Muscle Inflammation Susceptibility: A Prognostic Index of Recovery Potential after Hip Arthroplasty?

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ABSTRACT
While elective total hip arthroplasty (THA) for end-stage osteoarthritis (OA) improves pain, mobility function, and quality of life in most cases, a large proportion of patients suffer persistent muscle atrophy, pain, and mobility impairment. Extensive skeletal muscle damage is unavoidable in these surgical procedures, and it stands to reason that poor recovery and long-term mobility impairment among some individuals after THA is linked to failed muscle regeneration and regrowth following surgery; and that local muscle inflammation susceptibility (MuIS) is a major contributing factor. Here we present results of two integrated studies. In Study 1, we compared muscle inflammation and protein metabolism signaling in elective THA (n=15) vs. hip fracture/trauma (HFX, n=11) vs. non-surgical controls (CON, n=19). In Study 2, we compared two subgroups of THA patients dichotomized into MuIS(+) (n=7) or MuIS(-) (n=7) based on muscle expression of TWEAK receptor (Fn14). As expected, HFX demonstrated overt systemic and local muscle inflammation and hyper-metabolism. By contrast, no systemic inflammation was detected in elective THA patients; however, local muscle inflammation in the perioperative limb was profound in MuIS(+) and was accompanied by suppressed muscle protein synthesis compared to MuIS(-). Muscle from the contralateral limb of MuIS(+) was unaffected, providing evidence of a true inflammation susceptibility localized to the muscle surrounding the hip with end-stage OA. We suggest MuIS status assessed at the time of surgery may be a useful prognostic index for muscle recovery potential, and could therefore provide the basis for a personalized approach to post-surgery rehabilitation.
INTRODUCTION

Elective total joint arthroplasty is the last resort for end-stage osteoarthritis (OA) and, in most cases, total hip or knee arthroplasty (THA or TKA) significantly improves pain, mobility function, and quality of life (7); however, numerous THA/TKA patients suffer persistent muscle atrophy (22, 24), reduced muscle strength (14, 23), pain (27-29, 31), and mobility impairment (6, 28, 30). In fact, epidemiologic findings from our group reveal a troubling prevalence of significant mobility limitation (35%) and frank disability (13%) 5 y post-surgery (30). Given that THA/TKA volumes are increasing exponentially (32) with over 1.1 million in the US in 2009 (12), refractory mobility impairment is a major public health problem that is not understood.

Extensive skeletal muscle damage is unavoidable in these surgical procedures, and it stands to reason that successful muscle regeneration post-surgery is necessary to fully restore muscle function. Regeneration of damaged muscle requires a complex integration of coordinated processes involving acute inflammation, infiltration of immune cells (i.e. macrophages), proteolysis, protein synthesis, and the activation of resident muscle stem cells (i.e., satellite cells) (4, 25). Chronic, hyper-inflammation is a well known inhibitor of muscle regeneration; thus, elevated inflammatory signaling in the musculature surrounding and supporting a diseased joint would be expected to impair regeneration after arthroplasty. For example, chronic, local muscle infusion of IL-6 induces atrophy (11) and, while some TNF-α signaling appears to promote the initiation of muscle regeneration (5), chronic hyperactivity of the major transcription factor activated by TNF-α, nuclear factor kappa B (NFkB), leads to impaired muscle regeneration (1) and atrophy in mice driven in part by the E3 ubiquitin ligase MuRF1(3). Further, TWEAK impairs myogenesis (9) and induces muscle atrophy (15), also via NFkB, by binding to its receptor, TWEAK-R (aka fibroblast growth factor-inducible 14, Fn14), and degrading the inhibitor of κB (IκB) via the E3 ubiquitin ligase, TNF receptor associated factor 6 (TRAF6).

In a recent aging study, we identified and described a novel state of muscle inflammation susceptibility (MuIS), defined as a heightened basal state of pro-inflammatory signaling in skeletal muscle independent of circulating cytokine levels, as demonstrated by hyperactivity of the tumor necrosis factor α (TNF-α), TNF-like weak inducer of apoptosis (TWEAK), and/or interleukin (IL)-6 signaling pathways in muscle tissue. Additionally, satellite cells isolated from muscles of older individuals displaying MuIS (i.e. MuIS(+)) were found to have elevated basal pro-inflammatory signaling in vitro, along with cytokine hyper-sensitivity and impaired myogenic
potential when compared to satellite cells isolated from MuIS\(^{(+)\}}\) individuals, indicative of a true cellular phenotype beyond the niche. In a follow-up K-means cluster analysis, we identified adults of all ages who shared the MuIS\(^{(+)\}}\) phenotype (unpublished). We therefore suspect MuIS status may distinguish those who experience a poor regenerative response after surgery independent of age.

It is our overarching hypothesis that poor recovery and long-term mobility impairment among some individuals after THA or TKA is linked to failed muscle regeneration and regrowth following surgery, and that local MuIS is a major contributing factor. The primary objective of this initial investigation was to fully characterize the inflammatory burden in skeletal muscle of hip surgery patients, and to test whether MuIS status in elective THA patients can discriminate muscle anabolic potential. If so, MuIS status at the time of surgery may prove (in future studies) to be a promising biological, prognostic index that could predict long-term muscle regrowth potential and identify patients in need of a targeted, pro-anabolic rehabilitation program.

Here we present results of two coordinated studies. In **Study 1**, we compared local skeletal muscle inflammation and systemic inflammation across three groups: elective THA patients with end-stage OA vs. trauma victims with hip fracture (denoted HFX) vs. healthy, non-surgical controls (CON). We recruited the HFX patients specifically for the purpose of defining the most extreme state of muscle inflammation, which enabled us to better interpret findings in elective THA patients classified as MuIS\(^{(+)\}}\) vs. MuIS\(^{(+)\}}\) in Study 2. Comparisons included markers of systemic inflammation (serum cytokines), muscle tissue gene expression and protein cell signaling for pro-inflammatory and muscle protein turnover pathways, and rates of muscle protein synthesis and breakdown. Among THA and HFX, comparisons were made between perioperative samples of the major hip extensor (gluteus maximus) on the surgical side and vastus lateralis muscle samples from the contralateral thigh. Results were compared to vastus lateralis muscle tissue samples from CON. In **Study 2**, the THA patients from Study 1 were dichotomized into two groups – MuIS\(^{(+)\}}\) vs. MuIS\(^{(+)\}}\) – based on the inflammatory status of the skeletal muscle surrounding and supporting the diseased hip (i.e. perioperative gluteus maximus muscle). Differences between MuIS\(^{(+)\}}\) and MuIS\(^{(+)\}}\) in both ipsilateral (surgical) and contralateral muscles were then tested: (i) rates of muscle protein synthesis; (ii) cell signaling pathways that regulate inflammation and protein turnover; and (iii) associated transcriptional activity.

**METHODS**
Subjects

THA patients were recruited from the University of Arkansas for Medical Sciences (UAMS) Orthopedic Clinic once scheduled for elective THA (n=10), resurfacing (n=4), or THA revision (n=1). For this report, we collapsed all three of these elective surgery indications into a single group, noted as THA (n=15). For contrast purposes, 11 trauma patients with hip fracture (8 motor vehicle accident victims, 3 falls from height) undergoing emergency hip surgery were recruited from the UAMS Trauma Service, noted throughout as HFX. Subjects were excluded if they were taking insulin, thiazolidinedione drugs, or metformin; had a history of chronic renal insufficiency/disease or liver disease; had uncontrolled hypertension at the time of pre-surgical screening; had any history of hypo- or hyper-coagulation disorders including the taking of Coumadin; had a history of atrial fibrillation, angina or congestive heart failure; had recently (6 months or less) been treated for cancer other than basal cell carcinoma, or if they were pregnant. UAMS IRB-approved informed consent was obtained by the study nurse after the study was described and discussed in detail. Coded, de-identified muscle and serum samples from the UAMS hip surgery patients were sent to and analyzed in the Core Muscle Research Laboratory serving both the Birmingham VA Medical Center and University of Alabama at Birmingham (UAB). For a non-surgical comparison, muscle and serum samples – along with muscle mass and strength data – from 19 healthy subjects similar in age, gender, and BMI to the elective THA patients were used as controls (CON). These controls were drawn from the Core Muscle Research Laboratory’s de-identified human tissue and data bank.

Lower Limb Muscle Mass

Muscle mass was determined by dual-energy x-ray absorptiometry (DXA) prior to surgery in THA patients to compare surgical vs. contralateral lower limb muscle mass using a Hologic Discovery QDR DXA scanner (Hologic, Inc. Bedford, MA) according to manufacturer’s instructions.

Serum Cytokine Analysis

Circulating concentrations of proinflammatory cytokines IL-6, IL-1β, IL-8, and TNF-α were assessed using ELISA on 28 subjects (10 THA, 9 HFX; 9 CON) with MS2400 Human Pro-Inflammatory 4-Plex II Ultra-Sensitive Kits (Meso Scale Discovery, Gaithersburg, MD) and standard procedures. Samples were measured
in triplicate, and demonstrated CV’s of 3.05%, 5.47%, and 6.76% for IL-6, IL-8, and TNF-α, respectively. IL-1β levels were not sufficient to meet the minimum level of detection in some samples.

**Muscle Tissue Collection and Muscle Protein Metabolism Measurements**

For THA and HFX patients, a one-hour perioperative metabolic study was conducted to determine the fractional synthetic and breakdown rates (FSR/FBR) of skeletal muscle (gluteus maximus) protein in the surgical limb. For THA subjects only, FSR was also assessed in the contralateral thigh (vastus lateralis) prior to discharge. These contralateral muscle samples from THA patients were collected using our standard percutaneous needle biopsy procedure in the morning, fasted state under local anesthetic (1% lidocaine) with a 5-mm Bergstrom-type biopsy needle under suction as previously described (2). Any visible fat or connective tissues were dissected from muscle samples at the bedside. Identical procedures were used at UAB to collect the stored vastus lateralis samples of CON subjects. All muscle tissue samples were weighed, divided, and snap-frozen (25–35 mg/tube) in liquid nitrogen and stored at -80°C.

The method of determining FSR and FBR has been validated and described in detail (34). Briefly, this method involves a stable isotopic pulse tracer injection and the measurement of enrichment in arterial blood and muscle. Calculations of FSR are based on the precursor-product principle by calculating the rate of tracer incorporation from the muscle intracellular free pool to the protein bound pool. Calculations of FBR are also based upon the precursor-product principle; however, the precursor is now the unlabeled protein bound amino acid (phenylalanine), and the product the arterial dilution of a second tracer (15N-phenylalanine). Patients were anesthetized using a standardized anesthesia protocol of propofol, isoflurane, and an opioid of choice as deemed necessary by the anesthesiologist. Blood samples were drawn from an existing arterial line. Isotope infusions were given via an existing peripheral catheter separate from the one used for blood draws. A background blood sample was drawn before the isotope was injected. Once the surgeon had the subject prepped and draped for surgery, the stable isotope ring-13C₆-phenylalanine (35 µmol/kg) was given by IV push and the study timer started. Blood samples (~5ml) were drawn at approximately 5, 10, 15, 20, 30, 35, 40, 50, and 60 minutes on the timer (exact times were recorded). Samples of the gluteus maximus muscle (~100-200mg) were taken from the surgical site 5 and 60 min after tracer injection, subsequently cleaned of visible
fat, and snap frozen in liquid nitrogen. To measure FBR, a second tracer, $^{15}$N-phenylalanine (35 μmol/kg), was injected at the 30 minute time point and arterial blood was sampled as described above.

Prior to discharge from the hospital following THA surgery, patients were studied to determine 24 h FSR. Briefly, ring-$^2$H$_5$-phenylalanine infusion was started and the first muscle biopsy was taken from the vastus lateralis of the contralateral (non-surgical) leg approximately 2 h later. The following morning, after an overnight fast of 10 h, a second biopsy was taken from a different incision site in the same leg approximately 21-22 h after the first biopsy.

Blood and muscle samples were analyzed for isotope enrichment using methods previously described (8). Briefly, upon thawing, biopsy samples were precipitated with 800 μl of 14% perchloroacetic acid. Tissue was homogenized, centrifuged, and tissue free amino acids (labeled phenylalanine) were extracted from the supernatant by cation exchange chromatography (Dowex AG 50W-8X, 100–200 mesh H+ form; Bio-Rad Laboratories, Richmond, CA), and dried under vacuum (Savant Industries, Farmingdale, NY). The remaining muscle pellet was washed and dried, hydrolyzed in 6N HCl at 50°C for 24 h. Muscle free and protein-bound ring-$^{13}$C$_6$-phenylalanine enrichment were determined using the tert-butyldimethylsilyl derivative and GCMS (HP model 5973/5, Agilent Technologies) with electron impact ionization and selective ion monitoring for ions 234, 239, and 240.

**Muscle Protein and RNA Isolation**

As we have described in more detail elsewhere (19, 21), muscle samples (~30 mg) were homogenized after a 15 min pre-incubation in 6 μl/mg muscle of ice cold lysis buffer with protease and phosphatase inhibitors and then centrifuged at 15,000xg for 40 min at 4°C. Supernatant was stored at -80°C until assayed for protein content using the bicinchoninic acid technique with BSA as a standard. Total RNA was isolated and further purified from frozen muscle samples (~30 mg) using Tri-Reagent (Molecular Research Center, Cincinnati, OH) and RNeasy Mini Kits (QIAGEN Inc., Valencia, CA), respectively, following the manufacturer’s instructions. RNA quantity and quality were determined using a spectrophotometer (NanoDrop ND-1000, Thermo Scientific, Rockford, IL).

**qPCR**
Muscle transcript levels for 10 genes involved in pro-inflammatory, proteolytic, and anabolic processes were quantified via qRT-PCR using Taqman gene expression assays (Applied Biosystems, Foster City, CA) as we have described previously (21, 33). Briefly, cDNA was synthesized via reverse transcription using the SuperScript VILO cDNA Synthesis kit (Invitrogen, Carlsbad, CA). Specific mRNAs of interest assayed via qPCR included: IL-6 (Hs00985639_m1); IL-6 receptor (Hs00794121_m1); TNF-α (Hs00174128_m1); TNF receptor 1A (Hs00533560_m1); TWEAK (Hs00356411_m1); TWEAK receptor (Hs0017993_m1); atrogin-1 (Hs01041408_m1); MuRF1 (Hs00822397_m1); IGF-I (Hs01547656_m1); and IGF-I receptor (IGF1R, HS00609566_m1). GAPDH (Hs02758991_g1) expression served as internal control. All samples were run in triplicate. Relative amounts of target mRNA were determined using the comparative threshold cycle method using StepOne software version 2.2.2 (Applied Biosystems). All results are expressed as the relative fold difference compared to the non-surgical controls.

**Immunoblotting**

Immunoblotting was performed as we have detailed elsewhere (2, 20, 21) on 15 THA (muscle samples from both surgical and contralateral legs), 11 HFX, and 19 CON. Twenty-five µg of skeletal muscle mixed protein lysate were resolved on 4-12% SDS-PAGE gels (Invitrogen), and transferred to PVDF membranes. Protein cell signaling analysis included the pro-inflammatory pathways of interest (TWEAK/NFκB and IL-6/STAT3), along with key regulators of translation initiation / protein synthesis (p70S6K1 and 4EBP1) and protein catabolism (FOXO3a). Primary antibodies against TWEAK-R/Fn14 (#4403), TRAF6 (#8028), p-NFκB p65Ser536 (#3033), NFκB p50 (#3035), p-S6K1Ser421/Thr424 (#9204), p-4EBP1Thr37/46 (#9459), STAT3 (#4904), p-STAT3Ser727 (#9136), p-STAT3Tyr705 (#9138), p-FOXO3aSer253 (#9466), and FOXO3aThr32 (#9464) were purchased from Cell Signaling Technologies (Danvers, MA) and used at 1:1000 dilution in either 5% goat serum (monoclonal antibodies) or 2% milk/2% BSA (polyclonal antibodies). HRP-conjugated secondary antibody (Pierce Thermoscientific, Rockford, IL) was used at 1:50,000 (w/v) followed by chemiluminescent detection in a Bio-Rad (Hercules, CA) ChemiDoc imaging system with band densitometry performed using Bio-Rad Quantity One (version 4.5.1).

**Statistical Analysis**
Study 1. Among perioperative THA, HFX, and CON, group differences in muscle protein signaling, gene expression, and serum cytokine levels were tested by one-way, between-groups ANOVA. For measures that were collected only on two groups (e.g., THA and CON), tests of between-groups differences were made by independent t-tests. Within THA, surgical and contralateral limbs were tested for differences in DXA-derived muscle mass by a paired t-test.

Study 2. We conducted a secondary analysis in THA patients to explore the impact of MuIS status. THA patients were dichotomized into MuIS\(^{(+)}\) (n=7) and MuIS\(^{(-)}\) (n=7) based on gene expression of TWEAK-R in perioperative gluteus maximus muscle from the diseased hip. There was no bias among surgical indications or procedures in the subjects dichotomized as MuIS\(^{(+)}\) (4 THA, 2 resurfacing, 1 revision) vs. MuIS\(^{(-)}\) (5 THA, 2 resurfacing). Two-tailed, independent t-tests were used to assess differences between MuIS\(^{(+)}\) and MuIS\(^{(-)}\) in serum cytokines, muscle protein signaling and gene expression, and muscle protein synthesis. For all tests, Fisher’s least significant difference post hoc analysis was performed where appropriate. Results are expressed as mean ± SE. An alpha level of p<0.05 was considered statistically significant.

STUDY 1 RESULTS

Subject Characteristics

Descriptive characteristics of the three groups (CON, THA, HFX) are shown in Table 1. HFX patients were significantly younger, but no other group differences were noted. Among THA, muscle mass of the limb undergoing surgery was lower compared to the contralateral limb (p<0.05), indicating muscle atrophy associated with the disease process and/or reduced weight-bearing activity/loading.

Systemic Inflammation

Marked systemic inflammation was noted in HFX as expected. Significant group effects (p<0.01) for serum concentrations of IL-6 and TNF-α were driven by substantial hyper-inflammation in HFX. For example, serum IL-6 concentration in HFX was 40-fold higher than THA and 20-fold higher than CON (p<0.01, Fig. 1A), while circulating TNF-α concentration in HFX was 79% higher than THA and 45% higher than CON (p<0.01, Fig. 1B). IL-8 levels were not elevated in HFX (10.9 ± 1.7 pg/mL) compared to CON (9.7 ± 1.4 pg/mL) and, surprisingly, serum IL-8 was low (p<0.05) in THA (4.9 ± 1.0 pg/mL) compared to the other two groups (not shown). Because IL-1β levels were not sufficient to meet the minimum level of detection in several samples,
complete data were not available. Overall we found no indications of heightened systemic inflammation in THA vs. CON.

**Skeletal Muscle Inflammation**

The major hip extensor (gluteus maximus) surrounding the fractured hip in HFX at the time of emergency surgery was severely inflamed, as indicated by markedly elevated expression of pro-inflammatory genes and concordant elevations in pro-inflammatory signaling. In many cases, perioperative gluteus maximus samples from THA were also characterized by hyper-inflammation, albeit to a lesser degree than HFX. Results for each of the inflammatory pathways studied are summarized in Figure 2 and described here.

**IL-6 Pathway.** Main group effects (p<0.001) for the skeletal muscle expression of IL-6 and its receptor were driven by HFX. Muscle IL-6 mRNA was 14-fold higher in HFX vs. CON and THA (p<0.001), while levels in THA and CON were not different (Fig. 2A). IL-6 receptor mRNA was more than 17-fold higher in HFX vs. CON, and 4-fold higher in THA vs. CON (p<0.001, Fig. 2A). The greater than 4-fold difference between HFX and THA was also significant (p<0.001). Regarding IL-6/STAT3 signaling, STAT3 (S727) phosphorylation in HFX was 150% higher than CON and 78% higher than THA (p<0.05) (Fig. 2B-C); however, total STAT3 protein and STAT3 phosphorylation at Y705 were not significantly elevated in HFX (Fig. 2C).

**TNF-α and TWEAK Pathways.** Main group effects (p<0.001) were found for all gene transcripts assayed with the highest levels of expression noted in HFX. TNF-α mRNA levels were more than 8-fold higher (p<0.001, Fig. 2A) in HFX vs. both THA and CON (which were not different). Compared to CON, TNF-α receptor (TNF-R1) expression was 6-fold higher in HFX and 2-fold higher in THA (p<0.001, Fig. 2A). The 3-fold difference in TNF-α receptor expression between HFX and THA was also significant (p<0.001). Similarly, muscle TWEAK mRNA levels were significantly different (p<0.01) among all groups (Fig. 2A): nearly 4-fold higher in HFX, and 2-fold higher in THA vs. CON. TWEAK mRNA was also higher in HFX compared to THA. TWEAK-R expression was 12-fold higher in HFX vs. CON (p<0.001, Fig. 2A) and nearly 6-fold higher in HFX vs. THA (p<0.001), while levels did not differ between THA and CON. Regarding TWEAK/TRAF6/NFκB signaling, protein levels were remarkably elevated (p<0.05) in HFX compared to both CON and THA: TWEAK-R was 12-fold higher in HFX vs. CON; TRAF6 levels in HFX were 52% greater than CON and 105% greater
than THA (Fig. 2B-C); and the phosphorylation state of NFκB p65 (S536) was 83-fold higher than CON and 5- 
fold higher than THA (Fig. 2B-C). NFκB p50 levels were not different among groups (Fig. 2C).

**Skeletal Muscle Protein Turnover and Associated Regulation**

Results of muscle protein metabolism and putative regulatory pathways are shown Figure 3. The 
mixed muscle protein fractional synthetic rate (FSR) was higher in HFX vs. THA at the time of surgery 
(p<0.05), while fractional breakdown rate (FBR) did not differ between groups (Fig. 3A). Both groups were in 
negative protein balance, reflective of the fasted state and likely exacerbated by stress. Muscle protein 
metabolism studies via tracer infusions were not performed in CON. Regarding the expression of genes whose 
protein products modulate protein turnover, we analyzed transcript levels of the ubiquitin E3 ligases MuRF1 
and atrogin-1, as well as the anabolic IGF-I and its primary receptor (IGF1R), and main group effects (p<0.005) 
were found for 3 of 4 transcripts (atrogin-1, IGF-I, and IGF1R, Fig. 3B). Atrogin-1 mRNA was more than 2-fold 
higher in HFX vs. THA and CON (p<0.01), while MuRF1 expression did not differ between groups (not shown). 
Compared to CON, IGF-I expression was 3-fold higher in THA (p<0.005) and 5-fold higher in HFX (p<0.001). 
Also relative to CON, IGF1R expression was 1.7-fold higher in THA (p<0.05) and 2.6-fold higher in HFX 
(p<0.001). We also noted significant group differences in protein turnover signaling (Fig. 3C). The 
phosphorylation state of FOXO3a was highest in HFX at both phosphorylation sites assessed (T32 
phosphorylation shown). FOXO3a phosphorylation was 3-fold higher in HFX vs. CON at both sites (p<0.001) 
and, in HFX vs. THA, T32 phosphorylation was 106% higher (p<0.05) and S253 phosphorylation tended to be 
higher (80%, p=0.054). S6K1 phosphorylation (S421/T424) was also highest in HFX (113% higher than both 
CON and THA, p<0.01). No group differences in 4EBP1 phosphorylation were found.

**Effects of Fracture/Trauma on Muscle Inflammation are Systemic**

In addition to the perioperative muscle samples collected from the gluteal muscle of the surgical hip, in 
both HFX and THA patients we biopsied the vastus lateralis of the contralateral thigh just prior to discharge. 
Among HFX, this contralateral muscle showed elevated pro-inflammatory gene expression compared to CON, 
indicating a systemic inflammatory state (for at least a few days) in addition to the local trauma (Fig. 4). Pro-
inflammatory gene expression was heightened in the contralateral thigh of HFX for IL-6 (5-fold), IL-6R (4-fold), 
TNF-α (2.3-fold), TWEAK (2.2-fold), and TWEAK-R (11-fold) (p<0.05). By contrast, among THA, heightened
pro-inflammatory gene expression in the contralateral muscle (vs. CON) was noted only for IL-6R (3.4-fold) and TWEAK (2.1-fold) \((p<0.05)\), suggesting the inflammatory burden was largely localized to the ipsilateral limb (muscles surrounding the diseased hip). Further, for three target genes (IL-6, TNF-\(\alpha\), TWEAK-R), contralateral muscle expression was greater in HFX vs. THA \((p<0.05)\). Levels of gene expression for atrogin-1, MuRF1, IGF-I, and IGFR1 in the contralateral muscle did not differ among HFX, THA, and CON (not shown), suggesting the hypermetabolic effects were localized to the injured muscle.

**STUDY 2 RESULTS**

**Differentiating MuIS\(^{(+)}\) from MuIS\(^{(-)}\) among Elective THA Patients**

We dichotomized the elective THA patients into MuIS\(^{(+)}\) and MuIS\(^{(-)}\) based on TWEAK-R (aka fibroblast growth factor inducible-14 (Fn14)) gene expression in the muscle tissue surrounding the diseased hip. TWEAK-R gene expression averaged 5-fold higher in the 7 elective THA patients with the highest levels, which we identified as the MuIS\(^{(+)}\) group, while TWEAK-R expression in the MuIS\(^{(-)}\) group did not differ from CON (Fig. 5A). We verified an elevated pro-inflammatory and proteolytic gene expression profile in the gluteus muscle surrounding the diseased hip in the MuIS\(^{(+)}\) group (Fig. 5B). In MuIS\(^{(+)}\) vs. CON, IL-6 mRNA was 2-fold higher, IL-6R expression was elevated 6-fold, transcript levels for TNF, TNF-R, and TWEAK were \(~2.5\) fold higher, and MuRF1 expression was more than 3-fold higher \((p<0.05)\). Among MuIS\(^{(-)}\) THA patients, only IL-6R expression was higher than CON \((~2.7\)-fold, \(p<0.05)\).

**Among Elective THA Patients, MuIS is a Local, non-Systemic Phenomenon**

The impact of MuIS was largely localized to the muscles of the diseased hip. No differences in systemic inflammation (serum cytokines) were seen between MuIS\(^{(+)}\) and MuIS\(^{(-)}\) groups (Table 2), and in the contralateral thigh of MuIS\(^{(+)}\) only IL-6R \((+5\)-fold) and TWEAK \((+3\)-fold) mRNAs were elevated vs. CON (not shown). Follow-up immunoblotting revealed that TWEAK-related protein cell signaling was elevated in MuIS\(^{(+)}\) vs. MuIS\(^{(-)}\) and CON, but only in the muscle surrounding the diseased hip (Fig. 6A-B). Among MuIS\(^{(+)}\), muscle TWEAK-R protein was elevated 190\% on the surgical side compared to CON \((p<0.05)\), but not significantly different from CON on the contralateral side. Compared to CON, the phosphorylation of NF\(\kappa\)B p65 (S536) was 5-fold higher in MuIS\(^{(+)}\) on the surgical side \((p<0.05)\) but not different from CON in the contralateral muscle. While p-NF\(\kappa\)B p65 (S536) was also elevated on the surgical side in MuIS\(^{(-)}\) vs. CON \((p<0.05)\), the degree of
phosphorylation in MuIS(+) was significantly greater than in MuIS(-) (p<0.05). TRAF6 protein levels did not differ among groups or between limbs.

Skeletal Muscle Protein Synthesis

MuIS(+) patients displayed a markedly depressed rate of mixed muscle protein synthesis in the muscle tissue surrounding the diseased hip at the time of THA surgery compared to MuIS(-) patients (-32%, p<0.05) (Fig. 7). By contrast, muscle protein synthesis rates in the contralateral limbs did not differ between MuIS(+) and MuIS(-) patients. Direct, within-subjects comparisons of surgical vs. contralateral FSR could not be made because two different FSR assessment protocols were used (surgical = 1-hour fasting pulse tracer protocol; contralateral = 24-hour FSR protocol which included meals).

DISCUSSION

This is the first study to fully characterize the inflammatory burden and protein metabolism profile in skeletal muscle of hip surgery patients, and to examine whether MuIS status in elective THA patients can discriminate muscle anabolic potential. The HFX patients experienced blunt force trauma resulting in hip fracture and a host of other injuries and, as expected, they displayed overt systemic inflammation along with markedly elevated muscle pro-inflammatory gene expression (up to +17-fold) and protein signaling (up to +83-fold) profiles at the site of injury. Muscle surrounding the fractured hip was also found to be hypermetabolic, a well-known consequence of acute trauma (10). This heightened state of muscle protein turnover was accompanied by elevated expression of genes and protein signaling involved in anabolism and proteolysis. Interestingly, concurrent with hyper-phosphorylation of S6K1 which promotes protein synthesis, we noted hyper-phosphorylation of FOXO3a. The FOXOs promote the transcription of atrogens (atrogin-1, MuRF1) and subsequent proteolysis via the ubiquitin-proteasome pathway (35). Phosphorylation inhibits nuclear translocation and therefore squelches FOXO3a activity. The data therefore suggest a negative feedback response in HFX in an attempt to counteract the up-regulated proteolysis.

The overt hyper-inflammatory state of HFX muscle resulted at least in part from systemic inflammation and this is supported by up-regulation of all pro-inflammatory genes in muscle from the contralateral limb. While most of these genes were over-expressed at much lower levels in the contralateral vs. injured limb muscles, TWEAK-R was equally induced in both limbs (~12-fold). This suggests transcriptional regulation of
TWEAK-R is highly sensitive to stress, which led us to focus on its expression as the biomarker for MuIS status in the elective THA patients. In stark contrast to HFX, we found no indications of systemic inflammation in MuIS\(^{(t)}\) THA patients, indicating the heightened skeletal muscle inflammatory burden in peri-operative muscle among MuIS\(^{(t)}\) was not driven by circulating cytokines. This is supported by results in muscle from the contralateral limb showing a limited induction of only two pro-inflammatory genes, no elevation of TWEAK-R/NFκB signaling, and a normal rate of muscle protein synthesis.

Heightened inflammatory cytokine expression has been noted in the vastus lateralis of knee OA patients (16). However, in this prior work, differences between OA patients and matched controls were relatively modest and variability among OA patients was fairly high for some transcripts (e.g., TNF-α mRNA). It is possible, if not likely, that some of the OA patients in the report by Levinger et al. (16) expressed a high muscle inflammatory burden (i.e. MuIS\(^{(t)}\)) while others remained normal. Dichotomization in the current design enabled us to reveal a phenotype that may be particularly susceptible to a chronic inflammatory burden in skeletal muscle supporting the diseased joint. It is important to point out that, when analyzed as a single group, the current results in elective THA were in many cases not different from CON. However, when dichotomized by TWEAK-R expression, several key differences between MuIS\(^{(t)}\) and CON and/or MuIS\(^{(c)}\) were revealed. For example, only MuIS\(^{(t)}\) demonstrated heightened expression of all inflammatory genes in muscle of the surgical limb, with coordinate elevations in TWEAK-R signaling and suppressed muscle protein synthesis (i.e. FSR). The potential impact of these stark MuIS\(^{(t)}\) vs. MuIS\(^{(c)}\) differences on post-surgery recovery could be profound.

Hyperactivity of NFκB, the major downstream transcription factor of both TNF-α and TWEAK signaling, has previously been shown to reduce myogenic activity and skeletal muscle regeneration by multiple mechanisms (reviewed in (1)) and induce muscle atrophy (reviewed in (13, 17)) in part via in MuRF1(3), while NFκB inhibition can enhance myogenesis and improve muscle regeneration in vivo (18). Here we show the phosphorylation (activation) state of the NFκB p65 subunit was elevated 5-fold in the perioperative muscle of MuIS\(^{(t)}\) patients with a coordinate and nearly 2-fold elevation of TWEAK-R protein. Such heightened TWEAK signaling would certainly be expected to impair myofiber regeneration potential following surgery in the MuIS\(^{(t)}\) group. That the aberrant signaling was found only in the muscle surrounding the diseased hip (i.e. not in contralateral muscle) suggests a local muscle hypersensitivity to an inflamed OA joint among MuIS\(^{(t)}\).
individuals. A logical follow-up would be to use –omics approaches to explore molecular determinants of MuIS. Because the transcriptional regulation of, for example, TWEAK-R is incompletely understood, a muscle molecular profile may shed some light on how and why MuIS develops in some individuals but not others despite the presence of end-stage OA in both groups.

It is noteworthy that THA patients in this study were consuming various individually prescribed combinations of analgesics, muscle relaxants, glucocorticoids (dexamethasone), and/or NSAIDs before and after surgery; however, there was no distinct bias in the medication regimens of those classified as MuIS(+) vs. MuIS(-). While the medication regimens of these subjects may have helped attenuate systemic inflammation, the drugs apparently had little to no effect on attenuating intracellular inflammatory signaling within the muscle of the diseased hip in the MuIS(+) group.

The findings should be interpreted in the context of a few study limitations. A small sample size did not allow us to adjust for differences in age/gender/BMI. Our THA cohort was a bit younger than that reported frequently, which indicates that the present findings are applicable to younger patients; whether these can be generalized to older patients undergoing THA remains to be determined. Direct, within-subjects comparisons of surgical vs. contralateral FSR could not be made because two different FSR assessment protocols were used. FSR was determined by the 1-hour pulse tracer method in the surgical limb perioperatively in the fasted state, while FSR in the contralateral limb was assessed just prior to discharge via a 24-hour protocol. Despite limitations, the findings of these novel experiments may offer some biological insight toward understanding a profoundly important clinical problem. Epidemiologic findings indicate as many as 35% of elective THA patients experience persistent muscle atrophy (22, 24) and mobility impairment (6, 30) even several years post-surgery, and age > 70 y, female gender, BMI ≥ 30, and depression are each associated with increased relative risk (26, 30). Presumably the long-term mobility impairment results at least in part from impaired muscle regeneration and regrowth following THA surgery.

In summary, the results suggest MuIS status at the time of surgery may be a powerful determinant of recovery potential independent of age and BMI. While MuIS(+) and MuIS(-) groups did not differ in age or BMI and both groups were non-obese and well below 70 y of age, they displayed marked differences in peri-operative muscle protein synthesis and muscle inflammatory burden. Clearly the concept of MuIS is real. It is
measurable and, in the diseased limb, reflects an intracellular pro-inflammatory and proteolytic state that approaches the acute hyper-inflammatory state following hip fracture/trauma. Certainly long-term study is needed, but it is attractive to speculate that peri-operative MuIS status may be a useful prognostic indicator of long-term muscle regrowth potential. We suspect MuIS(+) patients may be in need of a rehabilitation program that is more intensive or more pro-myogenic than usual care to overcome MuIS. If this proves to be the case, identifying MuIS(+) patients at the time of surgery may enable clinicians to make an informed decision regarding post-surgery rehabilitation – essentially facilitating a personalized rehabilitation medicine approach.

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REFERENCES


FIGURE LEGENDS

Figure 1. Indices of systemic inflammation. Serum IL-6 (A) and TNF-α (B) concentrations in controls (CON) vs. patients undergoing elective total hip arthroplasty (THA) vs. trauma/hip fracture (HFX) victims. *Different from CON, p<0.05. †Different from THA, p<0.05. Values are mean ± SE.

Figure 2. Inflammatory profile of muscle surrounding surgical hip. A) Muscle gene expression in patients undergoing elective total hip arthroplasty (THA) vs. trauma/hip fracture (HFX) victims of three pro-inflammatory cytokines (IL-6, TNF-α, TWEAK) and their receptors relative to the control (CON) group (dotted line). *Different from CON, p<0.05. †Different from THA, p<0.05. Values are mean ± SD. B) Muscle protein signaling results. S727 phosphorylation of STAT3 is plotted. *Different from CON, p<0.05. †Different from THA, p<0.05. Values are mean ± SE. C) Representative immunoblots.

Figure 3. Muscle protein metabolism and putative regulators in muscle surrounding surgical hip. A) Muscle protein fractional synthesis (FSR) and breakdown (FBR) rates in patients undergoing elective total hip arthroplasty (THA) vs. trauma/hip fracture (HFX) victims. †Different from THA, p<0.05. B) Muscle gene expression in THA vs. HFX of atrogin-1, IGF-I, and IGF-I receptor relative to the control (CON) group (dotted line). *Different from CON, p<0.05. †Different from THA, p<0.05. C) Muscle protein signaling results. T32 phosphorylation of FOXO3a is plotted. *Different from CON, p<0.05. †Different from THA, p<0.05. All values are mean ± SE. D) Representative immunoblots.

Figure 4. Pro-inflammatory gene expression profile of contralateral muscle in patients undergoing elective total hip arthroplasty (THA) vs. trauma/hip fracture (HFX) victims relative to the control (CON) group (dotted line). *Different from CON, p<0.05. †Different from THA, p<0.05. Values are mean ± SD.

Figure 5. Inflammatory profile of muscle surrounding surgical hip in THA patients classified as MuIS(-) vs. MuIS(+). A) THA patients dichotomized into MuIS(-) vs. MuIS(+) based on TWEAK-R gene expression. TWEAK-R expression was 5-fold higher in MuIS(+) than both CON and MuIS(-). B) Muscle expression profile of proteolytic (MuRF1, atrogin-1) and inflammatory (IL-6, IL-6R, TNF, TNF-R, TWEAK) genes in MuIS(-) and MuIS(+) relative to the control (CON) group (dotted line). *Different from CON, p<0.05. †Different from MuIS(-), p<0.05. Values are mean ± SD. MuIS, muscle inflammation susceptibility.
Figure 6. Inflammatory TWEAK signaling in muscle surrounding surgical hip compared to contralateral limb in MuIS(+) vs. MuIS(-) THA patients. A) Protein levels of TWEAK-R, TRAF6, and the phosphorylation of NFκB p65 (S536). *Different from CON, p<0.05. †Different from MuIS(-), p<0.05. All values are mean ± SE. B) Representative immunoblots. MuIS, muscle inflammation susceptibility.

Figure 7. Muscle protein synthesis rates in muscle surrounding the surgical hip and in the contralateral limb of MuIS(+) vs. MuIS(-) THA patients. *Different from MuIS(-), p<0.05. Values are mean ± SE. MuIS, muscle inflammation susceptibility. FSR, fractional synthesis rate.
**Table 1.** Descriptive characteristics for the three groups in Study 1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Elective Total Hip Arthroplasty (THA)</th>
<th>Hip Fracture (HFX)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>54.6 ± 2.4</td>
<td>55.2 ± 1.9</td>
<td>44.1 ± 4.1†</td>
</tr>
<tr>
<td>Gender</td>
<td>12M, 7F</td>
<td>9M, 6F</td>
<td>7M, 4F</td>
</tr>
<tr>
<td>Body mass index (BMI, kg/m²)</td>
<td>25.6 ± 0.7</td>
<td>26.1 ± 1.2</td>
<td>30.5 ± 1.9</td>
</tr>
<tr>
<td>Lower limb muscle mass (g)</td>
<td>Surgical</td>
<td>Contralateral</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8554 ± 616*</td>
<td>9174 ± 680</td>
<td></td>
</tr>
</tbody>
</table>

*Different from contralateral, p<0.05. †Different from all other groups, p<0.05. Values are mean ± SE.

**Table 2.** Descriptive characteristics and serum cytokines for total hip arthroplasty (THA) patients dichotomized as MuIS(+) or MuIS(−) based on muscle TWEAK-R gene expression in Study 2.

<table>
<thead>
<tr>
<th>Variable</th>
<th>MuIS(−) (n = 7)</th>
<th>MuIS(+) (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>52.0 ± 1.8</td>
<td>58.9 ± 3.1</td>
</tr>
<tr>
<td>Body mass index (BMI, kg/m²)</td>
<td>26.4 ± 1.3</td>
<td>26.9 ± 2.0</td>
</tr>
<tr>
<td>Serum IL-6 (pg/mL)</td>
<td>1.32 ± 0.44</td>
<td>1.41 ± 0.23</td>
</tr>
<tr>
<td>Serum IL-8 (pg/mL)</td>
<td>4.45 ± 1.22</td>
<td>5.39 ± 1.67</td>
</tr>
<tr>
<td>Serum TNF-α (pg/mL)</td>
<td>2.55 ± 0.15</td>
<td>2.38 ± 0.15</td>
</tr>
</tbody>
</table>

MuIS, muscle inflammation susceptibility. No differences between groups. Values are mean ± SE.
mRNA Fold Diff. ($2^{-\Delta \Delta Ct}$) vs. CON

- IL-6
- IL-6R
- TNF
- TNF-R1
- TWEAK
- TWEAK-R

**Legend:***

- White bars represent THA
- Gray bars represent HFX

**Significance:**
- *: p < 0.05
- †: p < 0.01

**Note:** The graph shows the fold change in mRNA expression for various genes in response to THA and HFX treatment compared to the control condition (CON). The significance is indicated by stars and double stars, with the legend specifying p-values.