Proteins regulating cap-dependent translation are downregulated during total knee arthroplasty

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Ratchford SM, Bailey AN, Senesac HA, Hocker AD, Smolkowski K, Lantz BA, Jewett BA, Gilbert JS, Dreyer HC. Proteins regulating cap-dependent translation are downregulated during total knee arthroplasty. Am J Physiol Regul Integr Comp Physiol 302: R702–R711, 2012. First published December 28, 2011; doi:10.1152/ajpregu.00601.2011.—Total knee arthroplasty (TKA) utilizes a tourniquet to reduce blood loss, maintain a clear surgical “bloodless” field, and to ensure proper bone-implant cementing. In 2007, over 600,000 TKAs were performed in the United States, and this number is projected to increase to 3.48 million procedures performed annually by 2030. The acute effects of tourniquet-induced ischemia-reperfusion (I/R) on human skeletal muscle cells are poorly understood and require critical investigation, as muscle atrophy following this surgery is rapid and represents the most significant clinical barrier to long-term normalization of physical function. To determine the acute effects of I/R on skeletal muscle cells, biopsies were obtained at baseline, maximal ischemia (prior to tourniquet release), and reperfusion (following tourniquet release). Quadriceps volume was determined before and 2 wk post-TKA by MRI. We measured a 36% decrease in phosphorylation of Akt Ser473 during ischemia and 37% during reperfusion (P < 0.05). 4E-BP1 Thr37/46 phosphorylation decreased 29% during ischemia and 22% during reperfusion (P < 0.05). eEF2 Thr56 phosphorylation increased 25% during ischemia and 43% during reperfusion (P < 0.05). Quadriceps volume decreased 12% in the TKA leg (P < 0.05) and tended to decrease (6%) in the contralateral leg (P = 0.1). These data suggest cap-dependent translation initiation, and elongation may be inhibited during and after TKA surgery. We propose that cap-dependent translational events occurring during surgery may precipitate postoperative changes in muscle cells that contribute to the etiology of muscle atrophy following TKA.

OSTEOARTHRITIS (OA) OF THE KNEE is among the most prevalent chronic diseases in the United States (10) affecting 60% of adults over the age of 65 (44) and is nearly 40% more prevalent in women than in men (25). The impact on disability due to knee OA is fourth behind ischemic heart disease, cerebrovascular disease, and musculoskeletal disease (48). Total knee arthroplasty (TKA) is the most commonly performed surgical remediation for patients with long-standing OA. In 2005, nearly 500,000 TKAs were performed in the United States at a cost of over $11 billion (33), and the number of surgeries performed annually in the United States is projected to increase to 3.48 million by 2030 (30).

Despite the universal success of TKA in eliminating knee pain due to OA, it remains to be determined whether physical function normalizes over time. Physical function is directly linked to muscle mass and strength of the lower extremities, the quadriceps in particular. However, a key finding of several studies on TKA, is the persistent inability to restore quadriceps muscle volume and strength (20, 37, 38, 40, 57, 60), leading many to conclude that the single greatest clinical barrier following TKA is long-term muscle atrophy. Further, quadriceps muscle atrophy has been shown to be the single greatest contributor to strength deficits, explaining 77% of the strength loss, at 1 to 3 years post-TKA (36).

Ischemia/reperfusion (I/R) occurs whenever a tourniquet is used during surgeries, such as TKA, to control blood loss, maintain a clear surgical field, and facilitate proper bone-implant cementing. Unfortunately, there is a gap in our understanding of the acute effects of tourniquet-induced I/R injury on human skeletal muscle cells during TKA. Previous studies report that skeletal muscle is negatively affected by periods of ischemia lasting 15 (2), 30 (2, 51, 53, 54), and 60 min (2, 18, 46, 51, 52) with damage progressing as the duration of ischemia increasing. Among the cellular responses to ischemia is conservation of ATP for use to stabilize the cell. As such, protein synthesis, which uses significant amounts of ATP on a percentage basis, is acutely inhibited at protein control points, eukaryotic initiation factor 4E binding protein (4E-BP1) and eukaryotic initiation factor 2 (eIF2), which are primarily responsible for regulating cap-dependent translation initiation (59). Over 95% of mRNA is translated in a cap-dependent process (24, 26).

Assimilated, it is reasonable to conclude that tourniquet-induced I/R injury may precipitate a cascade of cellular events that, in part, help to explain the significant and rapid loss of muscle that is observed clinically following TKA. The overarching goal of this study was to characterize the cellular events during I/R that may help to explain postoperative changes in muscle cells that may contribute to the etiology of muscle atrophy following TKA. Specifically, the purpose of this study was to test the hypothesis that I/R in skeletal muscle would alter cellular events involved with controlling cap-dependent translation initiation and elongation such as 4E-BP1, eIF2α, and eukaryotic elongation factor-2 (eEF2), in a manner consistent with downregulation of protein synthesis.
Further, this study also sought to test the hypothesis that quadriceps muscle volume would decrease following TKA.

**METHODS**

**Ethics approval.** This study was approved by the PeaceHealth Institutional Review Board, Sacred Heart Medical Center, at Riverbend and the Biomedical Institutional Review Board for the University of Oregon and conducted in accordance with the Declaration of Helsinki. All subjects gave informed written consent prior to study participation. This study is registered with ClinicalTrials.gov (identifier: NCT00760383).

**Subjects.** We studied 13 subjects (10 females and 3 males) who were recruited from surgical candidates from the Slocum Center for Orthopedics and Sports Medicine. All subjects were between 60 and 80 years of age and scheduled to have primary TKA. Excluded from the study were patients with untreated endocrine disease, significant heart, kidney, liver, blood or respiratory disease, peripheral vascular disease, active cancer, recent treatment with anabolic steroids, or oral corticosteroids for greater than 1 wk, and alcohol or drug abuse. Details for each subject are provided in Table 1.

**Study design.** Two weeks prior to surgery, subjects had a MRI of the mid-thigh region at the Slocum Center for Orthopedics and Sports Medicine MRI facility equipped with a Philips Achieva 8 C 1.5 T system. A T1-weighted, fast spin echo-pulse sequence using the following parameters were used to obtain slice images of the mid-thigh region of interest: transmit times of ~500 ms, and a echo time of ~12 ms; fields of view were variable to match patient size, with slice thickness of 5 mm and acquisition matrix size of 256 × 512 mm. Two weeks after TKA, each subject returned to the Slocum Center for Orthopedics and Sports Medicine MRI facility for a repeat scan. The prescan image was used by the operator to align postscan parameters to determine changes in muscle tissue volume in the identical region of interest. Coded images were transferred electronically via encrypted electronic data transfer to the laboratory computer for analysis. Three preoperative mid-thigh slice images were matched to three identical postoperative midthigh slice images for analysis using Analyze v.10 software package with semiautomated delineation of muscle borders, and through the use of thresholding methods, the software can auto-differentiate muscle from fat using voxel intensity within each border region for quantitative determination of muscle volume. One researcher was responsible for analyzing each image. Interclass correlation coefficients for that operator are nonoperative (contralateral) quadriceps: rectus femoris (RF), ICC = 0.96, vastus (Vast), including all three vasti, i.e., intermedius, lateralis, and medialis, ICC = 0.99; and operative (TKA) quadriceps: RF, ICC = 0.96, vasti, ICC = 0.99.

On the day of surgery, each subject was admitted in the morning to Sacred Heart Medical Center, at Riverbend, in the fasted state. Following standardized preoperative procedures for TKA, each subject was prepped for surgery. Anesthesia was administered with either an epidural, spinal, or general anesthetic, along with a preoperative femoral nerve block placed for postoperative analgesia. Induction of general anesthesia was accomplished with intravenous propofol, and anesthesia maintenance was with inhalational anesthetic (either desflurane or sevoflurane), with or without muscle relaxant (rocuronium bromide) (Table 1). After induction of anesthesia in the operating room (OR), a 10-cm-wide Zimmer tourniquet was placed at the proximal third of the thigh (but not inflated), and standard lower extremity sterilization procedures were performed. This was followed by the first of three muscle biopsies obtained from the vastus lateralis muscle on the operative (TKA) leg, at the level of middle-third of the thigh, using a 5-mm Bergström biopsy needle with applied suction. After the first muscle biopsy was obtained, the operative leg was exsanguinated using an Esmarch bandage, and the tourniquet was inflated for the first time to 300 mmHg or greater depending on systolic blood pressure to ensure minimal blood flow to the operative (TKA) leg. Just prior to tourniquet deflation, following the main components of the surgery, a second muscle biopsy was obtained. After the second muscle biopsy was obtained the tourniquet was deflated for the later components of the surgery, allowing for reperfusion of the limb with blood. The third and final muscle biopsy was obtained during the reperfusion phase of the OR prior to being moved to postoperative recovery (Fig. 1). Muscle biopsy samples were immediately blotted, and adipose tissue was removed before being frozen in liquid nitrogen (<1 min) and stored at ~80°C until analysis.

**Muscle tissue homogenization.** Details of the homogenization procedures have been previously published (16), with specific modifications implemented for this study. Frozen muscle samples (20–30 mg) were ground using Heidolph Brinkmann’s Silent Crusher M in a nitrogen atmosphere, and stored at −80°C until analysis.

**Table 1. Subject characteristics and treatment**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age</th>
<th>Ht, cm</th>
<th>Wt, kg</th>
<th>BMI</th>
<th>Dx Medications</th>
<th>Tourniquet Time, min</th>
<th>Reperfusion Time, min</th>
<th>Anesthesia</th>
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<tbody>
<tr>
<td>F</td>
<td>70</td>
<td>174</td>
<td>73</td>
<td>24</td>
<td>OA Aspirin*, Osteo-Biflex, I-caps, Protonix, Crestor, Tylemol</td>
<td>42</td>
<td>16</td>
<td>FNB and Gen</td>
</tr>
<tr>
<td>F</td>
<td>76</td>
<td>173</td>
<td>59</td>
<td>20</td>
<td>Deg Arth Benazepril, hydrochlorothiazide, Diltiazem, aspirin, Lipitor, ibuprofen as needed, alendronate, NitroQuick</td>
<td>35</td>
<td>18</td>
<td>FNB and Gen</td>
</tr>
<tr>
<td>F</td>
<td>65</td>
<td>160</td>
<td>31</td>
<td>31</td>
<td>Deg Arth Aleve*, Lexapro, Wellbutrin</td>
<td>42</td>
<td>17</td>
<td>FNB and Spinal</td>
</tr>
<tr>
<td>M</td>
<td>66</td>
<td>185</td>
<td>101</td>
<td>29</td>
<td>OA Aspirin*, Tylemol, Glucosamine</td>
<td>52</td>
<td>16</td>
<td>FNB and Gen</td>
</tr>
<tr>
<td>M</td>
<td>73</td>
<td>183</td>
<td>122</td>
<td>36</td>
<td>OA Estradiol, Levorthoxrnine, Pirixicam, Potassium, Triamterene, Amlodipine, Darvocet</td>
<td>40</td>
<td>19</td>
<td>FNB and Gen</td>
</tr>
<tr>
<td>F</td>
<td>72</td>
<td>168</td>
<td>65</td>
<td>23</td>
<td>Deg Arth Advast, Ambien, aspirin*, Calcium, Cardizem, Fosamax, Lipitor, Miraxep, Paroxetine, Spironolactone, Triamterene, Vicodin, or Percocet</td>
<td>35</td>
<td>13</td>
<td>FNB and Gen</td>
</tr>
<tr>
<td>F</td>
<td>62</td>
<td>163</td>
<td>64</td>
<td>24</td>
<td>OA Amitriptyline, Zopidem, Vitamin D</td>
<td>47</td>
<td>16</td>
<td>FNB and Spinal</td>
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<tr>
<td>F</td>
<td>70</td>
<td>157</td>
<td>100</td>
<td>41</td>
<td>OA Bietmol, Caltrate, HCTZ, Levothoxrnine, Monopril, Tylemol, Naprosyn, Loratadine, Meclazine</td>
<td>49</td>
<td>13</td>
<td>FNB and Spinal</td>
</tr>
<tr>
<td>M</td>
<td>64</td>
<td>178</td>
<td>97</td>
<td>31</td>
<td>OA Diltiazem, Benazepril, Glucosamine, Buprophen*</td>
<td>48</td>
<td>16</td>
<td>FNB and Spinal</td>
</tr>
<tr>
<td>M</td>
<td>73</td>
<td>171</td>
<td>269</td>
<td>42</td>
<td>OA Calcium with vitamin D, Cymbial, Enalapril, Furocesside, Glucosamine, Norvasc, Prozac, Simvastatin, Truvatran, Vicodin</td>
<td>45</td>
<td>22</td>
<td>FNB, Gen, and muscle relaxant</td>
</tr>
<tr>
<td>F</td>
<td>76</td>
<td>165</td>
<td>88</td>
<td>32</td>
<td>OA Aspirin*, Coreg, Posinproin, Isosorbide, Lasix, Vytorin, Aleve*, Nitrolyciner</td>
<td>40</td>
<td>14</td>
<td>FNB and Spinal</td>
</tr>
<tr>
<td>F</td>
<td>71</td>
<td>147</td>
<td>77</td>
<td>36</td>
<td>OA Estradiol, Advair Disks, Triamterene/HCTZ, Celexa, Diclofenac, Prevacic, Glucosamine, Darvocet and Claritin as needed</td>
<td>32</td>
<td>15</td>
<td>FNB and Gen</td>
</tr>
<tr>
<td>F</td>
<td>73</td>
<td>170</td>
<td>73</td>
<td>25</td>
<td>OA Spiva, Advair Disks, Coreg, Simvastatin, Alendronate, vitamin D, Onemazole, Benicar, calcium, Albutoler inhaler as needed</td>
<td>35</td>
<td>13</td>
<td>FNB and Epi</td>
</tr>
</tbody>
</table>

*Medication withheld week prior to surgery. DX, diagnosis, Deg Arth, degenerative arthritis; Epd, epidural anesthesia; FNB, femoral nerve block; Gen, general anesthesia; OA, osteoarthritis; Spinal: spinal anesthesia. Femoral nerve block indicates a single injection of 30 ml of 0.25% to 0.5% bupivacaine or ropivacaine. For general anesthesia, intravenous administration of propofol was maintained by inhalation of either desflurane or sevoflurane. For spinal anesthesia, 0.75% bupivacaine + 20 μg of fentanyl was administered. For epidural anesthesia, 0.25% bupivacaine was administered. The muscle relaxant was administered by local injection of rocuronium bromide.
Tris-HCl, 250 mM mannitol, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1.0% Triton X-100, pH 7.4, 1 mM benzamidine, 1 mM DTT, 0.1 mM PMSF, and 5 μg/ml soybean trypsin inhibitor. Aliquots were collected after centrifugation at 6,000 rpm (2,817 g) at 4°C for 10 min. Separate aliquots to isolate 4E-BP1 were subjected to an additional 10 min of boiling at 100°C followed by centrifugation at 10,000 rpm (7,826 g) at 4°C for 30 min before being expelled of solid protein fragments. Protein concentration (mg/ml) was determined in duplicate using a Bradford assay (1.5 Bio-Rad protein assay concentrate) on a Bio-Rad SmartSpec Spectrophotometer. All samples were stored at −80°C until further analysis.

**SDS PAGE and immunoblotting.** Details of the immunoblotting procedures have been previously published (15, 16), with specific modifications implemented for this study. Homogenates were loaded in duplicate (equal micrograms per lane) into precast Bio-Rad Criterion Tris-HCL 7.5%, 15%, or TGX all kDa gels using electrode buffer (0.3% Tris base, 14.4% glycine, 1% SDS in dd-H2O) for analysis of various proteins of interest. A single muscle homogenate was repeatedly loaded onto each membrane as an internal control.

Following SDS PAGE, proteins were transferred to Bio-Rad Immuno-Blot PVDF membranes, which were soaked in 100% methanol for 1 min prior to transfer at 50 V for 60 min in transfer buffer for 7.5% and 15% gels and at 100 V for 60 min for TGX gels [10% N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer (2.21% CAPS and 2% 2 N NaOH in ultra-pure H2O) and 10% methanol in ultra-pure H2O].

Upon transfer, PVDF membranes were blocked in Blotto [5% nonfat dry milk powder in TBST] under constant agitation for 30–60 min. Following overnight incubation with primary antibodies, membranes were rinsed in TBS-Tween twice, followed by 60 min incubation in Blotto containing secondary antibody (donkey anti-rabbit) at room temperature. Following serial washes, the membranes were incubated in chemiluminescent reagent (ECL reagents, Amersham, Biosciences, Piscataway, NJ) for 5 min to detect horseradish peroxidase activity. Images were obtained with a ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA). Quantity One 1-D Software (version 4.6.9; Bio-Rad) was used to quantify the densitometry analysis of proteins. All results were made relative to our loading control (one lane per gel) to account for variance between gels and total actin, which was probed for in each lane, serving as an internal loading control.

**Antibodies.** The primary antibodies p-Akt Ser73 (no. 9271), Akt (no. 9272), p-4E-BP1 Thr37/46 (no. 9459), 4E-BP1 (no. 9452), p-4E-BP1 Ser473 (no. 2313), p-4E-BP1 Ser65 (no. 9453), p-eIF4E Ser209 (no. 9741), eIF4E (no. 9742), p-eIF4E Ser223 (no. 3957), p-eIF2a Thr56 (no. 9459), eIF2a (no. 9452), p-Mnk1 (no. 2111), and Mnk1 (no. 2195) were purchased from Cell Signaling (Beverly, MA). Additional primary antibodies, t-eIF2a (sc-11386), CREB-2/activating transcription factor 4 (ATF4) (sc-22800), and GADD34 (sc-824) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Monoclonal mouse anti-actin (no. 691002) was purchased from MP Biomedicals (Solon, OH) for use as our protein control. ECL anti-rabbit IgG and horseradish peroxidase from donkey and sheep were purchased from GE Healthcare (New York, NY) and used as our secondary antibodies.

**Total RNA isolation.** Details for RNA isolation have been previously published (17) with slight modifications implemented for this study. Skeletal muscle samples (20–30 mg) were homogenized in 1 ml TRI Reagent using Heidolph Brinkmann’s Silent Crusher M at 10,000–15,000 rpm in Eppendorf RNase-free tubes. Separation was achieved by adding 0.2 ml of chloroform and precipitated using 0.5 ml of isopropanol. The RNA pellet was washed twice and dried in 75% ethanol, and dissolved in 1.5 μl of 0.1 mM EDTA for each mg of starting tissue.

RNA concentrations were determined using an OD_{260/280} ratio on a Bio-Rad SmartSpec Spectrophotometer. All samples met the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines of OD_{260/280} with an average of 1.91 ± 0.03, indicating the RNA quality was without protein or phenol contamination (8, 55). Microfluidic capillary electrophoresis analysis (RNA standard sensitivity chips, Experion, software version 3.0; Bio-Rad) was used in accordance with RNA StdSens kit directions to establish an RNA quality index (RQI) based on the ribosomal RNA profile of each sample. Samples run in triplicate averaged an RQI of 8.52 ± 0.2 (highly degraded RNA to intact RNA on a 1–10 scale) and a 28S:18S ratio of 1.21 ± 0.02, a ratio between 1 and 2, indicating intact RNA.

**Reverse transcriptase and cDNA synthesis.** One microgram of RNA was reversed transcribed into cDNA on a CFX96 real-time PCR Detection System (Bio-Rad) using iScript Reaction Mix (Bio-Rad), according to manufacturer’s instructions. Samples were then stored at −80°C for future analysis.

**Oligonucleotide primers for quantitative PCR.** Oligonucleotide primers were designed using Beacon Design software (version 7.91), specific to our Bio-Rad CFX96 Real-Time PCR Detection System, based on NCBI Entrez Gene ID search results. Primer efficiencies were run in triplicate across four dilutions: 1 μg, 0.11 μg, 0.012 μg, and 0.0014 μg. In accordance with MIQE guidelines (8), primer efficiencies were 95–105% efficient when analyzed on the log linear scale: PCR efficiency = 10-1/slope-1. Primers were designed for GAPDH, NM_002046, forward: CTTGGTTAAAGTTGATATTGT and reverse: GTGGGAATCATATTGGGAACAC; EIF4G1, NM_001194946, forward: TATTCAGCCCACTTGTGC and reverse: CCACGCCTCCTTCTTAT; EIF4G2, NM_001042559, forward: CATACATGACGCTCTCAGAT and reverse: TTCCGGTTATCATAGACATCAAAC; EIF4G3, NM_001199881, forward: TTATAGCCGACTCTTCTTCTA and reverse: ATATTTCTTCATATCATGATCAAT and reverse: GADD45A, NM_001199741, forward: CAAAGGTATTTCA.

**mRNA quantitation by quantitative PCR.** Details on quantitative PCR (qPCR) have been previously published (17), with specific modifications employed for this study. Samples of cDNA were analyzed using SYBR Green fluorescence (iQ SYBR Green Supermix, Bio-Rad). Each reaction within a 96-well plate contained 12.5 μl SYBR Green, 9.5 μl of DEPC-treated, nuclease-free water, 0.5 μl forward, and 0.5 μl reverse primers, and 2 μl of cDNA template. A mastermix of SYBR Green, water, and primers was first made and distributed into each 96-well reaction tube before final addition of the cDNA template to diminish variance between samples. All samples...
were run in triplicate. An initial 5-min cycle at 95°C was used to
denature the cDNA. This was followed with 50 PCR cycles consisting of
denaturation at 95°C for 10 s followed by 30 s of primer annealing at
the optimized primer pair annealing temperature. All PCR cycles
were followed by a melt analysis. Each qPCR run had its cycle
threshold (CT) arbitrarily set to 200 RFU to account for intrarun
variability. All genes of interest (GOI) were normalized to GAPDH,
our gene of reference (GOR). mRNA results for all GOIs are ex-
pressed as fold changes relative to the GOR using the Livak method,
also known as the 2−ΔΔCT method (32): ΔCT(test) = CT(GOR test) −
CT(GOR, baseline), ΔCT(baseline) = CT(GOI, baseline) − CT(GOR, baseline), ΔΔCT =
ΔCT(test) − ΔCT(baseline), and 2−ΔΔCT = fold change from baseline.

Statistical analysis. All values are expressed as means ± SE.
Statistical evaluation of our data was performed using paired-samples
t-test to compare ischemic and reperfusion samples to baseline, which
is a test of gain scores (27). Differences between means were consid-
ered statistically significant at P ≤ 0.05. With 44 paired t-tests, we
expect between 0 and 5 false positives (95% confidence bound).
Specifically, we anticipate an 18% chance of four or more errors but
not different for age, height, weight, and body mass index (P >
0.05; data for all subjects are included in Table 1).

Results

Demographics. Male and female subject characteristics were
different for age, height, weight, and body mass index (P >
0.05; data for all subjects are included in Table 1).

Gene expression. Adequate tissue samples for mRNA anal-
alysis were available for 12 subjects. Compared with baseline,
eIF4G1 mRNA increased 19% [P = 0.048, 80% confidence
interval (CI) (9%, 30%)] during reperfusion (Fig. 2A). Com-
pared with baseline, eIF4G2 mRNA increased 19% [P = 0.04,
80% CI (10%, 29%)] during reperfusion (Fig. 2B). Compared with
comparison, eIF4G3 mRNA increased 28% [P = 0.01, 80% CI
(14%, 42%)] during reperfusion (Fig. 2C). Compared with
baseline, a downstream transcription target of ATF4, GADD45A,
increased 37% [P = 0.02, 80% CI (17%, 56%)] during isch-
emia and remained elevated by 30% during reperfusion, but
was not significant (P = 0.11) (Fig. 2D).

Cell signaling. Immunoblot data were assessed for Akt
Ser473, 4E-BP1 Thr37/46, eIF2α Ser209, eIF4E, and GADD34
using all 13 subjects. Adequate tissue sample quantities for
Mnk1, Thr179/202, eIF4E Ser1108, and eIF2 T56 were available for
for only 11 subjects.

Compared with baseline, Akt phosphorylation at Ser473
decreased by 35% [P = 0.01, 80% CI (−52%, −20%)] during
ischemia and 37% [P = 0.02, 80% CI (−48%, −26%)] during
reperfusion (Fig. 3A). Total Akt protein remained unchanged
(0.64 ± 0.13, 0.78 ± 0.09, and 0.62 ± 0.09, during baseline,
ischemia, and reperfusion, respectively; P > 0.05). Compared
with baseline, 4E-BP1 phosphorylation at Thr37/46 decreased by
29% [P = 0.04, 80% CI (−41%, −16%)] during ischemia
and 22% [P = 0.046, 80% CI (−42%, −3%)] during reper-
fusion (Fig. 3B). Similarly to Akt, total 4E-BP1 protein re-
mained unchanged [1.22 ± 0.25, 0.90 ± 0.19, and 0.84 ± 0.16
(AU), during baseline, ischemia, and reperfusion, respectively;
P > 0.05].

While phosphorylation of Mnk1 Thr179/202 did not signifi-
cantly change (1.91 ± 0.38, 1.68 ± 0.27, and 1.66 ± 0.27 AU,
during baseline, ischemia, and reperfusion, respectively; P >
0.05), compared with baseline its total protein content in-
creased 54% [P = 0.03, 80% CI (34%, 73%)] during ischemia
and returned to baseline during reperfusion (Fig. 4A). We did not
see a significant increase in phosphorylation of eIF4E
Ser1108 (2.86 ± 0.53, 2.57 ± 0.91, and 2.56 ± 0.69 AU,
during baseline, ischemia, and reperfusion, respectively; P >
0.05), although compared with baseline, total protein content of
eIF4E increased 51% [P = 0.03, 80% CI (18%, 84%)] during ischemia
and returned to baseline during reperfusion (Fig. 4B). We did not
see significant changes in phosphorylation of eIF4G
Ser1108 (0.79 ± 0.28, 0.48 ± 0.15, and 0.80 ± 0.16 AU,
during baseline, ischemia, and reperfusion, respectively; P >
0.05) or total protein of eIF4G (1.08 ± 0.20, 1.03 ± 0.17, and
0.80 ± 0.10 AU, during baseline, ischemia, and reperfusion,
respectively; P > 0.05).

Fig. 2. Increased gene expression of eIF4G and the activating transcription factor 4
(ATF4) transcription target growth arrest and DNA damage 45A (GADD45A). Transcripts
for eukaryotic initiation factor 4 gamma 1 (eIF4G1) increased 19% (P = 0.048) (A),
eIF4G2 increased 19% (P = 0.04) (B), and eIF4G3 increase 28% (P = 0.01) (C) from
baseline to reperfusion. The transcription target of ATF4, GADD45A mRNA, significantly
increased during ischemia 37% (P = 0.02) and showed a trend to increase, by 30% (P = 0.11)
above baseline, during reperfusion (D). Results were normalized to GAPDH. Data are ex-
pressed as mean fold change ± SE (n = 12). *P ≤ 0.05 vs. baseline.
Deactivation of the eEF2 was assessed by phosphorylation at eEF2 Thr56 which, compared with baseline increased 25% \( (P = 0.04, 80\% \text{ CI (12\%, 39\%)} \) during ischemia and 43% \( (P = 0.01, 80\% \text{ CI (50\%, 143\%)} \) during reperfusion (Fig. 5). Total protein for eEF2 was unchanged during the study (0.35 ± 0.07, 0.49 ± 0.09, and 0.41 ± 0.07 AU for baseline and ischemia, respectively; \( P > 0.05 \)).

Endoplasmic reticulum (ER) stress was assessed by the phosphorylation status of eIF2α Ser51 and its downstream targets. eIF2α phosphorylation and total protein content were not altered relative to baseline (phospho/total; 1.64 ± 0.31, 1.27 ± 0.18, and 1.83 ± 0.49, and total protein; 1.22 ± 0.22, 1.59 ± 0.39, and 0.95 ± 0.20 AU, during baseline, ischemia, and reperfusion, respectively; \( P > 0.05 \)) however, two downstream targets were upregulated during ischemia. ATF4 protein levels, compared with baseline increased 96% \( (P = 0.01, 80\% \text{ CI (50\%, 143\%)} \) during ischemia but returned to baseline during reperfusion (Fig. 6A), and GADD34 protein levels compared with baseline increased 83% \( (P = 0.03, 80\% \text{ CI} \) during ischemia and reperfusion.

Deactivation of the eEF2 was assessed by phosphorylation at eEF2 Thr56 which, compared with baseline increased 25% \( (P = 0.01) \) during ischemia and 37% \( (P = 0.02) \) during reperfusion (A). 4E-BP1 Thr46/147 was hypophosphorylated during ischemia by 29% \( (P = 0.04) \) and 22% \( (P = 0.046) \) during reperfusion (B). 4E-BP1 hypophosphorylation is indicative of blunted cap-dependent translation, which will block protein synthesis. Phosphorylation status was made relative to total protein. Actin served as a loading control. Data are expressed as means ± SE \( (n = 13) \). *\( P < 0.05 \) vs. baseline.

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Fig. 3. Dephosphorylation of Akt and 4E-BP1 during ischemia and reperfusion. Phosphorylation status of Akt at Ser473 was significantly decreased by 36% \( (P = 0.01) \) during ischemia and 37% \( (P = 0.02) \) during reperfusion (A). 4E-BP1 Thr37/46 was hypophosphorylated during ischemia by 29% \( (P = 0.04) \) and 22% \( (P = 0.046) \) during reperfusion (B). 4E-BP1 hypophosphorylation is indicative of blunted cap-dependent translation, which will block protein synthesis. Phosphorylation status was made relative to total protein. Actin served as a loading control. Data are expressed as means ± SE \( (n = 13) \). *\( P < 0.05 \) vs. baseline.

Fig. 4. Increased Mnk1 and eIF4E total protein during ischemia. Mnk1 total protein content significantly increased 54% \( (P = 0.03) \) during ischemia and returned to baseline during reperfusion (A). eIF4E total protein content significantly increased 51% \( (P = 0.03) \) during ischemia and returned to baseline during reperfusion (B). Actin served as a loading control. Data are expressed as means ± SE \( (n = 11) \). *\( P < 0.05 \) vs. baseline.
During ischemia, returning to baseline during reperfusion (Fig. 6B).

**Quadriceps atrophy.** Pre- and post-TKA MRIs were available for nine subjects. Compared with before surgery, midthigh quadriceps volume in the TKA leg decreased 12% \((P = 0.012, 80\% \text{ CI} \ (-17\%, -8\%))\) 2 wk after surgery. A similar reduction (6% decrease) was observed in the nonoperative (contralateral) midthigh quadriceps volume 2 wk after surgery, but was not significant \((P = 0.11)\) (Fig. 7). Before surgery, midthigh quadriceps volume was less 7% \((P = 0.05, 80\% \text{ CI} \ (12\%, -2\%))\) in the operative vs. nonoperative lower extremity; i.e., the TKA quadriceps volume was less than the contralateral leg.

**DISCUSSION**

This study reveals several novel and important findings regarding the acute effects of I/R in skeletal muscle on transcriptional, translational, and cell signaling pathways known to be involved in cap-dependent translation initiation and elongation. First, we report that 4E-BP1 was hypophosphorylated, which suggests that cap-dependent translation initiation was inhibited during I/R. Second, we determined that eEF2 phosphorylation was increased, supporting the hypothesis that translation elongation was similarly attenuated during I/R. Third, we measured significant quadriceps muscle atrophy occurring acutely, 2 wk post-TKA, in the operative \((-12\%)\) and a trend for similar declines in muscle volume \((-6\%)\) in the nonoperative lower extremity. To our knowledge, this study is the first to measure changes in key regulatory proteins important for controlling cap-dependent translation initiation and elongation during TKA surgery and subsequent muscle loss occurring within 2 wk of surgery.

Translation initiation begins with the formation of the ternary initiation complex (eIF4F), consisting of eIF4A, eIF4G, and eIF4E, which binds with the 40S ribosomal subunit, stabilizes the preinitiation complex, and binds to the 7-methylguanosine cap structure located at the 5’ end of eukaryotic mRNA. Although the formation of eIF4F is critical for cap-dependent translation initiation, the binding protein 4E-BP1 can inhibit formation of the eIF4F complex by binding to eIF4E, thus preventing cap-dependent translation initiation (21, 24). Activation of Akt has been shown to stimulate downstream effectors of the mTOR pathway, 4E-BP1, and S6K1, under anabolic conditions (16), while overexpression of a hypophosphorylation form of Akt has been shown to block...
Phosphorylation remained unaltered. Apart from changes in phosphorylation status and protein levels of eIF4E, the binding and unbinding from 4E-BP1 are the primary determinants for the availability of eIF4E to form the eIF4F complex (45). It remains to be determined the degree to which increases in Mnk1 and eIF4E levels play a role in skeletal muscle I/R during TKA.

Phosphorylation of eIF4G Ser1108 fully activates eIF4G and enhances the formation of the eIF4G-eIF4E complex (47). While we did not observe changes in phosphorylation or total protein of eIF4G, myocardial ischemia has been shown to upregulate caspase-3-induced cleavage of eIF4G (12), to suppress protein synthesis, and to attenuate an antiapoptotic response (11). It is possible that the increase in gene expression of all three isoforms of eIF4G (eIF4G1, eIF4G2, and eIF4G3) during reperfusion may be the cell’s response to proteolytic degradation of eIF4G and/or cleavage by caspases as Martin et al. (34) have shown that this disruption alters the ability for complex formation (eIF4F) and binding of the capped mRNA with the 40S ribosomal subunit, resulting in significant translation inhibition. However, while eIF4G1 and eIF4G3 promote translation, eIF4G2 may act as a translational repressor, and we have measured elevations in transcripts for all three. These findings potentially suggest that multiple levels of cellular responses are occurring that may involve transcription, translation, and/or posttranslational modifications. Further work is necessary to determine the contribution of each of these mechanisms to restore muscle cell homeostasis during I/R associated with TKA.

After eIF2 delivers the initiator methionyl tRNA (meth-tRNA) to the AUG start codon on the mRNA, eIF2 hydrolyzes GTP to GDP and dissociates from ribosomal complex. Phosphorylation of eIF2α blocks the GTP exchange for eIF2-GDP, effectively binding eIF2α with the guanine exchange factor eIF2B, and inhibits the formation of a ternary complex consisting of methionine-tRNA and eIF2 coupled to GTP (21). While eIF2α Ser51 phosphorylation was not altered in our study, this has been reported to be necessary for the acute response to hypoxic stress (29). It may be that eIF2α was phosphorylated at an earlier time point during ischemia, prior to the second biopsy. This is supported by our data showing an increase in ATF4 protein, a distal substrate of eIF2α whose cap-independent translation is upregulated during hypoxic stress, including ER stress, which can precipitate the unfolded protein response (4, 23).

Additional evidence for the potential involvement of eIF2α at an earlier time point is provided by the measured increase in GADD45A transcript levels, a downstream target of ATF4 (19), and is translated via cap-independent mechanisms, preferentially during cell stress (58). Further, the downstream target of eIF2α, GADD34, can increase in response to skeletal muscle cell stress (58). Additionally, eIF2α may not require complete phosphorylation to initiate down-regulation of translation initiation (59). This may explain our findings that downstream effectors of eIF2α were upregulated (i.e., ATF4 and GADD34), while phosphorylation of eIF2α at Ser51 remained unchanged during our sampling times. Alternatively, GADD34, which was upregulated during ischemia, may have been present in sufficient quantities to facilitate the assembly of an eIF2α phosphatase and subsequent dephosphorylation of eIF2α (7, 41), restoring cap-dependent initiation and cell homeostasis (42). Arsham et al. (3) were unable to detect a
change in eIF2α Ser51 following 30 min of hypoxia, despite significant downregulation of components of the mTORC1 pathway, including 4E-BP1. Lastly, eIF2α does not require complete phosphorylation to initiate downregulation of translation initiation due to the lower level of expression of eIF2B (59), and its activity may respond differently under anoxic (29) vs. hypoxic conditions (3, 31). Further work is required to unravel the potential contributions of eIF2α during I/R in our model.

Following initiation of cap-dependent translation, additional regulation of translation elongation can facilitate protein synthesis. The translocation of the peptidyl-tRNA and the growing polypeptide during the elongation phase of translation from the A-site to the P-site of the ribosome is promoted by eEF2, which loses its binding affinity for the ribosome when phosphorylated (9). The eukaryotic elongation factor-2 (eEF2) can thus attenuate translation, and subsequent protein synthesis, when phosphorylated (28). Our results for eEF2 are consistent with previous data (6) and provide further evidence for blunted translation during ischemia and reperfusion. Earlier work from rat models suggests that elongation is not impaired by sepsis in skeletal muscle (56); however, more recent work in myocardial I/R model has shown an increased phosphorylation of eEF2 at Thr56 during I/R (14). Our results show that phosphorylation of eEF2 at Thr56 increased during ischemia and reperfusion. Not only may translation elongation be inhibited during low energy levels, such as ischemia, but reperfusion failed to elicit a reversal of eEF2 phosphorylation in our clinical model of I/R in skeletal muscle. Further work is needed to determine whether phosphorylation of eEF2 at Thr56 is sufficient to attenuate translation elongation and if this translates to an attenuation of protein synthesis (6).

Typical of clinical translational research, our study design has inherent limitations. For example, we were unable to control for medications, physical activity, the anesthetics/muscle relaxants, and we do not have a direct measure of protein synthesis or degradation. As such, we acknowledge the critical need for further studies designed to quantify the magnitude of the contribution of I/R injury on muscle protein anabolism and catabolism during and after TKA.

**Perspectives and Significance**

By far, the most significant clinical barrier following TKA surgery is persistent muscle atrophy and weakness (20, 37, 57). Specifically, quadriceps muscle strength has been shown to be as much as 40% weaker than age-matched healthy controls 2 mo following surgery (43) and has been singled out as the main contributor to diminished strength and poor return of physical function after TKA (36). While there exists the strong possibility that the preoperative single injection femoral nerve block and/or the anesthetics used during surgery may be playing a role in altering proteins regulating cap-dependent translation, it stands to reason that acute alterations in muscle cells that we have measured in this study may initiate a cascade of events that culminate in the eventual loss of muscle that is a known sequela of TKA and are similar in extent to the rapid alterations measured in critically ill patients (13). Indeed, we have found that quadriceps muscle atrophy occurs acutely, within 2 wk post-TKA, in the operative leg (−12%), which is similar to what has been found by others at ~27 days post-TKA (39), but it was double that of the nonoperative leg (−6%), which is similar in the extent of atrophy observed by researchers modeling 2 wk of immobilization in healthy subjects (1). It remains that additional research is necessary to better delineate the potential impact of preoperative analgesics and tourniquet use on muscle metabolism and whether or not the clinical recommendation to apply continuous tourniquet for no more than 2 h needs to be revisited (5). Additionally, further research is necessary to elucidate the interactions of anesthetics and I/R injury and the evolution of atrophy in our clinical model, i.e., nonoperative disuse atrophy vs. operative disuse atrophy and I/R injury, we believe this work brings us one step closer to understanding the fundamental changes occurring acutely in muscle cells and begin the process of bridging the gap between cause and effect of muscle loss following an increasingly common surgery performed on older adults.

In the end, we found that key regulators of “global” translation initiation and elongation are altered in a way that is consistent with reductions in protein synthesis in skeletal muscle subjected to I/R during a commonly performed surgical procedure, which may exacerbate immobilization-induced reductions in muscle volume (Fig. 8).

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**Fig. 8.** Ischemia-reperfusion inhibits multiple proteins regulating cap-dependent translation. The schematic diagram of proteins regulating signaling pathways controlling cap-dependent mRNA translation initiation and elongation. Ischemia-reperfusion inhibits cap-dependent translation initiation via inhibition of Akt-mTOR pathway and availability of 4E-BP1 to bind to and inhibit eIF4E association with eIF4G to form an active mRNA cap-binding complex (eIF4F). Ischemia-reperfusion also activates endoplasmic reticulum (ER) stress via eIF2α, which inhibits cap-dependent translation initiation. ATF4 and GADD34, each downstream components of eIF2α, are proteins involved in recovery from cell stress and are upregulated during ischemia and provide inhibitory feedback onto eIF2α. Alterations due to ischemia/reperfusion (I/R) additionally stimulate mRNA for all three isoforms of eIF4G. Together, these data may potentially provide some insight into the dramatic quadriceps atrophy (−12%) occurring within 2 wk post-TKA.

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Further, multiple factors associated with translational regulation were found to be modified at both transcriptional and translational levels and lend support to our hypothesis that downregulation of cap-dependent translation initiation occurs in these patients. This study, to our knowledge, is the first to provide evidence of such events taking place in muscle cells of older adults during total knee replacement surgery.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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