Neuromuscular electrical stimulation increases muscle protein synthesis in elderly type 2 diabetic men

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1Department of Human Movement Sciences, NUTRIM School for Nutrition, Toxicology and Metabolism, Maastricht University, Maastricht, The Netherlands; 2Jessa Hospital, Heart Centre Hasselt, Hasselt, and Hasselt University, Faculty of Medicine, Diepenbeek, Belgium; and 3Department of Cardiac Intensive Care and Interventional Cardiology, Heart Centre, Hasselt, Belgium

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Wall BT, Dirks ML, Verdijk LB, Sijnders T, Hansen D, Vranckx P, Burd NA, Dendale P, van Loon LJ. Neuromuscular electrical stimulation increases muscle protein synthesis in elderly type 2 diabetic men. Am J Physiol Endocrinol Metab 303: E614–E623, 2012. First published June 26, 2012; doi:10.1152/ajpendo.00138.2012.—Physical activity is required to attenuate the loss of skeletal muscle mass with aging. Short periods of muscle disuse, due to sickness or hospitalization, reduce muscle protein synthesis rates, resulting in rapid muscle loss. The present study investigates the capacity of neuromuscular electrical stimulation (NMES) to increase in vivo skeletal muscle protein synthesis rates in elderly type 2 diabetes patients. Six elderly type 2 diabetic men (70 ± 2 yr) were subjected to 60 min of one-legged NMES. Continuous infusions with l-[ring-13C6]phenylalanine were applied, with blood and muscle samples being collected regularly to assess muscle protein synthesis rates in both the stimulated (STIM) and nonstimulated control (CON) leg during 4 h of recovery after NMES. Furthermore, mRNA expression of key genes implicated in the regulation of muscle mass was measured over time in the STIM and CON leg. Muscle protein synthesis rates were greater in the STIM compared with the CON leg during recovery from NMES (0.057 ± 0.008 vs. 0.045 ± 0.008%/h, respectively, P < 0.01). Skeletal muscle myostatin mRNA expression in the STIM leg tended to increase immediately following NMES compared with the CON leg (1.63- vs. 1.00-fold, respectively, P = 0.07) but strongly declined after 2 and 4 h of recovery in the STIM leg only. In conclusion, this is the first study to show that NMES directly stimulates skeletal muscle protein synthesis rates in vivo in humans. NMES likely represents an effective interventional strategy to attenuate muscle loss in elderly individuals during bed rest and/or in other disuse states.

aging; disuse; neuromuscular electrical stimulation; type 2 diabetes

AGING IS ASSOCIATED WITH A PROGRESSIVE LOSS of skeletal muscle mass that is often referred to as sarcopenia (22). The loss of muscle mass reduces strength, impairs functional capacity, and increases the risk of development of chronic metabolic diseases such as obesity and type 2 diabetes (22). Moreover, the onset of type 2 diabetes in elderly individuals generally further exacerbates sarcopenia (53). Sarcopenia is facilitated by a combination of factors, which include a reduced sensitivity to anabolic stimuli (15, 45), a less than optimal diet (20), and a more sedentary lifestyle (20). Indeed, elderly people who also suffer from type 2 diabetes tend to have an even more pronounced lower physical activity status (36). Furthermore, elderly individuals frequently experience short periods of muscle disuse following limb immobilization or bed rest due to injury or illness causing rapid muscle loss (26, 44), with elderly type 2 diabetes patients being particularly susceptible to periods of hospitalization due to complications (36). In addition, elderly individuals also display a reduced capacity to fully regain their muscle tissue during subsequent rehabilitation (61). Consequently, muscle loss during periods of disuse can contribute substantially to the loss of muscle mass and strength that is observed with aging.

Loss of skeletal muscle mass is secondary to an imbalance between muscle protein synthesis and breakdown rates. The loss of skeletal muscle mass observed during a period of bed rest (23, 44, 62) or lower limb immobilization (16, 26, 28, 30) has been attributed primarily to a reduction in basal and/or postprandial muscle protein synthesis rates. Preventing excessive muscle tissue loss (2, 12, 24, 47, 52, 64). However, under many clinical conditions (e.g., hospitalization due to illness or injury), maintenance of physical activity is often not practical or even feasible. Therefore, surrogate interventional strategies that stimulate muscle contraction may be applied to effectively stimulate muscle protein synthesis rates and, as such, attenuate skeletal muscle loss.

Neuromuscular electrical stimulation (NMES) has been proposed as an effective means to maintain some level of muscle function when habitual physical activity cannot be performed (19, 27, 70). Prolonged application of NMES has been reported to attenuate the loss of muscle mass and/or strength in patients recovering from surgery (60, 66, 69), patients suffering from severe cardiovascular complications (6, 58, 67), or patients treated in the intensive care unit (25, 32). Despite the proposed benefits of NMES in a clinical setting, there is little evidence for the proposed impact of NMES on muscle protein synthesis (27, 28).

The present study investigates the capacity of NMES as a means to increase skeletal muscle protein synthesis rates in vivo in older type 2 diabetes patients. We hypothesize that a single bout of NMES can directly stimulate muscle protein synthesis rates and, as such, can be effectively applied to compensate for the reduction in muscle protein synthesis during disuse.

We selected older type 2 diabetic patients since this population is generally more sedentary (36), suffers from accelerated muscle loss (53), and is more likely to be hospitalized for complications (36) when compared with age-matched normoglycemic controls. Neuromuscular electrical stimulation...
was performed for 60 min on the quadriceps of one leg, after which muscle protein synthesis rates were assessed in both the stimulated and nonstimulated leg. Continuous intravenous infusions with L-\([\text{ring-}^{13}\text{C}_6]\)phenylalanine were combined with frequent plasma and skeletal muscle tissue sampling to allow direct measurements of muscle protein fractional synthetic rates. This is the first study to show that NMES directly stimulates skeletal muscle protein synthesis rates in vivo in humans in the acute recovery phase immediately following stimulation.

**METHODS**

**Subjects’ characteristics.** Six elderly (age: 70.3 ± 2.4 yr), sedentary type 2 diabetic males were selected to participate in the present study. Subjects’ characteristics are presented in Table 1. Subjects had been diagnosed with type 2 diabetes for 7.8 ± 1.3 yr, and all were being treated with oral blood glucose-lowering medication [metformin only, n = 2; metformin in combination with sulfonylurea derivatives (SUDs), n = 3; metformin in combination with an SUD and dipeptidyl peptidase IV inhibitor, n = 1]. Five of the six subjects were also being treated with statins. Exclusion criteria were present use of insulin or antplatelet treatments, renal treatment, liver disease, morbid obesity (BMI >40 kg/m²), hypertension (>160 mmHg systolic and/or >100 mmHg diastolic), and a history of either wound-healing issues or severe cardiovascular problems (myocardial infarction within the last year or stroke). Furthermore, volunteers had not participated in any form of regular exercise training for ≥2 yr prior to inclusion. All subjects were informed of the nature of the study and possible risks before they gave their written informed consent to inclusion. All subjects were informed of the nature of the study and possible risks before they gave their written informed consent to participate and were aware that they were free to withdraw from the experiment at any time. The study was approved by the Medical Ethics Committee of Jessa Hospital (Hasselt, Belgium) and was conducted in accordance with the Declaration of Helsinki.

**Screening.** At least 1 wk prior to the study, each subject completed a routine medical screening and filled in a general health questionnaire. After height and weight were measured, body composition was assessed using dual-energy X-ray absorptiometry (Lunar DPXL; GE Medical Systems Benelux, Diegem, Belgium). All participants underwent an oral glucose tolerance test (OGTT). Oral glucose and lipid-lowering medications were maintained in the days preceding the OGTT. After an overnight fast, participants arrived at the laboratory at 08:00 by car or public transportation. A fasting blood sample was obtained, after which an OGTT was performed to determine type 2 diabetes according to World Health Organization criteria (3). Finally, volunteers were familiarized with the NMES protocol (described below).

**Diet and physical activity prior to the experiment.** Volunteers were instructed to abstain from their oral blood glucose and lipid-lowering medications, alcohol, and strenuous exercise for 3 days prior to the experimental visit. In addition, volunteers were instructed to maintain their diet and physical activity as constantly as possible for 3 days prior to the experiments. On the evening prior to the experimental visit, all subjects were provided with the same standardized meal (3.7 MJ, consisting of 62% energy content from carbohydrate, 16% from protein, and 12% from fat), which they consumed at home.

**Experimental protocol.** Volunteers traveled by car or public transport to the Jessa Hospital at 08:00 on a single occasion following an overnight fast. The experimental protocol is depicted in Fig. 1. On arrival, subjects rested in a semisupine position on a bed, after which a Teflon catheter was inserted into an antecubital vein for stable isotope infusion. A second Teflon catheter was inserted into a heated dorsal hand vein of the contralateral arm, and the hand was placed in a hot box (60°C) for arterialized blood sampling (1). Basal arterialized venous blood samples were collected (t = −120 min), after which a single intravenous dose of L-\([\text{ring-}^{13}\text{C}_6]\)phenylalanine (2 μmol·kg⁻¹·min⁻¹; Cambridge Isotopes, Andover, MA) was administered to prime the plasma and muscle free phenylalanine pools. Thereafter, a continuous L-\([\text{ring-}^{13}\text{C}_6]\)phenylalanine infusion (0.05 ± 0.001 μmol·kg⁻¹·min⁻¹) was started (t = −120 min) using a calibrated IVAC 598 pump (Cardinal Health), which ran for 360 min. Arterialized venous blood samples were collected 60 min after the start of the infusion (t = −60 min), after which the NMES protocol was applied to one leg (described below) for 60 min. During NMES, arterialized venous blood samples were collected every 30 min (t = −30 and 0 min). The NMES protocol was terminated at t = 0 min, after which a muscle biopsy sample was collected from the vastus lateralis muscle of both the stimulated (STIM) and nonstimulated control (CON) leg within ~5 min of cessation of the NMES. Thereafter, arterialized venous blood samples were collected at t = 30, 60, 90, 120, 150, 180, 210, and 240 min. Furthermore, muscle biopsy samples were collected from both the STIM and CON leg at t = 120 and 240 min.

**NMES protocol.** Four bipolar self-adhesive neuromuscular stimulation electrodes (10 × 4.5 cm; Enraf Nonius, Delft, The Netherlands) were placed over the distal medial, distal lateral, proximal medial, and proximal lateral areas of the quadriceps muscle groups (to target primarily the vastus lateralis and vastus medialis as well as the rectus femoris) of both legs of each subject, with the subject lying semisupine. However, stimulation pulses were delivered to one leg only (randomized and counterbalanced for left and right) via an En-Stim 4 stimulation device (Enraf Nonius). A biphasic rectangular waveform electrical current was selected with pulse duration of 500 μs, pulse frequency of 60 Hz, and a 3-s contraction/3-s relaxation time that was based on previous recommendations (31). This protocol was selected to ensure that 60 min of NMES was achievable in our subjects before the onset of fatigue (31). The intensity of the stimulus was regulated voluntarily but increased steadily for the first 30 min to maintain proper (visible) muscle contractions and maintained as constantly as possible thereafter while complete contractions were maintained. Volunteers were encouraged to select the maximal intensity that they felt comfortable with. Surface pressure analysis was performed (Microfrod 2; Hoggan Health Industries) with the device fixed against a custom-made leg positioner throughout the stimulation protocol to ensure that the muscle was continually stimulated and that the pressure did not decline over time. Both the STIM and CON legs were fixed to the bed to ensure that no additional voluntary contractions could take place. Both legs were treated identically (except for the electrical stimulation) to prevent any confounding effects due to

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Table 1. **Subject characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tr>
<td>Age, yr</td>
<td>70 ± 2</td>
</tr>
<tr>
<td>Time since diagnosis of type 2</td>
<td>7.8 ± 1.3</td>
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<tr>
<td>diabetes, yr</td>
<td>79.3 ± 2.9</td>
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<tr>
<td>Body mass, kg</td>
<td>26.5 ± 1.0</td>
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<tr>
<td>BMI, kg/m²</td>
<td>29.9 ± 2.2</td>
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<tr>
<td>Body fat, %body mass</td>
<td>55.2 ± 2.2</td>
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<tr>
<td>Lean body mass, kg</td>
<td>6.9 ± 0.6</td>
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<tr>
<td>Basal plasma glucose, mmol/l</td>
<td>13.3 ± 1.5</td>
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<tr>
<td>Plasma glucose OGTT (t = 120 min), mmol/l</td>
<td>16.2 ± 1.6</td>
</tr>
<tr>
<td>Plasma insulin, μmol/l</td>
<td>54.7 ± 13.0</td>
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<tr>
<td>Plasma insulin OGTT (t = 120 min), μmol/l</td>
<td>62.2 ± 0.3</td>
</tr>
<tr>
<td>Hb A₁c, %</td>
<td>52.5 ± 0.9</td>
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<tr>
<td>Oral glucose-lowering medication</td>
<td>Metformin only, n = 2; metformin + SUD, n = 3; metformin + SUD + DPP IV inhibitor, n = 1</td>
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</tbody>
</table>

Values represent means ± SE (n = 6). BMI, body mass index; OGTT, oral glucose tolerance test; HOMA-IR, Homeostasis model assessment of insulin resistance, SUD, sulfonylurea derivatives; DPP IV, dipeptidyl peptidase IV.
handling of the legs (i.e., putting on electrodes, wiring, touching the leg, etc.).

Sample collection and analysis. Articularized venous blood samples (8 ml) were collected into EDTA-containing tubes and immediately centrifuged at 1,000 g for 10 min at 4°C. Aliquots of plasma were then frozen in liquid nitrogen and stored at −80°C for subsequent analysis. Muscle biopsy samples were obtained from the middle region of the vastus lateralis (15 cm above the patella and ~3 cm below entry through the fascia) using the percutaneous needle biopsy technique under local anesthesia (8). Muscle samples were dissected carefully and freed from any visible nonmuscle material. The muscle sample was immediately frozen in liquid nitrogen and stored at −80°C until subsequent analysis. The biopsies collected at t = 0 and 120 min were taken through the same incision, and the biopsy at t = 240 min was taken from a new incision ≥3 cm away from the original site. Biopsies collected from the same incision were taken with the needle in a distal and proximal direction.

Plasma analyses. Plasma glucose concentrations were determined using a commercially available radioimmunoassay kit (InsulinRIA kit; Linco Research, St. Charles, MO), and plasma insulin was measured using a COBAS-FARA semiautomatic analyzer (using a Unit Kit III, 07367204; Roche, Basel, Switzerland), and plasma insulin was measured using a commercially available radioimmunoassay kit (Insulin RIA kit; Linco Research, St. Charles, MO). One aliquot of plasma (100 µl) was deproteinized on ice using 10 µg of dry 5-sulfosalicylic acid, and after centrifugation the supernatant was used to determine amino acid concentrations using HPLC after precolumn derivatization with o-phthalaldehyde analysis (65). For plasma phenylalanine enrichment measurements, plasma phenylalanine was derivitized to its r-butyldimethyl-silyl (TBDMS) derivative, and the enrichment in mixed muscle protein, as described previously (41). Briefly, L-[ring-13C6]phenylalanine was derivitized into the ethoxy-carbonyl ethyl esters (33), and the ratio of labeled to unlabeled derivatives were determined by GC-C-IRMS (MAT 252; Finnigan, Bremen, Germany). Standard regression curves were applied to assess the linearity of the mass spectrometer and to control for the loss of tracer. The coefficient of variation for the measurement of L-[ring-13C6]phenylalanine enrichment in mixed muscle protein averaged 1.1 ± 0.1%.

Total RNA was isolated from 10–20 mg of frozen muscle tissue using Tri Reagent (Sigma-Aldrich) according to the manufacturer’s protocol. Total RNA quantification was carried out spectrophotometrically at 260 nm (Nanodrop ND-1000 Spectrophotometer; Thermo Fisher Scientific), and RNA purity was determined as the ratio of readings at 260/280 nm. Thereafter, first-strand cDNA was synthesized from 1 µg of RNA sample using random primers (Promega) and PowerScript Reverse Transcriptase (Applied Biosystems). Taqman PCR was carried out using an ABI Prism 7000 sequence detector (Applied Biosystems) with 2 µl of cDNA, 18 µl of each primer, 5 µl probe, and Universal Taqman 2 × PCR master mix (Eurogentec) in a 25-µl final volume (Table 2). Each sample was run in duplicate in duplex reactions. The housekeeping gene hydroxymethylbilane synthase was used as an internal control since it was unaffected by the treatments (mean CT values were unaffected by time or across legs; data not shown) and has been used previously in similar studies (14). Taqman primer/probe sets were obtained from Applied Biosystems (Foster City, CA) for the following genes of interest: mammalian target of rapamycin (mTOR), p70 ribosomal protein S6 kinase (p70S6K), myogenin, MyoD, myostatin, muscle atrophy F-box (MAFbx), muscle RING finger 1 (MuRF1), forhead

Fig. 1. Schematic representation of the study protocol. NMES, neuromuscular electrical stimulation.
Table 2. Probes and primer sequences for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Probe</th>
<th>Primer Sequence</th>
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<td>FAK</td>
<td>Hs01056457_m1</td>
<td>GCCAGACCACACAGAGATGGAGA</td>
</tr>
<tr>
<td>FOXO1</td>
<td>Hs01054576_m1</td>
<td>GGCCTAGAACATATTGGCTGAT</td>
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<td>HMBS</td>
<td>Hs00609297_m1</td>
<td>ATCTTGCCACCGGCGCAAGAAA</td>
</tr>
<tr>
<td>MAFbx</td>
<td>mTOR</td>
<td>TGGCGACGGGACAGTCCACAAAGCT</td>
</tr>
<tr>
<td>MuRF1</td>
<td>Hs00261590_m1</td>
<td>AGAGAAGAGACCACTGAAAGGA</td>
</tr>
<tr>
<td>MyoD</td>
<td>Hs00159025_m1</td>
<td>GCAGCGCCAGACAGCCGCGCGC</td>
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<tr>
<td>Myogenin</td>
<td>Hs00231167_m1</td>
<td>CAGCCAGGCGCTCAGCAAGAATG</td>
</tr>
<tr>
<td>Myostatin</td>
<td>Hs00165063_m1</td>
<td>AAAGAGGCTGGATCAAGCTTTTAAAGA</td>
</tr>
<tr>
<td>p70S6kin</td>
<td>Hs01773577_m1</td>
<td>AAGACAGCTGCTGCTTTTACTTGGC</td>
</tr>
</tbody>
</table>

FAK, focal adhesion kinase; FOXO1, forkhead box class O transcription factor 1; HMBS, hydroxymethylbilane synthase; MAFbx, muscle atrophy F-box; MuRF1, muscle RING finger 1; p70 ribosomal protein S6 kinase.

box class O transcription factor 1 (FOXO1), and focal adhesion kinase (FAK). All genes of interest were labeled with the fluorescein reporter 6-carboxyfluorescein. The thermal cycling conditions used were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Ct values of the target gene were normalized to Ct values of the internal control hydroxymethylbilane synthase, and relative fold changes in the CON and STIM legs were calculated at each time point.

Muscle samples (~40 mg) for Western blotting analyses were freeze-dried, and collagen, and blood, and other nonmuscle fiber materials were removed from the muscle fibers under a light microscope. The isolated muscle fiber mass (6–10 mg) was weighed, and seven volumes (7 × dry weight of isolated muscle fibers × wet/dry ratio) of ice-cold buffer (in mmol/l: 20 Tris, 5 EDTA, 10 NaPyrophosphate, 100 NaF, 2 Na3VO4, 1% Nonidet P-40, 3 benzamidine, and 1 PMSF plus 10 μg/ml aprotinin and 10 μg/ml leupeptin) were added (11). The tissue was then homogenized, after which homogenates were centrifuged for 5 min at 1,000 g and 4°C. Thereafter, the supernatant was centrifuged at 10,000 g at 4°C for 10 min, resolved in 1× SDS buffer, and boiled for 5 min at 100°C. Polyclonal primary phosphospecific antibodies [anti-phospho-mTOR (Ser2448), anti-phospho-S6 kinase (Thr223/ Ser244), anti-phospho-S6 kinase (Ser235/240), anti-phospho-eukaryotic initiation factor 4E-binding protein (4E-BP1) (Thr37/46), anti-S6 kinase, anti-RS6, and anti-4E-BP1] were purchased from Cell Signaling Technology (Beverly, MA). Equal amounts of protein (40 μg/lane) were run on either 10% (S6 kinase and RS6) or 15% (4E-BP1) SDS-polyacrylamide and 4–15% Tris-acetate gel (mTOR) (200 V, miniprotein 3 cell; Bio-Rad), and proteins were transferred (2 h, 250 mA,Criterion blotter; Bio-Rad) to 0.45-mm nitrocellulose membranes. After Ponceto S staining and destaining, membranes were blocked for 1 h in 5% nonfat dry milk power (NFDM; Bio-Rad) in Tris-buffereed saline containing 0.1% Tween-20 (TBST). Thereafter, a 1:1,000 dilution of the primary (phospho)specific antibody (all from Cell Signaling Technology) in 5% NFDM-TBST was added and incubated overnight at 4°C on a shaker. After the membranes were washed four times for 5 min in 15 ml of 5% NFDM-TBST, the membranes were incubated with a 1:10,000 dilution of the horseradish peroxidase-conjugated secondary antibody (Pierce) in 5% NFDM-TBST. Thereafter, the membranes were washed in 25 ml of TBST for 5, 15, 5, and 5 min. The Gel-Doc station (Bio-Rad) was used to detect immunoreactive bands using chemiluminescent substrate (SuperSignal CL; Pierce). Pictures were scanned densitometrically, and quantification was performed with the program Quantity One version 4.2.1 (Bio-Rad). α-Actin was used to standardize for the amount of protein loaded. Phosphorylation of mTOR, 4E-BP1, S6K1, and RS6 were expressed relative to the total amount of each protein. Because of the availability of muscle tissue following other analyses, only a complete data set for four subjects was available for the Western blot analyses.

Calculations. The present study involved the infusion of L-[ring-13C6]phenylalanine combined with muscle biopsy and arterialized venous blood sampling to determine the fractional synthesis rate (FSR) of mixed muscle protein. Fractional rate of mixed muscle protein synthesis (FSR) was calculated by dividing the increment in enrichment in the product (i.e., protein-bound L-[ring-13C6]phenylalanine) by the enrichment of the precursor. Plasma L-[ring-13C6]phenylalanine and muscle free L-[ring-13C6]phenylalanine enrichments were used to provide an estimate of the lower and higher boundaries of true FSR, respectively. The formula used was FSR = [ΔEp/Eprecursor × 0] × 100, where ΔEp is the delta increment of protein-bound L-[ring-13C6]phenylalanine during incorporation periods, Eprecursor is the enrichment of the precursor used during the time period for amino acid incorporation determination (i.e., average plasma L-[ring-13C6]phenylalanine, the muscle free L-[ring-13C6]phenylalanine enrichment, or the muscle free L-[ring-13C6]phenylalanine enrichment corrected for the contribution of intracellular water) (7, 71), and t denotes the time duration (h) between biopsies while the equation is multiplied by 100 to express FSR as percentage per hour.

Statistics. All data are expressed as means ± SE. A one-way analysis of variance (ANOVA) was applied to detect changes in plasma glucose, insulin, and amino acid concentrations over time. mRNA and protein expression were analyzed using a repeated-measures ANOVA. When a significant main effect was detected, Bonferroni’s post hoc test was applied to locate the differences. For non-time-dependent variables, Student’s t-test was used to compare differences in treatment effects (STIM vs. CON leg). Statistical significance was set at P < 0.05. Statistical calculations were performed using GraphPad Prism 5.

RESULTS

Plasma glucose and insulin. Plasma glucose and insulin concentrations throughout the experimental protocol are illustrated in Fig. 2, A and B, respectively. From a baseline value of 8.6 ± 0.9 mmol/l, blood glucose concentrations decreased significantly throughout the experiment to 6.6 ± 0.5 mmol/l (P < 0.01). Baseline plasma insulin concentrations averaged 19.5 ± 2.4 mU/l and did not change during the experiment.

Plasma phenylalanine concentration and enrichment. The time course of plasma phenylalanine concentration and plasma L-[ring-13C6]phenylalanine enrichments are reported in Fig. 3, A and B, respectively. Plasma phenylalanine concentrations averaged 72.8 ± 1.2 μmol/l and did not change significantly during the experiment (P = 0.95). In line with this, plasma L-[ring-13C6]phenylalanine enrichments did not change over time and averaged 0.0982 ± 0.0027 [tracer/tracee ratio (TTR)] during the experiment (P = 0.63).

Muscle tracer enrichment. Mean plasma L-[ring-13C6]phenylalanine enrichments, δ-muscle free L-[ring-13C6]phenylalanine enrichments, and δ-muscle protein-bound L-[ring-13C6]phenylalanine enrichments during the first 2 h (0–2 h), the second 2 h (2–4 h), and the entire 4-h (0–4 h) poststimulation period are presented in Table 3. δ-Muscle free L-[ring-13C6]phenylalanine enrichments did not differ between the STIM and CON leg at any time point. δ-Muscle protein bound L-[ring-13C6]phenylalanine enrichments tended to be 16% greater in the STIM compared with the CON leg during the 0–2 h poststimulation period (P = 0.06) and were 53% greater in the STIM compared with the CON leg (P < 0.01).
Muscle protein synthesis rates. Mixed muscle protein FSRs, with the mean plasma L-[ring-13C6]phenylalanine enrichment as precursor, are displayed in Fig. 4. Mixed muscle FSR tended to be 25% greater during the 0- to 2-h poststimulation period in the STIM compared with the CON leg (0.064 ± 0.014 and 0.051 ± 0.009%/h, respectively, \( P = 0.06 \)) and was 26% greater in the STIM compared with the CON leg during the 2- to 4-h poststimulation period (0.049 ± 0.008 and 0.039 ± 0.008%/h, respectively, \( P < 0.05 \)). Overall muscle protein FSR over the entire 4-h poststimulation period was 27% greater in the STIM compared with the CON leg (0.057 ± 0.008 and 0.045 ± 0.008%/h, respectively, \( P < 0.01 \)).

mRNA expression. Figure 5 displays the fold changes in skeletal muscle mRNA expression of selected genes of interest in the CON and STIM leg immediately following stimulation (0 h) and after 2 and 4 h of recovery. Relative mRNA expression of the CON leg at \( t = 0 \) was set as 1, and all other values are expressed as fold changes compared with the CON leg at \( t = 0 \). Muscle mTOR (Fig. 5A) and p70S6K expression (Fig. 5B) demonstrated a significant effect over time (\( P < 0.05 \)), but no treatment effects or individual differences were detected. No significant differences in FAK (Fig. 5C), FOXO1 (Fig. 5D), MAFbx (Fig. 5E), MuRF1 (Fig. 5F), or myogenin (Fig. 5H) expression were observed over time or between groups. Myostatin (Fig. 5G) expression tended to be higher (\( P < 0.07 \)) immediately following stimulation in the STIM compared with the CON leg (1.63- vs. 1.00-fold, respectively), after which levels declined significantly in the STIM leg after 2 and 4 h of recovery from stimulation (\( P < 0.05 \)). MyoD expression was increased significantly after 2 h of recovery in the STIM leg (0.72- vs. 1.55-fold).

**DISCUSSION**

In the present study, we demonstrate that 60 min of one-legged NMES increases skeletal muscle protein synthesis rates in the stimulated compared with the nonstimulated leg during 4 h of recovery in older type 2 diabetic men. Importantly, this is the first study to show that NMES directly stimulates skeletal muscle protein synthesis.
Interestingly, muscle protein-bound muscle protein synthesis rates in vivo in humans in the acute recovery phase immediately following stimulation.

Disuse muscle atrophy can contribute significantly to the loss of muscle with aging. Elderly individuals have been reported to lose substantial amounts of muscle tissue during only short periods of muscle disuse (17, 21, 44) and generally show a reduced ability to regain muscle tissue lost during subsequent rehabilitation (34, 61). Furthermore, elderly type 2 diabetes patients commonly have an accelerated loss of muscle mass when compared with healthy elderly individuals (53). Clearly, interventional strategies should be developed to offset muscle loss during such short periods of disuse atrophy. Muscle loss during disuse has been attributed primarily to decreased postabsorptive muscle protein synthesis rates (23, 26, 44). NMES may provide an effective surrogate for voluntary muscle contraction and, as such, stimulate muscle protein synthesis rates. We assessed the impact of a 60-min one-legged NMES stimulation protocol on subsequent muscle protein synthesis by comparing fractional muscle protein synthesis rates for 4 h in the stimulated and nonstimulated control leg. In the control leg, muscle protein-bound l-[ring-13C6]phenylalanine enrichment increased to 0.000103 ± 0.000021 after 2 and 4 h, respectively (Table 2), resulting in average muscle protein synthesis rates of 0.045 ± 0.008%/h over the entire 4-h period (Fig. 4). These data are in line with previous findings on resting muscle protein synthesis rates in the postabsorptive state (10, 59). Interestingly, muscle protein-bound l-[ring-13C6]phenylalanine enrichment showed a substantially greater increase in the stimulated leg despite similar plasma and free muscle l-[ring-13C6]phenylalanine enrichments (Table 3). Consequently, muscle protein synthesis rates were ~25% greater in the stimulated leg when compared with the control leg during the first 2 h after electrical stimulation (0.064 ± 0.014 vs. 0.051 ± 0.009%/h). These differences were maintained for the entire 4-h period following stimulation, resulting in 27% greater muscle protein synthesis rates in the stimulated vs. nonstimulated leg (0.057 ± 0.008 vs. 0.045 ± 0.008%/h, respectively; Fig. 4). These data clearly show the advantage of the chosen study design in which each individual served as their own control. It is important to acknowledge that an apparent decline over time (albeit nonsignificant) in the nonstimulated leg may have contributed to the observed positive effect of NMES; however, it is also clear that the temporal response of the stimulated leg was greater at each time point. The difference in muscle protein synthesis rates in the stimulated vs. nonstimulated leg is of a similar value as previous work in our laboratory comparing postprandial muscle protein synthesis rates in an exercised vs. nonexercised leg (71). Of course, the average muscle protein synthesis rates in the stimulated leg remain well below (~40%) the values we reported previously following 4–6 h of recovery from exhaustive resistance-type exercise (40, 42, 43). Nevertheless, the substantial changes in muscle protein synthesis rates in the stimulated vs. nonstimulated leg were much greater than anticipated and highlight the strong anabolic properties of NMES. This suggests that NMES can indeed act as a surrogate for normal physical activity, and therefore, it is of considerable clinical relevance. Because muscle disuse has been reported to reduce both basal (16, 30) and postprandial (9, 30) muscle protein synthesis rates by a magnitude similar to the increase that we report following NMES, it could be speculated that NMES may be able to maintain proper muscle protein synthesis rates and, as such, attenuate the loss of muscle mass during short periods of muscle disuse.

To support the in vivo measurements of muscle protein synthesis, we used the remaining muscle tissue to also assess changes in gene expression of a number of key genes implicated in the regulation of skeletal muscle mass (Fig. 5) and the phosphorylation status of several proteins thought to acutely regulate muscle protein synthesis (Fig. 6). The activity of the signaling cascade controlling muscle protein synthesis is presumed to be determined by the phosphorylation of mTOR and its subsequent downstream activation of p70S6K and RS6. For instance, pharmacological

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**Table 3. Tracer data**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Plasma Amino Acid Enrichment</th>
<th>Muscle Free Amino Acid Pool Enrichment</th>
<th>ΔEnrichment Muscle Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–2 h</td>
<td>l-[ring-13C6]phenylalanine (CON) 0.102 ± 0.009</td>
<td>0.022 ± 0.005</td>
<td>0.000103 ± 0.000021</td>
</tr>
<tr>
<td></td>
<td>l-[ring-13C6]phenylalanine (STIM) 0.102 ± 0.009</td>
<td>0.052 ± 0.006</td>
<td>0.000119 ± 0.000029</td>
</tr>
<tr>
<td>2–4 h</td>
<td>l-[ring-13C6]phenylalanine (CON) 0.093 ± 0.009</td>
<td>0.020 ± 0.003</td>
<td>0.000058 ± 0.000018</td>
</tr>
<tr>
<td></td>
<td>l-[ring-13C6]phenylalanine (STIM) 0.093 ± 0.009</td>
<td>0.020 ± 0.003</td>
<td>0.000089 ± 0.000012*</td>
</tr>
<tr>
<td>0–4 h</td>
<td>l-[ring-13C6]phenylalanine (CON) 0.098 ± 0.008</td>
<td>0.021 ± 0.004</td>
<td>0.000173 ± 0.000029</td>
</tr>
<tr>
<td></td>
<td>l-[ring-13C6]phenylalanine (STIM) 0.098 ± 0.008</td>
<td>0.022 ± 0.003</td>
<td>0.000225 ± 0.000034**</td>
</tr>
</tbody>
</table>

Values are means ± SE. STIM, stimulated leg; CON, nonstimulated control leg. Enrichment expressed as tracer/trace ratio. Significant difference compared with corresponding CON value: *P < 0.05; **P < 0.01.
blockade of the mTOR-signaling pathway prevents the normal obligatory exercise-induced increase in muscle protein synthesis rates (18). In keeping with this, we observed trends for mTOR (Fig. 6A), S6K1 (Fig. 6B), and RS6 phosphorylation (Fig. 6C) to increase immediately after stimulation in the stimulated compared with the control leg. Furthermore, these data revealed that this increase in phosphorylation status of key anabolic signaling proteins in the stimulated leg appeared to persist at 2 and/or 4 h following stimulation. These data are consistent with an early molecular signal to stimulate muscle protein synthesis. Here, the absence of a clear statistically significant effect is most likely due to the low power involved, because there was minimal tissue available for the Western Blotting, meaning only n = 4 are included in these analyses. Although, it is generally accepted that the acute regulation of muscle protein synthesis is posttranscriptional (38, 39, 68), specific gene transcriptional activation after acute exercise of any nature is involved in restoring homeostasis in the muscle and contributes at least in part to skeletal muscle adaptation. Therefore, we also determined mRNA expression of mTOR (Fig. 5A) and P70S6K (Fig. 5B) but, perhaps unsurprisingly, did not observe any differences in either the stimulated or control leg following NMES. In support of this observation, the protein abundance of these intramuscular anabolic signaling proteins tends to remain static after acute anabolic stimuli in younger subjects (29), and muscle protein synthesis rates have been reported to increase in response to physical exercise well before any measurable changes in total mRNA content (68). However, we provide additional evidence in a sedentary population of type 2 diabetics that the transcriptional regulation of these signaling proteins has little influence on the acute stimulation of muscle protein synthesis rates.

Although it is generally believed that the loss of muscle mass during a short period of disuse is attributed primarily to reductions in basal and/or postprandial muscle protein synthesis rates (9, 16, 30), it is likely that changes in muscle protein breakdown also occur and contribute to muscle loss (13, 63). The latter, of course, represents an understatement under more compromised clinical conditions such as cancer cachexia, sepsis, or type 2 diabetes (35). Although the present study focuses on the impact of NMES on the stimulation of muscle protein synthesis rates, we also wished to gain some insight into the potential for a single bout of NMES to alter muscle protein breakdown. Muscle protein breakdown in humans is thought to be mediated primarily by the ubiquitin proteasome system (51). Specifically, the transcriptional regulation of the ubiquitin ligases atrogin-1 (MAFbx) and MuRF1 is presumed to be integral in regulating muscle protein breakdown (35, 51). Here, we did not observe any changes in the muscle mRNA expression of either MAFbx (Fig. 5E) or MuRF1 (Fig. 5F) or their

Fig. 5. Muscle mRNA expression of selected genes in elderly type 2 diabetic men (n = 6) at t = 0, 2, and 4 h following 60 min of 1-legged NMES in both the STIM and CON legs. Relative mRNA expression of the CON leg at t = 0 min was set as 1, and all others are expressed as fold changes compared with the CON leg at t = 0. Values represent means ± SE. Data were analyzed with a 2-way ANOVA (time × leg). †Significantly different from corresponding baseline at t = 0 min (P < 0.05). mTOR, mammalian target of rapamycin; FAK, focal adhesion kinase; FOXO1, forkhead box class O transcription factor 1; MAFbx, muscle atrophy F-box; MuRF1, muscle RING finger 1.
upstream regulator FOXO1 (Fig. 5D) following NMES, which may suggest that muscle protein breakdown was not altered substantially following NMES. This would of course suggest that, clinically, NMES would potentially be an effective modulator of muscle protein synthesis without a parallel (undesired) rise in muscle protein breakdown also being elicited. However, it should be noted that a lack of transient changes in the expression of these genes does not necessitate an absence of alterations in muscle protein breakdown, especially since it has been shown recently that the functional role(s) of MuRF1 likely extends beyond the regulation of muscle protein breakdown (5).

Myostatin is known to be a negative regulator of muscle mass in animals (48, 49) and humans (57) and is thought to act by negatively influencing mTOR signaling (56) and the differentiation of satellite cells via the myogenic regulatory factors (i.e., myogenin, MyoD, MRF4, and Myf5) (4, 50). Consistent with the proposed functional role of myostatin, we report that myostatin mRNA expression declined significantly over 2 and 4 h of recovery in the stimulated leg (Fig. 5G), which coincided with a significant increase in MyoD mRNA expression at 2 h (Fig. 5I). This is in line with previous findings that demonstrate a reduced myostatin (2.2-fold) and increased MyoD (2.0-fold) mRNA expression 4 h after a bout of resistance exercise (55) and decreased myostatin expression (~45%) 24 h after a bout of exercise. It should be noted that we also observed a trend for a somewhat paradoxical increase in myostatin expression (1.6-fold) immediately following stimulation in the stimulated leg. This suggests that the subsequent downregulation of myostatin may actually be a normalization of its expression. Moreover, this may also indicate that muscle anabolism only began to increase following the cessation of NMES. Taken together, our data indicate that a single session of 60 min of NMES is capable of inducing molecular changes that are consistent with an anabolic stimulus.

In the present study, we selected a more compromised subpopulation of elderly men who are more likely to be confronted with a period of hospitalization. Even in these compromised individuals, we have demonstrated the efficacy of NMES in stimulating muscle protein synthesis. Although we venture that similar or even greater effects would be observed in healthy elderly men, we can only speculate on this since we did not include a nondiabetic control group. Another consideration of the present study was that we assessed mixed muscle protein synthesis. It would be of interest for future studies to determine the effect of NMES on the specific synthesis of subfractions of proteins within skeletal muscle. Most notably, given the proposed application of NMES in attenuating disuse muscle atrophy, it would be of benefit to determine whether NMES specifically stimulates the synthesis of the myofibrillar proteins in skeletal muscle. Finally, it is important that future studies also closely address various different NMES protocols to elucidate the optimal intensity and duration of the stimulation to achieve the desirable effects on muscle anabolism.

Our data provide a rationale for the clinical use of NMES (25, 28, 54) by demonstrating a direct stimulation of muscle protein synthesis. As such, electrical stimulation may be applied effectively to compensate for detriments in basal and/or postprandial muscle protein synthesis that occur during brief periods of muscle disuse. The prolonged benefits of NMES to attenuate the loss of muscle mass and strength during a period of disuse require further investigation. However, the present study clearly lays the mechanistic foundation for the successful clinical use of NMES. Potential applications of NMES are far reaching within as well as beyond our healthcare system, such as hospitalization, postsurgery rehabilitation, recovery from sports injury, convalescence from illness, or any other situation leading to insufficient physical activity and consequent muscle loss. Of particular relevance to the aforementioned conditions...
is the elderly individual who may be more vulnerable to muscle disuse atrophy.

In conclusion, NMES increases skeletal muscle protein synthesis rates in vivo in older type 2 diabetes patients. This is the first study to provide proof of principle that NMES stimulates skeletal muscle protein synthesis in the acute recovery phase immediately following stimulation and, as such, may be used effectively to attenuate muscle loss in elderly individuals during periods of bed rest and/or immobilization.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES

ELECTRICAL STIMULATION AND MUSCLE PROTEIN SYNTHESIS


