Low-frequency stimulation regulates metabolic gene expression in paralyzed muscle

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Petrie M, Suneja M, Shields RK. Low-frequency stimulation regulates metabolic gene expression in paralyzed muscle. J Appl Physiol 118: 723–731, 2015. First published January 29, 2015; doi:10.1152/japplphysiol.00628.2014.—The altered metabolic state after a spinal cord injury compromises systemic glucose regulation. Skeletal muscle atrophies and transforms into fast, glycolytic, and insulin-resistant tissue. Osteoporosis is common after spinal cord injury and limits the ability to exercise paralyzed muscle. We used a novel approach to study the acute effect of two frequencies of stimulation (20 and 5 Hz) on muscle fatigue and gene regulation in people with chronic paralysis. Twelve subjects with chronic (>1 yr) and motor complete spinal cord injury (ASIA A) participated in the study. We assessed the twitch force before and after a single session of electrical stimulation (5 or 20 Hz). We controlled the total number of pulses delivered for each protocol (10,000 pulses). Three hours after the completion of the electrical stimulation (5 or 20 Hz), we sampled the vastus lateralis muscle and examined genes involved with metabolic transcription, glycolysis, oxidative phosphorylation, and mitochondria remodeling. We discovered that the 5-Hz stimulation session induced a similar amount of fatigue and a five- to sixfold increase (P < 0.05) in key metabolic transcription factors, including PGC-1α, NR4A3, and ABRA as the 20-Hz session. Neither session showed a robust regulation of genes for glycolysis, oxidative phosphorylation, or mitochondria remodeling. We conclude that a low-force and low-frequency stimulation session is effective at inducing fatigue and regulating key metabolic transcription factors in human paralyzed muscle. This strategy may be an acceptable intervention to improve systemic metabolism in people with chronic paralysis.

spinal cord injury; health quality; diabetes; metabolic syndrome; electrical stimulation

An inflexible muscular system is a primary characteristic of the diabetic phenotype (5, 34, 36, 59), as up to 70% of glucose utilization may occur at the skeletal muscle level (5). People with spinal cord injuries have an impaired ability to activate their skeletal muscle voluntarily, which contributes to their overall poor metabolic state. Confounding their condition is the severe osteoporosis, which affects the ability to apply high-frequency evoked muscle forces via electrical stimulation.

Neuromuscular electrical stimulation is a time-honored method that challenges the physiological state of skeletal muscle tissue. Historically, a primary goal of electrical stimulation training is to increase the size (hypertrophy) of the underlying skeletal muscle (20, 41, 55). Recent emerging evidence for nondisabled people supports that long-duration, low-force physical activity may have a greater stabilizing influence on a muscle’s metabolic state compared with short-duration, high-intensity activity, even if the total energy expenditure is identical (10, 21, 31).

We sought to understand whether the same amount of electrical stimulation energy (no. of pulses at the same intensity) across two different frequencies would yield different fatigue and regulation of metabolic genes in skeletal muscle. For example, at a low frequency of stimulation (5 Hz), paralyzed muscle is not fused so that the force is low; however, at a higher stimulation frequency (20 Hz), paralyzed muscle is fused, and a higher muscle force develops. We controlled the peak muscle force for each frequency by testing the muscle in a shortened position, assuring the safety of the underlying skeletal system. Because a higher stimulation frequency sustains the calcium transient compared with the lower frequency stimulation, this study probed the question as to whether the frequency of calcium release (and subsequent muscle force) regulates key genes associated with muscle metabolism (11, 42, 51). To date, one study suggests that a high-intensity muscle force is a prerequisite to modulate peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), a major transcription factor regulating muscle metabolism through mitochondrial biogenesis (24).

Our goal was to examine key groups of genes known to be associated with the diabetic phenotype of skeletal muscle (5, 44, 47). Therefore, we focused on acute stress genes associated with oxidative transcription [PGC-1α, nuclear receptor subfamily 4 group A member 3 (NR4A3), interferon-related developmental regulator 1 (IFRD1), and actin-binding rho-activating protein (ABRA)] (24, 38, 44, 49). Next, we examined genes that are typically longer adapting that are known to regulate muscle contractile speeds [fast: MYL5, MYL6, ACTN3, and parvalbumin; slow: (MYH)6, MYH7, MYL2, and MYL3](45, 49, 50). Finally, we focused on genes that regulate

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metabolic pathways, such as glycolysis [pyruvate dehydrogenase kinase 4 (PDK4), PDHA1, PDHB, and PDHX] (7, 49), fatty acid oxidation (ACADVL, ACADL, ACAD8, and ACAD9) (49, 58), tricarboxylic acid cycle (BRP44, BRP44L, OGDH, and SDHB) (6, 49), oxidative phosphorylation (NDUFB1, NDUFA2, CYC1, and COQ10A) (27, 49), and mitochondrial remodeling (MFF, OPA1, MFN1, and MFN2) (7, 35, 46, 49).

Accordingly, the purpose of this study was to compare the effect of low- and high-frequency stimulation on muscle fatigue and metabolic gene expression in human paralyzed muscle. We expected that the low-force stimulation (5 Hz) would induce significant fatigue and upregulate key genes associated with metabolism. If supported, this intervention offers a strategy to regulate skeletal muscle in people with severe osteoporosis from spinal cord injury.

METHODS

Subjects. Twelve subjects with chronic (>1 yr) and complete spinal cord injury (ASIA A) had the quadriceps twitch force assessed before and after electrical stimulation with a 5- or 20-Hz protocol on 2 separate days (Table 1 and Fig. 1, sessions A and B). A subset of six randomly chosen subjects participated in two additional sessions where a vastus lateralis muscle biopsy was performed bilaterally 3 h after a unilateral session of 5 or 20 Hz of electrical stimulation; each session only one leg was stimulated, and the opposite leg served as a control. No subject reported previous participation in electrical stimulation for 6 mo prior to this study. Subjects with a history of lower-limb fracture, peripheral nerve injury, pressure ulcers, or history of muscle injury were excluded. Informed consent was obtained from all subjects as per the University of Iowa Institutional Review Board’s approval.

Twitch force assessment of fatigue. The muscle twitch force before and after each stimulation protocol (5 or 20 Hz) was measured using a custom apparatus, as described previously (48). A lower-stimulation frequency of 3 Hz was used to assess the single-twitch properties before and after the 5- and 20-Hz protocols, as depicted in Fig. 1, sessions A and B. The 3-Hz stimulation frequency was used because the muscle remains unfused at this frequency even after fatigue has induced significant change in the muscle contractile speed (48). All 3-Hz-generated muscle forces were obtained with the subject positioned in a chair, with the hip, knee, and ankle flexed to 90° and the ankle secured in series to a load cell (1500ASK0299; Interface, Scottsdale, AZ). Two 7 × 13 cm oval carbon electrode pads (EMPI, St. Paul, MN) were placed over the proximal and the distal anterior thigh to deliver stimulus pulses to the quadriceps muscle, as described previously (17, 18, 48). A constant current stimulator (Digitimer model, DS7A; Digitimer, Welwyn Garden City, Hertfordshire, UK) delivered 200-μs pulses under software control. A data acquisition board (NI USB-6221-BNC; National Instruments, Austin, TX) digitized the analog signals from the load cell and stimulator at a sampling rate of 2,000 Hz.

To warm up the muscle and establish the supramaximal stimulation intensity, single stimulus pulses were delivered at increasing intensities (maximal capacity: 300 mA). When there was no further increase in muscle twitch force, with increased stimulation intensity we increased the stimulation current an additional 25% to assure supramaximal muscle stimulation. We assumed that nearly all muscle fibers were activated as supported by our previous M-wave analysis (1, 12, 20, 52, 53). Three minutes after the supramaximal stimulation, a single bout of 200 stimulus pulses was delivered at a 3-Hz stimulation frequency (48). This protocol was delivered 5 min before and immediately after the 5- or 20-Hz protocol on different days, as shown in our protocol timeline (Fig. 1, sessions A and B). The peak twitch force was assessed to determine the physiological influence of the 5- and 20-Hz stimulation.

Test stimulation protocols (5 and 20 Hz). The knee was extended (10° short of being straight) for the delivery of the 5- and 20-Hz protocols. This position enabled us to minimize the overall peak force that develops at the 20-Hz stimulation condition to study people with chronic paralysis (and subsequent osteoporosis) (43). Figure 2 illustrates an example of the force-time curves with the stimulation pulses of one contraction using the 5- and 20-Hz stimulation protocols.

The 5- or 20-Hz electrical stimulation intervention consisted of 20 bouts of 50 contractions. Each contraction consisted of 10 stimulus pulses delivered at a frequency of either 5 or 20 Hz. A work/rest ratio of one on and two off was maintained for both stimulation protocols. The total number of stimulus pulses (10,000) and relative time...
between contractions was identical between the 5- and 20-Hz protocols. The 5-Hz stimulation session (1.6 h) was four times longer than the 20-Hz stimulation session (0.42 h). A 1-min rest followed each bout of 50 contractions.

**Muscle biopsy and exon microarray protocol.** A subset of subjects (n = 6) underwent bilateral percutaneous muscle biopsies 3 h after the 20- and 5-Hz electrical stimulation protocols on separate days (Fig. 1, sessions C and D). All gene expression signals from the stimulated leg were normalized to the same subject’s opposite leg muscle that did not receive the stimulation on that day (20 or 5 Hz). Upon return ≥1 mo later, the control leg from the first session received the stimulation protocol (20 or 5 Hz), and the opposite leg served as the control. The leg that received the stimulation was always biopsied first, followed by the control limb that did not receive stimulation.

We have described in detail the biopsy procedure in a previous report (1). Briefly, percutaneous muscle biopsies were taken from both the intervention and control vastus lateralis muscle of each subject using a Temno biopsy needle (T1420; Cardinal Health) under ultrasound guidance within a sterile field. Five passes of the needle were made to assure a wide sampling range within the muscle. Each pass of the needle was through the same incision site, but we altered the needle angle to sample a different part of the muscle. Because of severe muscle atrophy, each pass generated less than ~20 mg of muscle tissue. Following harvest, we placed the muscle biopsy samples in RNALater (Ambion) and stored them at -80°C until further use.

The RNA extraction procedure has been described previously (48). Briefly, RNA was extracted using the RNeasy Fibrous Tissue Kit (Qiagen) with DNase to remove genomic DNA from final samples. Microarray hybridizations were performed at the University of Iowa DNA Facility, as reported previously (1, 48). Briefly, 50 ng total RNA was converted to SPiA amplified cDNA using the WT-Ovation Pico RNA Amplification System version 1 (cat. no. 3300; NuGEN Technologies, San Carlos, CA) according to the manufacturer’s recommended protocol. The amplified SPiA cDNA product was purified through a Qiagen MinElute Reaction Cleanup column (cat. no. 28204; Qiagen) according to modifications from NuGEN. Four micrograms of SPiA amplified DNA was used to generate ST-cDNA using the WT-Ovation Exon Module version 1 (cat. no. 2000; NuGEN Technologies) and again cleaned up with the Qiagen column, as described above. Five micrograms of this product was fragmented (average fragment size = 85 bases) and biotin labeled using the NuGEN FL-Ovation cDNA Biotin Module version 2 (cat. no. 4200; NuGEN Technologies) as per the manufacturer’s recommended protocol. The resulting biotin-labeled cDNA was mixed with Affymetrix eukaryotic hybridization buffer (Affymetrix, Santa Clara, CA), placed onto Human Exon 1.0 ST arrays (part no. 900650), and incubated at 45°C for 18 h with 60 rpm rotation in an Affymetrix Model 640 Genechip hybridization oven. Following hybridization, the arrays were washed, stained with streptavidin-phycocerythin (Molecular Probes, Eugene, OR), and signal amplified with anti-streptavidin antibody (Vector Laboratories, Burlingame, CA) using the Affymetrix Model 450 Fluidics Station. Arrays were scanned with the Affymetrix Model 3000 scanner with 7G upgrade, and data were collected using the GeneChip operating software version 1.4. All microarray data are minimal information about microarray experiments compliant and have been submitted to the Gene Expression Omnibus (accession no. pending).

**Exon microarray analysis.** The Affymetrix Human Exon 1.0 ST arrays were normalized using a Robust Multi-array Average (RMA) and transformed into a log2 hybridization signal using Partek Genomic Suites (version 6.6; Partek, St. Louis, MO). The log2 hybridization signals were analyzed using an expression-profiling technique. All mRNA transcripts with log2 hybridization signals less than two standard deviations below the mean signal intensity for all subjects were discarded from the analysis, restricting the analysis to only those mRNA transcripts with a high signal relative to background. A paired sample t-test was used to find mRNA transcripts, with a significant difference between the stimulated and nonstimulated limb with a stimulation frequency. All mRNA transcripts with a P value of <0.05 were used to assess fold change differences. The fold change was calculated by subtracting the log2 hybridization intensity of the nonstimulated control limb from the log2 hybridization intensity of the stimulated limb. Any mRNA transcripts with a mean fold change (FC) >2.0 or <0.667 were considered differentially expressed. If the FC was <1, then the negative inverse of the FC is reported to indicate that a gene is less expressed in the stimulated limb compared with the nonstimulated limb.

**Quantitative PCR procedure and analysis.** The muscle samples were homogenized in lysis buffer using a tissue homogenizer and hard tissue grinding tip (Omni), followed by a column-based RNA extraction using the RNeasy Fibrous Tissue Kit (Qiagen). DNAse was used to ensure absence of genomic DNA in the samples. The RNA samples were eluted in water and quantified via the nanodrop method. Each sample was assayed using the Agilent 2100 Bioanalyzer and reverse transcribed using high quality RNA samples from an iScript supernormix (Bio-Rad). All input quantity of RNA for all reactions was standardized (500 ng each). At the time of quantitative (q)PCR plate preparation, we diluted the cDNA samples fivefold in water and analyzed them via SYBR green technology using a custom PrimePCR plate (Bio-Rad), followed by an analysis in duplicate at a final concentration of 0.5 ng/μl cDNA per 10-μl reaction using an ABI 7900 machine. All expression levels were converted to a relative FC of the acutely stimulated limb to the control limb using the comparative Ct method. β2-Macroglobulin served as the reference gene because it demonstrated a consistent expression across all limbs. qPCR was used on a subset of genes, including NR4A3, ABRA, and myostatin (MSTN), to validate the microarray data.

**Statistical analysis.** A two-way analysis of variance with repeated measures was used to compare the maximum twitch force generated before and after the 5- and 20-Hz exercise. Stimulation frequency (5 or 20 Hz) and time were independent factors. The Tukey procedure was used for all post hoc analysis. A t-test to determine whether the intervention increased or decreased gene expression was employed. Regression analysis was used to calculate coefficients of determination (R-squared) to assess any relationships between fatigue and gene regulation. An α-level of 0.05 was used to test for significance. Data are reported as means ± SD.
RESULTS

Effect of stimulation protocol on muscle fatigue. The peak muscle force for the cohort was 2.68 ± 0.27 times higher for the 20-Hz stimulation compared with the 5-Hz stimulation (P < 0.001). The peak single twitch forces before and after the 20-Hz sessions were 30.92 ± 5.52 and 12.22 ± 4.13 N, respectively (P < 0.001), whereas the peak single-twitch forces before and after the 5-Hz sessions were 27.58 ± 4.42 and 12.06 ± 3.3 N (P < 0.001), respectively (Fig. 3). The magnitude of fatigue was not different between the two stimulation frequencies (P = 0.31), supporting that a low-force stimulation protocol (5 Hz), distributed over time (1.6 h), induced a similar level of muscle fatigue to that of a high-force protocol (20-Hz) delivered in a short time (0.42 h). Importantly, the number of pulses (energy) delivered to the muscle was constant.

Highest regulated gene expression after 5- or 20-Hz stimulation. We analyzed more than 17,000 genes 3 h after a session of 5 and 20 Hz of electrical muscle stimulation; 1,084 and 748 genes had unadjusted P values <0.05 for the 5- and 20-Hz training groups, respectively. Of those, 29 were upregulated with a FC ≥2.0, and 66 were downregulated with a FC less than −1.5 in the 5-Hz group. In contrast, 48 were upregulated with a FC ≥2.0, and 16 were downregulated with a FC less than −1.5. Seventeen genes were upregulated in both groups with NR4A3, PGC-1α, EGR1, ABRA, and GEM in the top 10 most upregulated genes in both groups. The FCs for NR4A3, PGC-1α, EGR1, ABRA, and GEM were 12.40 ± 2.93, 5.09 ± 0.71, 4.87 ± 2.11, 4.56 ± 0.87, and 3.24 ± 0.81, respectively, after 5-Hz muscle stimulation. The FCs for NR4A3, PGC-1α, EGR1, ABRA, and GEM were 9.72 ± 2.79, 4.97 ± 1.44, 9.09 ± 3.61, 3.51 ± 0.66, and 5.77 ± 2.52, respectively, after 20-Hz muscle stimulation. There was no significant difference in the FC between the 5- and 20-Hz exercise sessions for NR4A3, PGC-1α, EGR1, ABRA, and GEM (P = 0.513, P = 0.948, P = 0.553, P = 0.285, and P = 0.238, respectively). Two genes (ABCA10 and TXNIP) were the only downregulated genes common to both groups.

Metabolic transcription and fiber type gene regulation. We investigated the influence of 20- and 5-Hz stimulation on a subset of genes typical of a diabetic phenotype. As described above, there was a significant increase in the expression of major muscle metabolic transcription factors, including PGC-1α, NR4A3, and ABRA, and a significant decrease in the expression of MSTN after 20- and 5-Hz stimulation (Fig. 4, A–D); however, there was no difference between the 20-Hz and 5-Hz condition. This finding is congruent with the similar physiological level of fatigue induced by each protocol despite a 40% lower force generated by the 5-Hz protocol. The acute bout of stimulation did not regulate the fast- or slow-twitch genes for either the 20- or 5-Hz condition (Fig. 4, E–L).

Glycolytic and fatty acid metabolism gene regulation. Both the 20- and 5-Hz condition showed a trend for an increased PDHK4 expression following the stimulation; however, only the 5-Hz condition was significant (P < 0.05; Fig. 5A). There was a significant decrease in PDHA1, but only after the 5-Hz stimulation condition (Fig. 5B). There were no differences detected in any genes associated with fatty acid oxidation.

Oxidative phosphorylation and mitochondrial gene regulation. There were no differences detected in gene expression after 20- or 5-Hz stimulation for the tricarboxylic acid cycle and oxidative phosphorylation genes (Fig. 6); however, there was a trend for a decrease in the expression of COQ10A (P = 0.087), but only after 20-Hz stimulation (Fig. 6H). MFN2 gene was repressed after 20-Hz stimulation (P = 0.038) but was unchanged after 5-Hz stimulation (Fig. 6L). MFN2 gene was repressed after 5 Hz (P = 0.021) but was unchanged after 20 Hz (Fig. 6K). There were no other mitochondrial remodeling genes modulated by the 20- and 5-Hz training (Fig. 6, I–K).

Relationship between gene expression and fatigue. In an effort to understand whether the amount of fatigue among subjects was able to explain the magnitude of change in gene expression, we assessed the relationship between the change in twitch force (fatigue) and the change in gene expression. The coefficients of determination were 0.29, 0.03, 0.10, and 0.69 for PGC-1α, NR4A3, MSTN, and ABRA, respectively. The fatigue after the 20- and 5-Hz protocol was internally consistent among all subjects (Fig. 3).

qPCR validation. A subset of representative genes that regulate metabolism, hypertrophy, and atrophy was analyzed. In all cases, the qPCR analysis confirmed the microarray effects, although the FCs were greater with qPCR. There was a 12.4 ± 2.4 and 200.9 ± 50.4 FC for NR4A3, a 6.0 ± 0.4 and 21.6 ± 6.3 FC for ABRA, a and 0.56 ± 0.06 and 0.42 ± 0.2 FC for MSTN. No differences in gene expression were detected between the 5- and 20-Hz conditions using qPCR analysis, supporting the results obtained from the microarray analysis.

DISCUSSION

We examined the effect of low- (5 Hz) and high-frequency (20 Hz) stimulation on muscle fatigue and mRNA expression in human paralyzed muscle. The objective of the study was to determine whether low-frequency stimulation and the associated low force challenge skeletal muscle to trigger fatigue and the expression of key metabolic gene regulators. The low-
force, 5-Hz stimulation protocol induced a similar amount of fatigue (examined using a 3-Hz single-twitch assessment) as the 20-Hz protocol. Both stimulation protocols caused a robust upregulation of major muscle transcription factors associated with muscle metabolism (PGC-1α, NR4A3, and ABRA). This finding is important, because the 20-Hz protocol induced nearly three times more force than the 5-Hz protocol and suggests that a low-force protocol may be effective in regulating metabolism of skeletal muscle in people with a weakened skeletal system.

Skeletal muscle fibers are generally highly adaptable and capable of altering their metabolic and contractile properties based on the dose of physiological stress (exercise). A skeletal muscle that is regularly stressed will trigger the transformation from fast glycolytic muscle to oxidative fatigue-resistant muscle (1, 12, 20, 52, 53). In the absence of regular physical activity, skeletal muscle becomes less able to metabolize sugar and fat, leading to a diabetic phenotype that contributes to an altered blood/glucose regulation pathway (5, 59). Not surprisingly, individuals with chronic spinal cord injury commonly develop insulin resistance and diabetes (14, 15) and a muscle phenotype that resembles that of nonparalyzed people with diabetes (59). It is logical that people with spinal cord injury may promote improved systemic metabolic health by regular use of their paralyzed musculature; however, the strategy used to activate the muscle is complex because of the muscle force applied to the weakened skeletal system after spinal cord injury. The important finding from this study is that major metabolic gene transcription pathways are regulated at low-force levels compared with high-force levels (5 and 20 Hz).

Skeletal muscle operates at a mechanical disadvantage within the human body. To offset the high external torques from gravity, skeletal muscle must generate high force when moving a limb against gravity. When we consider the limited length of the anatomic lever arm for muscle, it is common for a muscle force to be in excess of six times body weight, a stress that can damage osteoporotic bone (30). Previously, we quantified that people with spinal cord injury lose between 2 and 4% of their bone each month after a spinal cord injury (20), and it is understood that a fracture can be life threatening for people with chronic spinal cord injuries (8). As clinical scientists search for methods to normalize the metabolic state through skeletal muscle activity, they realize that the force should be low to protect the underlying bone. The findings of this study...
support that a low-force exercise, induced through a low-frequency electrical stimulation, is effective at physiologically challenging the skeletal muscle (fatigue) and increasing the expression of several stress response genes, specifically PGC-1α, NR4A3, and ABRA. These genes play a key role in the initiation of transcription and translation of proteins required for oxidative metabolism and are known to induce long-term oxidative muscle adaptations (9, 25, 37, 44).

Exercise is a well-known stimulus of PGC-1α expression in skeletal muscle (23, 24, 40); however, there is a knowledge gap about the actual dose of muscle force necessary to optimally regulate this gene. Previous reports support the finding that high-intensity exercise increases PGC-1α expression (23, 24, 40) to a greater extent than lower-intensity exercise (23, 40). However, these studies generally measure the intensity of whole body exercise while on a stationary bicycle using a percentage of the synergistic muscle workload (oxygen consumption). An inverse relationship between PGC-1α expression and whole body high-intensity exercise, as reported recently, supports that there may be methodological issues, like incomplete muscle fiber recruitment, affecting the biopsy sample in humans (23). Because we control the input to the vastus lateralis through supramaximal electrical stimulation, we were confident that nearly every fiber was activated, reducing the risk of us sampling muscle that had not been exercised. We also delivered the identical number of stimulation pulses (10,000) but manipulated the duration of the exercise (time). By positioning the limb in a less than optimal length (knee extension), we could generate a differential force between the 20- and 5-Hz conditions, but at an acceptable stress for people with a fragile skeletal system. To our knowledge, no previous studies have directly evaluated a low-force (long duration) or high-force (shorter duration) session of exercise with a controlled stimulation intensity (supramaximal) or pulse number on gene expression in human skeletal muscle.

In people without spinal cord injury, a long-duration, low-intensity voluntary exercise performed throughout the day caused an improved sensitivity to insulin compared with a high-intensity exercise even at similar levels of energy expenditure (2, 21, 22). We designed this study using several important translational principles to assess the effect of a low-force to a high-force muscle contraction on gene regulation. First, we reduced the total shear force of the muscle on the skeletal system by extending the knee and by placing the quadriceps in a less than optimal length (43). Second, we preserved a force differential between the 20-Hz condition and the 5-Hz condition in the knee extension position. On average, the 5-Hz condition generated around 40% of the force produced at 20 Hz, although both muscle forces were nearly 25% lower with the knee in the more extended position (compared with the 90° flexed position in pilot data from our laboratory). Third, we maximally activated nearly all fibers of the muscle to eliminate any issue of biopsy sampling error from incomplete muscle fiber recruitment. Finally, we discovered that we induced a similar level of muscle fatigue when we delivered a long-duration, low-force exercise compared with a high-force, short-
er-duration exercise. Taken together, these findings are novel because they represent the first study to control for several factors that are essential to understanding gene regulation in human muscle. However, these findings are not without important methodological considerations. For example, we used just one time point (3 h) to assess the effects on gene regulation, an approach necessary to limit tissue injury in people with spinal cord injury but one that limited our ability to generalize these findings to other time points.

In people without spinal cord injury, muscle fibers are recruited only as they are needed to complete a given task (3, 4, 32). Low-intensity exercise requires fewer muscle fibers, whereas high-intensity exercise recruits nearly all muscle fibers. Because fewer muscle fibers are active during low-recruitment exercise, the ratio of activated to nonactivated muscle fibers is often uncertain in the absence of electromyogram recordings. Therefore, the high-intensity exercise and subsequent full muscle recruitment typically increases the probability that biopsied fibers will demonstrate the response to exercise. Because we maximally activated the entire muscle at a low frequency (low force), we were able to examine a novel “physiological condition” that does not occur during voluntary activation of human muscle. Indeed, nearly 100% recruitment of a muscle at a low force is novel and enabled us to address the question of whether peak force is an integral part of regulating these major metabolic gene transcription pathways. Our findings suggest that low force over a long duration is a robust modulator of these metabolic genes, a new finding that has important implications today for people with spinal cord injury.

The absolute mechanism contributing to the signaling of these pathways remains elusive. Calcium release and sequestration within a muscle fiber determines the number of cross-bridges activated and force developed during a muscle contraction. The CaMK II pathway, which is triggered by calcium, is thought to signal the transcription and translation of PGC-1α and other metabolic regulators (11, 24, 39, 51). However, the mechanical transduction stimulus has also been hypothesized to be essential to trigger muscle signaling pathways (28), perhaps from the drop in ATP and increase in AMP (26). In this study, we controlled the amount of energy delivered to the muscle between the low- and high-force exercises. The number of stimulus pulses delivered during each condition was constant; therefore, the number of times the muscle released calcium was consistent. The major difference between the 5-Hz and 20-Hz conditions was the time required to deliver the stimulus pulses and the resulting contractile force. Taken together, our results support that the mechanical load differen-

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**Fig. 6.** Expression of tricarboxylic acid cycle, oxidative phosphorylation, and mitochondrial fission/fusion genes following acute or chronic stimulation. A–D: genes associated with the tricarboxylic acid cycle were unaltered 3 h after low- and high-frequency muscle stimulation. E–H: most genes associated with oxidative phosphorylation were unaltered 3 h after either low- or high-frequency muscle stimulation; however, COQ10A was decreased after high- but not low-frequency electrical muscle stimulation. I and J: most genes associated with the mitochondrial fission or fusion were unaltered 3 h after either low- or high-frequency muscle stimulation. However, MFF was decreased in most subjects after low-frequency muscle stimulation, and MFN2 was decreased in most subjects after high-frequency muscle stimulation. Fold change values represent the mean of the exercised limb compared with the opposite (nonexercised) limb from the same subjects. †P < 0.05 for a within-group paired *t*-test; ‡P < 0.10 for a within-group paired *t*-test.
tial delivered in this protocol had a minimal influence on regulating the metabolic gene transcription factors in paralyzed muscle.

Summary and conclusions. Oxidative skeletal muscle is a critical tissue for systemic metabolic health. After a spinal cord injury, oxidative skeletal muscle transforms into a glycolytic muscle consistent with a diabetic phenotype, and the skeletal system becomes osteoporotic within the first year after injury. We demonstrated that a low-muscle force exercise caused significant muscle fatigue and a robust increase in the expression of PGC-1α and other metabolic transcription factors that transform skeletal muscle into an oxidative phenotype. The findings from this study hold promise that this intervention may be an effective training protocol that does not increase the risk of bone injury in people suffering from chronic spinal cord injury. Future studies are also underway to understand the full impact of daily low-force muscle training on overall systemic metabolic health in people with paralysis.

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DISCLOSURES
The authors report no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

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