed to produce hypertrophy, and, in the follow-up paper (145), hypertrophy is produced only in the eccentrically contracting antagonist with poor control over both the load and electrical input. Adams et al. (2) indicate that neither load nor movement type (concentric, isometric, eccentric) is important for hypertrophy. In a follow-up study (63), they, however, find changes in the hypertrophy-related signaling molecules myostatin and insulin-like growth factor-I after four training bouts, and interpret these to be differentially affected by movement type.

We here provide evidence that, under identical neuronal activity conditions, differences in mechanical conditions have a major effect on the number of myonuclei and fiber size but not on fiber type. The mechanotransponder effect seems related to myosin mRNAs (137) and decreases proteolysis (138).

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METHODS

Animal experiments. Animal experiments were approved by the Norwegian Animal Research Authority and were conducted in accordance with the Norwegian Animal Welfare Act of December 20, 1974. The Norwegian Animal Research Authority provided a warrant to ensure that facilities and experiments were in accordance with the Act, National Regulations of January 15, 1996, and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes of March 18, 1986. Male Sprague-Dawley rats housed in standard cages with an enriched environment (4 rats/cage) weighing 316–354 g in the beginning of the experiment were used. Inhalation gas anesthesia with 1.2–2% isoflurane in air (MSS Isoflurane with the Surgivet Active Evacuation System) was used for all noninvasive training experiments, and the rats were continuously monitored. For terminal experiments, 2–3% isoflurane was used to maintain a deep anesthesia that was confirmed by pinching the metatarsus region of a limb. Rats were killed by neck dislocation while deeply anesthetized.

Training. The skin of the lower leg of anesthetized rats was shaved, and two platinum skin electrodes measuring 5 × 6 mm covered with electrode gel (Spectra 360; Parker Laboratories) were placed on the skin over the dorsiflexor muscle group [tibialis anterior (TA) and extensor digitorum longus (EDL)] of the right leg about 5 mm apart. The electrodes were connected to a pulse generator (Pulsar 6bp-a/s; HFC).

Each training day, the animals were subjected to a 28-min-long session where the muscles received a total of 900 short tetanic contractions, each contraction lasting about 120 ms. This resulted in the muscles contracting ~6% of the total training time. The training scheme is illustrated in Fig. 1A. Stimulation was delivered in trains of 10 consecutive 0.5-ms symmetrical bipolar pulses delivered at an instantaneous frequency of 150 Hz at amplitude 30–45 V. The force output during the training period was similar to the force produced by supramaximal stimulation directly on the peroneal nerve after the trifurcation of the sciatic nerve, as measured in invasive pilot experiments (Fig. 1B). During a training session, each pulse train was delivered each 600 ms repeated 30 times. Each such series was repeated every 40 s six times. Each such session was delivered every 360 s five times. All rats were trained 3–4 days/wk (on average every other day) for 6 wk for histological analysis. Before this, a 4-day adaptation period with two training sessions consisting of a voltage of 15–20 V with two to three “sessions” with the respective high- and low-load regime was performed. For molecular analyses only one acute training session was performed.

During training, the right leg was attached to a footplate connected to a DC motor (Dual lever-arm system 305C-LR connected to a 600A Digital Controller; Aurora Scientific, Aurora, ON, Canada) allowing the load to be varied during contraction. The left leg was not stimulated and served as an untrained control.
Two groups of 10 rats in each were both given identical electrical stimulation patterns, but one group performed pure isometric contractions (high load) while the other group performed mainly high-velocity (550°/s) concentric contractions (low load) with 50–60% of the peak force produced in the high-load group. The timeframe from 1/2 peak force to 1/2 relaxation was about 60–70 ms. The low-load group performed a short isometric phase in the beginning and end of each contraction with a force of about 1/2 of the peak force during its concentric phase, and thereby about 1/4 of the peak force produced in the high-load group. The high-load and low-load groups were always trained alternately to minimize circadian differences in training response, and the training was always performed during the rats’ dark cycle. The foot was positioned at an angle of 90° relative to the tibia trained alternately to minimize circadian differences in training response in the high-load group. The foot was positioned at an angle of 90° relative to the tibia trained alternately to minimize circadian differences in training response, and the training was always performed during the rats’ dark cycle. The foot was positioned at an angle of 90° relative to the tibia trained alternately to minimize circadian differences in training response, and the training was always performed during the rats’ dark cycle.

**Immunohistochemistry.** Histological analyses were performed on animals subjected to 6 wk of training, and all muscles were taken out 2 days after the last training session, weighed, and frozen by submersion in melting isopentane in a slightly stretched condition. Samples were stored at −80°C followed by cryosectioning at 10 μm.

For myonuclear number and cross-sectional area (CSA) analyses, sections were blocked in 1% bovine serum albumin; EDL was stained with a rabbit anti-laminin polyclonal antibody (L9393; Sigma) while TA was stained with a rabbit anti-dystrophin polyclonal antibody (AB15277; Abcam). The secondary antibody, anti-Rabbit IgG-FITC (F9887; Sigma), was used for both EDL and TA. All three antibodies were used at a dilution of 1:100. Nuclei in TA were finally costained using Hoechst dye 33342 (0.1 μg/ml in PBS; Invitrogen). As discussed previously (16, 57), to ensure that only the nuclei inside muscle fibers were included in the analysis, myonuclei were defined as nuclei with their geometrical center inside the inner rim of the dystrophin ring. For the EDL, a grid was placed over the whole muscle section, and the CSA was determined for each fiber that was located at grid intersections. For the TA, a randomized set of images was chosen, and all fibers in each image were analyzed. A total of 5,744 fibers (97–212/muscle) for TA and 5,687 fibers (49–239/muscle) for EDL were included. Comparable number of fibers was always analyzed for the comparison of trained with contralateral leg.

To investigate whether an increased level of myogenic regulatory factor 4 (MRF4) would lead to hypertrophy in EDL, fibers transfected with expression plasmids containing MRF4 and LacZ were stained for β-galactosidase, and neighboring sections were stained for myosin heavy chains. To correct for variance resulting from differential stretch of the individual muscles, measurements were normalized to those from fibers adjacent to the transfected fibers of the same fiber type.

Analysis of myosin heavy chain fiber type was performed as described previously (89) using the following panel of monoclonal antibodies: BA-D5 (I), SC-71 (Iia), 6H1 (Iix), and BF-F3 (Iib). All were grown in-house from hybridoma stocks obtained from ATCC/LGC Standards. Fiber type composition in EDL was determined from the same fibers as in the CSA analysis (5,167 fibers in total), except from a few fibers that were excluded due to damaged sections.

**RNA analysis.** For the RNA analysis using quantitative reverse transcriptase-PCR (qPCR), muscles were excised 4 h after subjection to one acute training session and stored in RNAlater. Gene-specific primers for reference and target genes were designed as previously described (36, 37), using Primer3 Plus (136). To avoid genomic residuals from affecting gene expression analyses, each primer pair was either located to across an exon-exon boundary containing a large genomic intron (typically >1,000 nucleotides) or made to include at least one primer positioned in an exon-exon boundary, whenever possible. HPLC-purified primers were purchased from Thermo Scientific (Waltham, MA). All primer pairs were tested with primer concentrations of 100 nM and annealing temperature of 60°C. Primer pairs showing the lowest cycle threshold values (Ct), while at the same time showing distinct melting curves, were selected. Primer sequences are given in Table 1.

Total RNA was extracted from TA and EDL muscle tissue using TRIzol reagent (Invitrogen), as previously described (36). RNA quantities were obtained using Nanodrop (Thermo Scientific), whereupon reverse transcription was performed on 1,000 ng total RNA using Superscript III Reverse Transcriptase (Invitrogen, Life technologies, Carlsbad, CA), primed with both random hexamers (Ambion, Life technologies) and oligo(dT) (Ambion, Life technologies), according to the manufacturer’s protocol. cDNA syntheses were performed in duplicates for each sample.

qPCR was performed on 1:25 dilutions of cDNA using Fast SYBR Green Master Mix (Applied Biosystems, Life technologies) and the 7500 Fast Real-Time PCR System (Applied Biosystems, Life technologies), according to the manufacturer’s protocol. Cycling consisted of initial UDG activation at 50°C for 2 min, followed by denaturation at 95°C for 2 min and 39 repeats of 94°C for 3 s and 60°C for 30 s. One qPCR reaction was performed per gene per cDNA synthesis, meaning that two qPCR reactions were performed per gene per muscle biopsy. To avoid artifacts caused by run-to-run differences in qPCR performance, the four experimental groups were always repeated on each qPCR plate (exploring the expression of a particular set of genes). Ct was calculated using the 7500 Fast Real-Time PCR System software or the LinRegPCR software (115), and priming efficiencies were calculated using the LinRegPCR software (115). For final calculations of target gene expression, average priming efficiencies were used, calculated separately for each primer pair.

Target gene expression was calculated using GeNorm (139), making use of normalization factors calculated from the two most stable reference genes, as determined from data on the five frequently used reference genes β2-microglobulin (β2m), peptidylprolyl isomerase A (PPIA, cyclophilin A), β-actin, polymerase (RNA) II (DNA directed) polypeptide A, and ribosomal protein L32 (RPL32). In TA, β2m and RPL32 were found to be most stable. In EDL, β2m and PPIA were found to be most stable. M-values (0.314 and 0.312, respectively) were well below the limit set by Vandesompele et al. (139).

All qPCR statistical tests were performed on log-transformed relative fold changes between the trained leg and contralateral nontrained control. The log transformation was chosen to stabilize variance and equalize the signals from up- and downregulation responses. One-sample tests were used to analyze contrasts between trained and nontrained legs while two-sample tests were used to compare relative fold changes between the high- and low-load treatment groups. All tests used the multiple testing procedures function from the Bioconductor package multtest (104), with default settings: one- or two-sample t-tests with bootstrap-based null hypothesis distributions and the single-step maxT procedure for family-wise error rate control (=0.05 in all tests). Two-sample tests used the Welch adaptation, covering for the possibility of unequal variances between groups. The R statistical computing environment, version 3.1.1 (107), was used for all calculations.

**Protein analyses.** For protein extraction, muscles were excised and snap-frozen in liquid nitrogen immediately after or 6 h after the muscles were subjected to one acute training session. Protein was prepared essentially as described previously (128). In brief, frozen rat TA muscles from the acute training experiment were crushed with a mortar cooled on dry ice, and PBS-based lysis buffer [PBS, pH 7.4, 1% Triton X-100 (Sigma), 0.1% Tween 20 (Sigma) with protease (Complete EDTA-free tablets; Roche) and phosphatase (PhosSTOP tablets; Roche) inhibitors] was added. Muscles were homogenized two times using a Polytron 1200 (IKA Labortechnik T25 basic; Tamirol Lab) and incubated on ice for 30 min, and the supernatant was collected, separated into aliquots, and stored at −80°C after centrifugation.

Protein concentrations were measured using Bio-Rad (5000-0006) or Pierce/Thermo Scientific (Micro BCA Protein Assay Kit) assays according to protocols. For analysis of syndecan-4 protein, muscle
Table 1. Primers used for gene expression analyses

<table>
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<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<td>PPIA</td>
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<tr>
<td>Notch1</td>
<td>ACATGCAGGAGCCCTGTATG</td>
<td>ACTGACAGCAGCGACACGC</td>
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qRT-PCR, quantitative reverse transcriptase-PCR. Primers are listed in the 5′→3′ direction. β2m, β2-microglobulin; PPIA, peptidylprolyl isomerase A; PolR2A, polymerase (RNA) II (DNA-directed) polypeptide A; RPL32, ribosomal protein L32; MyHC, myosin heavy chain; PGC1α s1 and 4, peroxisome proliferator-activated receptor-γ, coactivator-1α, splice variants 1 and 4; TTN, titin; SYNE1, synaptic nuclear envelope protein 1/myosin repeat-containing, nuclear envelope 1; NF-κB1, nuclear factor-xB p105 subunit; PAX7, paired box 7; MCAD, M-cadherin; LMNA sA and sC, lamin splice variants A and C; ITGA7, integrin, α7; Ankrd2, ankyrin repeat domain 2; MLIP, muscular LMNA-interacting protein; ACTA1, actin, α1, skeletal muscle; MYOG, myogenin/myogenic factor 4; MYOD, myogenic differentiation 1/myogenic factor 3; MRF4, muscle regulatory factor 4, also known as myogenic factor 6; MYF5, myogenic factor 5; SYND4, syndecan 4.

Lysates were treated with heparan sulfate-degrading enzymes essentially as described (19, 128). In brief, proteoglycans from 10 μg of muscle lysate were precipitated in methanol overnight at −20°C, and the pellet was washed in acetone and resuspended in heparitinase buffer (50 mM HEPES, pH 6.5, 50 mM NaOAc, 150 mM NaCl, and 5 mM CaCl2) with four enzymes as follows: heparitinase I, heparitinase II, heparitinase III, and chondroitinase cABC (all from AMSBIO, Abington, UK). Samples were incubated two times for 2 h and overnight at 37°C following addition of a second and third aliquot of primary antibodies and, subsequently, with species-appropriate horseradish peroxidase-conjugated secondary antibodies diluted in blocking solution. Blots were developed using ECL Prime (Amersham/GE Healthcare) and visualized in Las-4000 (Fujifilm) or Kodak Image Station 4000R Pro. Membranes were reprobed after stripping using the Restore Plus Western Blot Stripping Buffer (PI-46430; Thermo Scientific) according to protocol. Processing and quantification of blots were performed using Adobe Photoshop CS5 and ImageJ (National Institutes of Health), respectively.

Primary antibodies used were anti-phospho-Akt (Ser473, catalog no. 9271; Cell Signalling), anti-pan-Akt (catalog no. 9272; Cell Signaling), anti-phospho-S6K1 (Thr389, catalog no. 9206; Cell Signaling), anti-pan-S6K1 (catalog no. 2708; Cell Signaling), anti-syndecan-4 cytoplasmatic epitope (mapped to amino acids 175–198 in NP_035651, custom made; Genscript) (41), and anti-phospho-syndecan-4 (Ser79, custom made; Genscript) (41). To study cellular shedding of syndecan-4, the antibody detecting the cytoplasmic epitope of syndecan-4 was used to detect the 10- to 15-kDa syndecan-4 fragment remaining in the cell after shedding of the extracellular heparan sulfate-substituted fragment. This approach has been validated previously (109, 128). Positive controls for immunoblotting consisted of Akt Control Cell Extract (9273; Cell Signaling Technology) (data not shown) and HEK-293 cells transfected with an NH2-terminally tagged syndecan-4 (NP_035651) in the pcDNA3.1 vector (custom made by Genscript, Piscataway, NJ) using Lipofectamine, as described previously (41, 128). Coomassie Brilliant Blue R (27816; Sigma) staining was used as loading control.

MRF4 overexpression. To identify transfected muscle fibers in vivo, pAP-lacZ was used to overexpress β-galactosidase. The plasmid contains the Escherichia coli β-galactosidase coding sequence driven by the Rous sarcoma virus promoter (78). For overexpression of MRF4, the MRF4
coding sequence was cloned into the pAP-vector by removing the β-galactosidase coding sequence, cutting it with BamHI and HindIII. To test for expression of MRF4, human embryonic kidney cells (HEK-239) were transfected with pAP-MRF4+pAPlacZ (MRF4) or pAP-lacZ (sham), using the Lipofectamine2000 kit (Invitrogen). The MRF4 protein was extracted from these cells and visualized by Western blotting with a MRF4 specific antibody (kindly donated by A. Buamano) and a secondary goat anti-mouse IgG antibody (Southern Biotech).

In vivo transfection of the plasmids was performed as previously described (89, 94, 106). Briefly, the EDL was exposed surgically, and 100 μl DNA mixture were injected through a Hamilton syringe to the center of the muscle. The DNA mix contained 50 μg pAP-LAcZ and 50 μg pAP-MFR4 for MRF4 overexpression, or 50 μg pAP-Lacz only for sham transfection. The muscle was then subjected to an electrical field created by a pulse generator (Pulsar 6bp-a/s; Frederick Haer). Two silver electrodes placed 3 mm apart were moved along the muscle, five trains of bipolar pulses (200 μs) with a peak-to-peak voltage of 50 V were run across the muscle, and the rat was sutured and placed in a cage for recovery.

**Statistics.** All statistical analyses except the qPCR analysis (see qPCR methods section) were done using GraphPad Prism6. Repeated-measures two-way ANOVA with Tukey’s multiple-comparisons test was performed to compare fiber type distributions between experimental groups. One-way ANOVA with Holm-Sidak’s test for multiple comparisons was performed on all other tests between the trained leg and contralateral nontrained control, between the high-load and low-load group and in MRF4 overexpression data.

**RESULTS**

Load had an effect on muscle mass, fiber size, and the number of myonuclei. Rat body weight increased by an average 30% over the training period, with no significant differences
from untrained age-matched controls or between the two experimental groups (Fig. 2A).

There was a significant increase in muscle wet weight of the EDL and TA for the high-load group, when compared with the untrained contralateral leg (Fig. 2B). On average low-load-trained EDL and TA muscles were 6% heavier. High-load training increased the mass in the EDL by 12% and TA by 15%. Thus, high load doubled the effect of the training on muscle mass.

Low-load training had no effect on fiber size in EDL, but in TA the fiber CSA was increased by 18%. High-load training had a larger effect, and fiber size increased by 16 and 33% in EDL and TA, respectively (Fig. 2C).

In the TA we also counted the number of myonuclei per fiber on cross sections. The number of nuclei was increased by 18 and 37% in the low- and high-load groups, respectively (Fig. 2D). Thus, the increase in the number of nuclei was quantitatively similar to the increase in cytoplasmic volume, suggesting that cytoplasmic domain volumes remained constant in our model. Representative micrographs illustrating effects on fiber size and number of myonuclei are shown in Fig. 2, E and F.

Training, but not load, affected muscle fiber type. Due to its size and fast fiber type composition, the EDL was chosen for analysis of changes in fiber type. Training affected fiber type composition in the slow direction (Fig. 3); in the trained EDL, the number of 2b fibers was reduced from 31–41% to 3–5% while the number of 2x/2b hybrids and pure type 2x fibers increased in both training groups. There was no significant difference in fiber type composition between the low- and high-load groups. Thus, while the training shifted the fiber type profile from 2b to 2x (but not 2a), this was not affected by load.

Training induced Akt and S6K1 phosphorylation independent of load. The protein kinase Akt and its downstream effector S6K1 are activated by phosphorylation and thought to be key molecules regulating muscle hypertrophy through the Akt/mTOR/S6K1 pathway (11, 56, 121). Moreover, both Akt and S6K1 have previously been suggested to be activated by mechanical stress (70). We thus hypothesized that there would be a stronger activation of Akt and S6K1 in the high-load group. Consistent with Akt and S6K1 activation, in muscles excised immediately after an acute training session with our hypertrophy model, the level of phosphorylated Akt increased 2.7- and 3.5-fold in the low- and high-load group, respectively, while S6K1 increased 12- and 15.8-fold in the low- and high-load group, respectively. The levels of phosphorylated Akt and S6K1 were still elevated 6 h after the training session, although only S6K1 in the low-load group significantly so, Akt 1.5- and 1.4-fold and S6K1 3- and 1.7-fold in the low- and high-load group, respectively (Fig. 4). The low- and high-load-training regime had no significant differential effects on the total Akt or S6K1 protein levels. Thus, training activated Akt and S6K1, and more so in the high-load group, but there was no statistically significant effect of the increased mechanical conditions.

Syndecan-4 expression, phosphorylation, and shedding were not influenced by training or load. Syndecan-4 is a transmembrane proteoglycan providing a bridge between the extracellular matrix and cytoskeleton at focal adhesion points (143). It is localized to costameres and Z-discs (140), and it acts cooperatively with integrins (117). Syndecan-4 has been suggested as a mechanosensor in cardiac muscle, regulating hypertrophy and nuclear factor of activated T cells (NFAT) activation (41), and has been shown to activate the Akt/mTOR pathway (35). However, training had no significant effect on syndecan-4 protein levels in TA (Fig. 5, A and C). We then investigated whether load affected syndecan-4 phosphorylation, since phosphorylation of Ser179 in its cytoplasmic domain is known to regulate downstream calcineurin-NFAT activation in heart muscle (41). In contrast to findings in cardiac muscle, we were unable to detect the inactive phosphorylated form in skeletal muscle (Fig. 5B). The phosphorylated form was readily detected in our positive control, lysates from HEK-293 cells overexpressing syndecan-4. We also investigated whether there was shedding of the syndecan-4 ectodomain, an enzymatic process believed to be important for syndecan function (93, 129). We did not observe shedding in our experiments (Fig. 6B).

Training increased levels of several mRNAs while load differentially affected only MRF4 and myogenin. To investigate the effect of training and load on gene transcription, we selected 27 genes and measured mRNA levels with qPCR. The selection represented genes that we hypothesized could be important in signaling cascades coupling mechanical factors to hypertrophy such as proteins connected to the extracellular matrix (79), satellite cells (74, 124), costameres (8, 28, 30, 38), the nuclear envelope (52, 92, 99, 141), and transcription factors involved in muscle plasticity (56). Four hours after a single training session, a majority of the selected genes were upregulated, 3 (EDL) and 10 (TA) of them significantly (Fig. 6A). Among those were the transmembrane receptor syndecan-4, ankrd2, laminA, and several transcription factors, including the myogenic factors myogenin and MRF4.

It was a general trend that most of the RNAs we had selected were upregulated in the high-load group compared with the low-load group. MRF4 and myogenin were among the most
highly affected in both EDL and TA, but only the 2.1-fold increase of MRF4 in EDL and 2.3-fold increase of myogenin in TA reached statistical significance (Fig. 6B).

Overexpression of MRF4 did not induce hypertrophy. The RNA analysis pointed to myogenin and MRF4 as transcription factors possibly inducing hypertrophy. In previous publications we have overexpressed myogenin in transgenic mice (71) and in adult muscle fibers after electroporation (33), and this led to a slight atrophy. We thus electroporated an expression vector for MRF4 into muscle fibers of rat EDL muscle and compared with sham electroporation. After 14 days, there was no effect of MRF4 overexpression on fiber size in any fiber type (Fig. 7).

DISCUSSION

Mechanical factors are important for hypertrophy. We show for the first time that, under identical activity conditions, mechanical factors play a major role for the effect of training on muscle hypertrophy, indicating the existence of mechanorelated activity-independent signaling pathways.

Our findings are supported by recent data from a complex experimental intensive care unit (ICU) model (76, 108) where sedated mechanically ventilated rats were treated with α-cobratoxin to silence the neural input. Passive unilateral maximal ankle joint flexion-extension cycles partly counteracted the inactivity-induced atrophy. After 14 days, there was no effect of MRF4 overexpression on fiber size in any fiber type (Fig. 7).

Mechanical factors are important for myonuclear recruitment. It is well established that satellite cell activation and recruit-

ment of myonuclei take place during a variety of hypertro-

phy conditions (17, 69, 75, 90, 110, 111, 119, 120, 126). Our data suggest that mechanical factors per se contribute to satellite cell activation independent of the activity pattern due to the concurrent standardization of the neural input and differentiation of the mechanical parameters/output. Mechanical factors activating satellite cells might therefore be a prerequisite for the following increase in myonuclear number and fiber size (Fig. 2).

Mechanical load is not important in exercise-induced fiber type switches. In the present study, varying degrees of mechanical load had no effect on muscle fiber type distribution. This is in contrast to the conclusions of several previous studies where immobilization of fast muscles in a lengthened position (48–50, 87, 101), or overload elicited by synergist ablation (53, 54, 72, 77, 97, 100, 102, 113, 134), supposedly induced changes in the slow direction. However, as discussed in the Introduction, in all these cases the pattern of electrical activity was most likely also altered, and there is substantial evidence that electrical activity influences fiber type (12, 56).

In agreement with previous experimental findings demonstrating that increased amounts of electrical activity induced a shift toward slow fiber types (55, 58), we observed that training had an effect in the slow direction. Although we cannot exclude that mechanical conditions other than those applied in the present study might influence muscle fiber type, there is to our knowledge currently no strong in vivo evidence (1, 20–22)
for a mechano-dependent regulation of myosin heavy chains, and we suggest that such properties are mainly regulated by electrical activity.

Validity of experiment compared with previous reports: the importance of load. A major difference between the current work and previous strength training experiments using electrical stimulation (6, 22, 46, 63, 146) is the short duration (10 pulses, 150 Hz) of each individual contraction, which maximizes the difference in peak force development between our two loading groups. A certain peak force threshold might therefore be one of the factors initiating hypertrophy through mechanotransduction in our experiment. In line with the belief that load per se is important for hypertrophy (see Introduction), we postulate that load is in fact the basis for the observed differences between our loading groups rather than the isometric vs. concentric contraction mode. This is supported by a study that found similar anabolic signaling responses to strength training comparing eccentric, isometric, and concentric contractions with all modes of contraction having equal electrical activation and similar force integral (46). Results from Wong and Booth (146) indicate that differences in lengthening velocity during eccentric contractions in TA did not affect the hypertrophic response. This indicates that mode of contraction might be less important than load for inducing hypertrophy. Some early works using different combinations of denervation, synergist ablation, and tenotomy also indicate that mechanical load per se is an important factor for hypertrophy (62, 83, 91, 112, 123).

Some molecular pathways involved and not involved in mechanical signaling. Akt has been regarded as a key regulator of muscle hypertrophy by increasing translation via GSK3, mTOR, and S6K1 and by decreasing proteolysis via forkhead box O (1, 39, 56, 96, 116, 121). It has also been suggested that S6K1 in addition can be activated by mechanical stress independent of Akt (70, 80, 127, 148, 149). We found a strong activation of both Akt and S6K1 by phosphorylation right after a single training session, but there was no significant difference between the low- and high-load groups. Thus, while Akt and S6K1 might well be involved in muscle hypertrophy, they seem not to be vital for the differential effect of low and high load on the hypertrophy observed in the present study. It can, however, not be excluded that a larger difference in load or measurements at different time points could reveal a significant role for Akt and S6K1 in load-mediated hypertrophy, given the trend toward a higher activation in the high-load group. This is supported by the results from Kalmagi et al. (76), who found both phosphorylated and total Akt upregulated by passive mechanical stimuli per se in their rat ICU model. Importantly, they see these changes after 9–14 days of mechanical loading, but not at earlier time points.

Our findings suggest the existence of a mechanosensing mechanism related to the hypertrophic response in skeletal muscle. While such mechanisms have been poorly investigated in skeletal muscle, they have been more extensively studied in the heart, where it has been suggested that syndecan-4 acts as a molecular mechanosensor mediating the hypertrophic response to overload in this organ (9, 42). Syndecan-4 null mice displayed attenuated cardiac hypertrophy (41), and in hypertrophic heart tissue from pressure-overloaded mice (42) and patients (41) syndecan-4 protein levels were elevated. Syndecan-4 has also been shown to act as an immediate-early gene in smooth muscle cells following changes in mechanical stress (84). In skeletal muscle the protein is strongly reduced after denervation in vivo, and by nerve tetrodotoxin-impulse block of myotubes in vitro (135). Satellite cells, which are involved in muscle hypertrophy, express syndecan-4 at high levels (26).
Fig. 6. Training and load affect TA and EDL mRNA expression 4 h after an acute training session. *A*: relative mRNA fold changes in trained vs. nontrained contralateral leg. $\log_{10}$, $\log_{10}$ (trained/control). Blue circles denote statistically significant difference in trained compared with nontrained contralateral control.

*B*: relative mRNA fold changes in low- vs. high-load-trained muscles. Log$_{10}$/Log$_{10}$ (trained high load/control)/Log$_{10}$/Log$_{10}$ (trained low load/control). Red circles denote statistically significant differences in high load compared with low load after correction against their respective contralateral controls. $P < 0.05$; $n = 8$. 

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**Trained versus contralateral control**

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**Low versus high load**

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Our data provide no evidence for the involvement of syndecan-4 in the early events of training-induced hypertrophy, since protein levels were unchanged 6 h after a single training session although mRNA levels were increased. There were, however, no significant differences between the low- and high-load groups.

Syndecan-4-dependent signaling is rendered inactive by phosphorylation of the cytoplasmic domain, and this phosphorylation is reduced in hypertrophic heart tissue (41). We did not detect the phosphorylated form in skeletal muscle, precluding this as a relevant regulating mechanism in this tissue. Another posttranslational modification mechanism of syndecans that occurs in the heart is the ectodomain shedding that seems to cause reduced focal adhesion (128). We did not detect the shedded form in skeletal muscle. We conclude that the syndecan-4 biology of skeletal muscle and heart seems to be very different and that syndecan-4 does not seem to be involved in load signaling in skeletal muscle.

The RNA analysis pointed to the involvement of myogenic factors in the differential effects of load on hypertrophy, in particular myogenin and MRF4. This could either be related to activation of these genes in myofibers or in satellite cells. Myogenic factors are potent in selectively activating muscle-specific genes during development, and also seem to take part in regulating adult gene expression and phenotype (33, 34, 61, 71). We have previously shown that, when helix-loop-helix factors are inhibited by overexpressing Id-1 in muscle fibers of transgenic mice, fibers become atrophic in a dose-dependent manner (60), and it was suggested that this was caused by inhibiting the myogenic factors. However, when myogenin was overexpressed with the same expression cassette in transgenic mice (71), or in adult muscle fibers after electroporation (33), fiber size was reduced. Moreover, all of the myogenic factors are strongly upregulated during denervation atrophy (18, 23, 31). When we overexpressed MRF4 in the present study, it had no effect on fiber size. Negative results are, however, hard to interpret; myogenic factors might act in concert or they can be rendered inactive by posttranslational modifications, for example, overexpression of MyoD altered fiber type only when phosphorylation was prevented (34).

The myogenic factors are expressed in activated satellite cells, and the number of myonuclei was higher in the high-load group compared with the low-load group. Myogenin and MRF4, which was significantly elevated at the RNA level in the high-load group, are particularly high in satellite cells during fusion and in myotubes after fusion (114, 150). Also, when satellite cells were ablated by irradiation in quail muscles stretch overloaded for 3 days, myogenin mRNA level from total muscle homogenates fell to about 1/4 of nonirradiated muscles (88), indicating that a substantial amount of the myogenin mRNA present in hypertrophying skeletal muscle comes from satellite cells. We therefore speculate that the high levels of myogenin and MRF4 reflect a mechanoeffect on late stages of satellite cell activation that contributed to the hypertrophy.

Conclusion and future perspectives. In conclusion, we demonstrate that mechanical stimuli per se enhance the hypertrophic response to electrically stimulated strength training. We suggest that the concurrent increase in myonuclear number is important in this response through satellite cell activation via myogenin and MRF4 signaling. In contrast to hypertrophy, fiber type seems to be determined by the neural input independent of load. The present strength-training model can be further used to delineate important mechanotransduction pathways.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


