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Tourniquet Use during Knee Replacement Surgery May Contribute To Muscle Atrophy in Older Adults

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Abstract

Muscle atrophy after total knee arthroplasty (TKA) occurs at a rate of 1% per day for the first two weeks. Our hypothesis is that tourniquet-induced IR injury occurring during TKA influences metabolism and may contribute to atrophy. Identifying pathways that are up regulated during this critical “14-day window” after surgery may help us to delineate therapeutic approaches to avoid muscle loss.

Keywords

aging; clinical; hypoxia; stress; FOXO; mTORC1; TKA

INTRODUCTION

In 2008, more than 650,000 primary TKAs were performed in the U.S. at a cost of \$9 billion, and it is estimated that the number of annual cases will increase to 3.48 million by 2030 (22). Despite the success of TKA in mitigating the chronic pain associated with OA and restoring functional mobility for many older adults, there are residual consequences, such as persistent muscle atrophy, which may limit the ability of patients to achieve long-term functional mobility and quality of life compared to healthy peers (28).

Using MRI, we have measured a 14% reduction in quadriceps volume within 2 weeks of TKA (9). This corresponds to a loss rate of 1% per day. In contrast, at 6 weeks after surgery we have measured a total change in quadriceps volume of –18% in the same patients (9). In other words, ~80% of the muscle atrophy occurred within the first 14 days of surgery (Figure 1). Muscle atrophy approaching 1% per day suggests that active catabolic processes may be taking place, as opposed to a reduction in the rate of muscle protein synthesis at rest and in response to protein ingestion, which are estimated to decrease by as much as –60% and –50%, respectively (35). Our interpretation is that muscle atrophy following TKA is influenced by several factors ranging from post-operative reductions in ambulation and energy intake to local and systemic effects of inflammation triggered by surgery, which in our view is exacerbated by IR injury (Figure 2). We also suspect that obesity, reduced

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activity levels caused by OA-induced knee pain, and the older age of this patient cohort make muscle cells particularly sensitive to IR injury.

Insight into the mechanisms of atrophy following TKA is essential, considering that muscle loss in older patients is likely permanent (10). Age-associated muscle dysfunction results from reduced muscle mass, strength, and functional capacity (i.e., sarcopenia). Sarcopenia occurs with normal healthy aging and is associated with elevated risk for falls, physical disability, and loss of independence (10). The estimated healthcare costs due to sarcopenia in the U.S. in 2000 was \$18.5 billion (15). Reducing the prevalence of sarcopenia by 10% could save \$1.1 billion/year (15). While sarcopenia progresses at a rate of 1%/year in healthy older adults, surgeries such as knee replacement accelerate sarcopenia (9). Although eliminating knee pain with surgical remediation (i.e., TKA) is the most appropriate course of action and clinically necessary, available evidence suggests that long-term functional gains are hampered by the inability to regain muscle mass (40). Our view is that minimizing or preventing muscle loss during this critical “14-day window” allows for maximal recovery. Based on work from our laboratory and others, we have developed a potential rationale for our hypothesis that tourniquet-induced IR injury occurring in older adults during and after TKA alters cell and tissue(s) metabolism in a way that may contribute to muscle atrophy (Figure 3).

Ischemia-reperfusion Injury

Cells respond to acute oxygen deprivation by activating an evolutionarily conserved molecular cascade that works to stabilize the microenvironment until oxygen concentration is normalized. The series of molecular events begins with immediate reductions in cell metabolism, favoring decreased energy demand, as well as activation of cell signaling pathways that regulate proteins and gene clusters associated with the cell stress response(s). The degree of cell stress increases as the duration of O₂ deprivation continues. Once O₂ levels are reestablished, a second stage of injury occurs during reperfusion when metabolic byproducts that have accumulated during ischemia are released systemically, and oxygen rich blood is made available to the previously anoxic cells and reactive oxygen species (ROS) generation temporarily exceeds the capacity of endogenous antioxidants to neutralize them. The combined effect of ischemia (or anoxia) followed by reperfusion is referred to as ischemia reperfusion (IR) injury.

IR injury can occur during organ transplantation, limb reattachment, general surgery, stroke and head injury, myocardial, kidney, liver and intestinal infarction, and, more frequently, as a consequence of tourniquet use during orthopedic surgeries. It is estimated that a tourniquet is used on average 20,000 times per day globally during routine surgeries (27). Tourniquets are employed by surgeons to prevent bleeding into the surgical site in order to maximize visualization of the wound and prevent blood loss. During surgeries involving joint arthroplasty, tourniquet use also helps prevent bleeding from the resurfaced bone, allowing for proper bone-implant cementing. Orthopedic surgeons routinely use tourniquets during procedures involving the upper and lower extremities. Examples of lower extremity use are foot and ankle surgeries, anterior cruciate ligament (ACL) reconstruction, meniscal repair, open reduction internal fixation (ORIF) of fractures, and total knee arthroplasty (TKA). This

review will primarily focus on the effects of tourniquet use in older patients having TKA, however, the potential carryover to many orthopedic settings is significant.

The Two-hour Window: Is it really benign?

It is generally accepted that tourniquet times of 2 hours or less are not associated with permanent damage. This “2-hour window” was derived from a considerable body of evidence from animals and clinical studies, which clearly show that tourniquet use produces morphological, electrophysiological, and functional impairments that are significant and increase in severity as tourniquet duration increases, but appear, in general, to be reversible with time (12). A review from 2012 entitled “Safe Tourniquet Use: A Review of the Evidence” included eight “high-quality clinical studies” to support the recommendation that, “while early electrophysiological changes and muscle atrophy are detectable, long-term functional differences are rare, and outcomes between standard tourniquet and no-tourniquet groups are equivocal” (see Fitzgibbons *et al.*, 2012 and references therein). However, a more critical evaluation of the existing data is needed. Animal data may not translate to humans, and results from a few human studies may not necessarily translate to all settings. This review is not intended as a deconstruction of papers cited by Fitzgibbons *et al.*, but argues that these eight studies should not be used as “evidence” of the safety of tourniquet for all surgeries—in particular, TKA in older adults. For younger adults having ACL reconstruction, ORIF, or meniscectomy, tourniquet-induced IR injury may resolve with time and therefore be clinically acceptable. For example, of the eight studies included as evidence to support the clinical view that tourniquet use has no lasting negative impact, Fitzgibbons and colleagues cite only one study conducted in older TKA patients. Main findings showed that pain was greater at 6 hours post-TKA in the tourniquet group but were similar at 24 and 48 hours. Flexion range of motion was greater in patients without tourniquet at 5 days post-TKA but was not different at 10 days and 3 months post-TKA. Measures of atrophy, strength and functional mobility were not performed (38). The remainder of studies cited were conducted with younger adults during and after ACL reconstruction (6), with meniscectomy patients (39), with young patients having ORIF (19), and with subjects requiring surgery for femoral shaft fractures (control group) and tibial and ankle fractures (tourniquet group) (24) that reported changes in measures of pulmonary function.

Tourniquet use is a specific concern in older adults having TKA because their muscles may be sensitive to tourniquet-induced IR injury, but the literature about this patient population is unclear. There is evidence that tourniquet vs. non-tourniquet groups do not differ in older adults having TKA (5, 38). However, many studies find clinically meaningful differences favoring the no-tourniquet group, such as less pain (4, 11), better range of motion (4, 11, 38), less limb swelling (4), greater strength (8), and faster recovery (11). To gain a better understanding of the changes in skeletal muscle occurring during and after TKA at the molecular and cellular levels, our research group has collected muscle biopsy samples in the operating room (OR) before tourniquet inflation (baseline), during maximal ischemia, and during reperfusion, usually within 10 to 25 minutes. Our most recent data (33) examines changes in cell size and gene expression profiles from muscle samples collected about 2.3 hours post-TKA.

Our research focuses on mitigating muscle atrophy and functional mobility deficits that result from TKA. This surgery involves replacing the proximal tibial plateau, posterior patella, and distal femur with prosthetic components in order to eliminate the source of pain caused by chronic osteoarthritis (OA), which is the leading diagnosis for TKA. Currently, OA affects 60% of adults over the age of 65 in the U.S. and is the leading cause for hospitalization of adults aged 45–84 (34). OA of the knee is an insidious condition characterized by progressive loss of cartilage and bone, changes to tendon and ligaments, joint swelling, and painful ambulation. Inevitably, the pain from bone-on-bone contact necessitates surgical remediation via TKA, but the time from onset of symptoms to joint replacement may take years, during which health and activity are negatively impacted. Thus, it is not surprising that patients with OA have about two-thirds the muscle mass of older adults without OA (28).

The following is a brief review of the cell physiology of ischemia and reperfusion injury, along with results from our studies with older adults during and immediately after TKA surgery showing that cell signaling of muscle cells is altered. Included are brief reviews of the mechanisms governing muscle protein synthesis and degradation as well as cell and endoplasmic reticulum (ER) stress responses. To supplement this brief review, readers are encouraged to take advantage of the many excellent published reviews which cover relevant areas: immune and complement activation of IR injury (3), signaling pathways controlling translation (36), mitochondrial reactive oxygen species generation (31), ER stress and mitochondrial involvement in cell survival (26), ER stress in skeletal muscle (7), clinical perspective on safe tourniquet use (12), and cell biology of IR injury (18).

Ischemia

Under normoxic conditions basal cell metabolism relies primarily on complete oxidation of long-chain fatty acids in the mitochondria through beta-oxidation generating acetylCoA followed by citric acid cycling, electron transport chain (ETC) pumping of hydrogen ions, and ATP Synthase transferring an inorganic phosphate to ADP to form ATP. Oxygen's role is to accept electrons from the ETC, which combine with two hydrogen ions (H^+) to form molecular water, allowing the flow of electrons (and H^+ pumping) to proceed. When blood flow is cut off, oxygen tension and myoglobin saturation falls, resulting in tissue ischemia/anoxia regionally depending on local metabolic and cellular adjustments. It has been shown that in human skeletal muscle, molecular oxygen (O_2) levels decrease by 50% within 5 minutes of ischemia and are reduced further after 15 and 30 minutes, reaching levels of 28% and 19% of baseline, respectively (29).

Several mechanisms are available in muscle cells to buffer ATP levels. The phosphagen system is the immediate go-to source for energy via the coupled creatine kinase (CK) reaction ($\text{Creatine phosphate} + \text{ADP} + H^+ \rightarrow \text{Creatine} + \text{ATP}$) and adenylate kinase (AK; referred to as myokinase in muscle cell metabolism) activity ($2\text{ADP} \rightarrow \text{AMP} + \text{ATP}$). NMR spectroscopy has shown that PCr concentrations fall by half within 14 minutes of ischemia (25). This and another study (29) have shown that ATP concentration remains steady throughout 2.5 hours of ischemia while the concentrations of ADP, AMP, IMP, and Pi are elevated. However, other researchers have measured a decrease in ATP concentration in

skeletal muscle under ischemic conditions (17). In general, once ATP levels are depleted, cell death and necrosis result.

The phosphagen system is sensitive to alterations in the concentrations of substrate and products and acts in synergy with glycogenolysis during ischemia. AMP generated by the AK reaction increases the activity of phosphorylase, which breaks down glycogen for ATP production (limited to the cytosol during O₂ lack). In addition to stimulating phosphorylase, AMP increases the activity of phosphofructokinase (PFK) by allosteric regulation and enhances the activity of the purine nucleotide cycle (PNC). The PNC converts AMP to IMP, which combines with the amino acid aspartate to form ribose 5-P adenylosuccinate and the anaplerotic (increase in citric acid cycle intermediates) formation of fumarate by adenylosuccinase. The PNC is highly active in Type II muscle fibers during intense muscle activity, which parallels metabolic activity during O₂ lack.

During the shift from oxidative to O₂-depleted energy metabolism, the cell becomes acidic due to the accumulation of protons (H⁺) from excessive reliance upon glycolytic ATP hydrolysis to meet energy demands. In one study, glucose levels decrease significantly within 15 minutes of ischemia and drop to less than 50% of basal levels within 30 minutes, reaching nadir levels of ~35% of basal within 45 minutes (20). Lactate concentrations from this study showed inverse reciprocal increases, greater than 150% of baseline at 15 minutes and greater than 250% at the end of the ischemic period, ~75 minutes, indicating a rapid increase in anaerobic glycolysis. In response, the cell removes protons from the cytoplasm by co-export with lactate and by increasing Na⁺-H⁺ exchange. Thus there is an accumulation of intracellular Na⁺, which in the ischemic heart has been shown to increase linearly by fourfold within 30 minutes (31). Na⁺ accumulation may also occur because of a decrease in efflux secondary to reduced ATP availability to drive Na⁺/K⁺ exchange via the Na⁺/K⁺ ATPase pump. Intracellular concentrations of Ca²⁺ are kept several orders of magnitude lower than the SR (ER) and extracellular levels under homeostatic conditions. This is accomplished by several pumps, including the SR Ca²⁺ATPase, which pumps Ca²⁺ into the SR; the sarcolemmal Ca²⁺ATPase, which pumps Ca²⁺ out of the cell; and the Na⁺-Ca²⁺ exchanger, which exchanges intracellular Ca²⁺ with extracellular Na⁺. These pumps rely on a constant supply of energy in the form of ATP, which may be limited during ischemia (31). The accumulation of positively charged ions in the cytoplasm attracts negatively charged chloride ions. The combined increase in ion concentration within the cytoplasm causes bulk movement of water from the cell exterior to the cell interior by osmosis, facilitated by ubiquitously expressed aquaporin channels within the sarcolemma. As sarcoplasmic levels of lactate accumulate, it progressively inhibits glycolytic ATP production and exacerbates acidosis, further impairing the ability of the above-mentioned energy requiring pumps to maintain ion gradients, resulting in cell swelling and rupture.

Reperfusion

The duration of ischemia is the primary factor mediating the magnitude of cellular injury, however, reperfusion with oxygen-rich blood exacerbates initial damage caused by ischemia. Although reperfusion is obligatory for cell survival, it creates two immediate challenges for the patient. First, metabolic byproducts that accumulated during the ischemic

phase are now disseminated systemically via circulation, which is of considerable concern to physicians as this may lead to remote organ involvement/injury. Second, the previously anoxic cells and tissues are now exposed to oxygen, which can lead to oxidative damage in myocytes by promoting formation of reactive oxygen species (ROS) generation (18).

Under basal conditions, most oxygen entering cells is reduced to molecular water in the mitochondrial matrix. However, highly reactive superoxide anion ($O_2^{\cdot-}$) can be formed when a single electron attaches itself to the antibonding orbital of molecular oxygen (32). A primary site of $O_2^{\cdot-}$ production during ischemia reperfusion is the mitochondrial respiratory chain, particularly NADH-ubiquinone oxidodehydrogenase (complex I) and ubiquinone-cytochrome c oxidoreductase (complex III). Superoxide is also formed enzymatically by the membrane-bound enzymes nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidases and xanthine oxidase, and uncoupled nitric oxide synthase (NOS). If not enzymatically converted to hydrogen peroxide (H_2O_2) by endogenous superoxide dismutase (SOD), superoxide will react with cellular proteins, membrane phospholipids, RNA and DNA, contributing to cellular injury. Superoxide dismutase enzymes exist in two forms: copper zinc (CuZn) SOD expressed in the cytosol and interstitial space, and manganese (Mn) SOD located in the matrix of the mitochondria. SODs represent the first line of defense against oxygen radicals and under resting and exercise conditions are capable of mediating ROS production. Once generated, H_2O_2 can easily diffuse through cell membranes (e.g., mitochondrial, nuclear envelope, out of the cell) and react with transition metals Fe^{2+} and Cu^{2+} to form the highly reactive hydroxyl radical ($\cdot OH$) unless it is reduced to H_2O and O_2 by catalase or glutathione peroxidase (32). Under basal conditions, endogenous antioxidant enzymes can keep pace with ROS formation. However, following ischemia, when metabolic byproducts dilate blood vessels and oxygen is made available to the extremity, local ROS generation is accelerated at a rate that outpaces the capacity for antioxidant enzymes to neutralize them and damage occurs.

Muscle Protein Synthesis during TKA-induced IR Injury

Protein turnover is regulated in skeletal muscle cells by transcriptional and translational signaling mechanisms. A balance between anabolic and catabolic processes is primarily coordinated through the phosphoinositol 3-kinase (PI3K)/Akt/forkhead boxO3a (FOXO3a) pathways. During anabolic conditions, such as with insulin-like growth factor-1 (IGF1) stimulation, Akt activation of the mammalian target of rapamycin complex 1 (mTORC1) stimulates the activation of proteins controlling translation initiation and elongation. Translation initiation is the processes whereby mRNA destined for expression as a protein is attached to a mature ribosomal complex. Elongation denotes the process of peptide elongation when each amino acid is attached to the next via disulphide bonds according to the nucleotide sequence within the mRNA. In all cells, the process of protein synthesis is highly regulated and requires significant energy, accounting for 20% to 30% of total energy consumption of a cell (36). The stoichiometry works out to 2 ATP hydrolyzed for each amino acid activated and bound to t-RNA, and 2 GTP for synthesis of each peptide bond formed during elongation. Thus, for every 1 mole of amino acids incorporated into polypeptides, 4 moles of ATP are used. Under conditions that significantly reduce ATP

supply, energetic processes such as protein synthesis are inhibited until O₂ supply is normalized.

Translation initiation starts with the formation of the ternary initiation complex consisting of eIF4A, eIF4G, and eIF4E (forming the eIF4F complex). The eIF4F complex binds with the 40S ribosomal subunit and stabilizes the pre-initiation complex before it binds to the 7-methylguanosine cap (m⁷G) structure located at the 5' end of the mRNA transcript. The regulation of eIF4E availability is a primary mechanism for controlling eIF4F complex formation. Formation of eIF4F is blocked by the translational repressor 4E-BP1 (eIF4E-binding protein 1), which inhibits eIF4E from complexing with eIF4A and 4G. Like turning a switch on and off, phosphorylation of 4E-BP1 attenuates its ability to bind eIF4E while desphosphorylation does the reverse, allowing 4E-BP1 to bind eIF4E tightly and inhibit eIF4F complex formation. Thus, under ischemic conditions, 4E-BP1 becomes dephosphorylated, inhibits eIF4F complex formation, and reduces the rate of translation initiation. Additional regulation involves phosphorylation of eIF4E at Ser-209, which decreases the binding affinity of eIF4E for capped mRNA. Stress- and/or cytokine-activated signaling regulates phosphorylation at Ser-209 to a greater extent than anabolic stimuli (36) and suggests multiple levels of local control during cell stress. We have shown that during TKA phosphorylation of eIF4E at Ser-209, the target of the translational repressor protein 4E-BP1, is increased relative to pre-surgery (1). 4E-BP1 is a downstream target of mTORC1, which is regulated by Akt. mTORC1 inhibition by down regulation of Akt activates autophagy and inhibits translation initiation. We have demonstrated that during and after TKA, Akt and 4E-BP1 are hypophosphorylated, indicating a down regulation of translation initiation (1, 14, 37). In addition to initiation, further regulation of translation occurs via proteins controlling elongation. Eukaryotic elongation factor-2 (eEF2) promotes the translocation of the peptidyl-tRNA from the A-site to the P-site of the ribosome. eEF2 is inhibited by phosphorylation, which is controlled by eEF2 kinase (eEF2K), which itself is controlled by mTORC1. Thus, reduced activity of Akt down regulates mTORC1 signaling and results in decreased phosphorylation of 4E-BP1, which inhibits translation initiation as previously described, and increases phosphorylation of eEF2, which attenuates translation elongation. We have shown that eEF2 is phosphorylated during and after TKA. Assimilated, during TKA, three significant control points regulating protein synthesis are altered: Akt, 4E-BP1, and eEF2 (1, 14, 37). These data suggest that during TKA-induced IR injury muscle protein synthesis is down regulated by reducing translation initiation and elongation (Figure 4).

Muscle Protein Degradation and Cell Stress during TKA-induced IR Injury

The process of muscle protein degradation is initiated, in part, by down regulation of Akt signaling (Akt dephosphorylation). Reduced Akt activity dephosphorylates the transcription factor, FOXO3a, which becomes activated and translocates from the cytosol to the nucleus. Nuclear FOXO3a binds to the promoter region and up regulates the transcription of proteins involved in ubiquitination and the 26S proteasome (23). The 26S ubiquitin-proteasomal complex catabolises soluble and myofibrillar (actin, myosin and titin, for example) proteins (23). The ubiquitin-proteasomal pathway is rapidly up regulated by two muscle-specific E3 ubiquitin ligases, muscle atrophy F-Box (MAFbx) and muscle RING finger 1 (MuRF1).

MAFbx and MuRF1 are transcriptional targets of FOXO3a, which is regulated by Akt activity. The ubiquitin-proteasomal pathway can also be activated by the transcription factor NF- κ B, which is a known target of TNF- α , a cytokine that can bind to muscle cell membrane receptors. We have measured increases in protein and transcripts for MAFbx and MURF1 during TKA, suggesting that Akt down regulation may allow for catabolism to occur (1).

FOXO3a may also be acted upon by the stress-activated protein kinase (SAPK)/JNK pathway. We have measured an increase in SAPK/JNK transcript levels and protein concentrations in the cytoplasm during TKA (1). We have also measured an increase in p38 mitogen-activated protein kinase (MAPK) during TKA (1). Activation of p38 MAPK has been shown to occur in cardiac cells following IR injury (2). Stimulation of the SAPK/JNK/MAPK pathway during TKA suggests a role for oxidative stress as a potential factor stimulating the activation of the catabolic pathway.

BCL-2 proteins are important for regulating the permeability of the outer mitochondrial membrane. If the outer mitochondrial membrane becomes permeabilized, proteins within the inner mitochondrial membrane leak out and can activate pro-apoptotic pathways, notably, caspase-3. We have measured a trend ($p = 0.054$) for BCL-2 protein to accumulate in the cytoplasm during ischemia (1). We have additionally measured a decrease in nuclear levels of the BCL-2 19-kDa-interacting protein 3 (BNIP3) during TKA. BNIP3 is a pro-cell death member of the BCL-2 family of proteins that govern mitochondrial homeostasis, and has been shown to stimulate autophagy in myocardial cells following IR injury. Further, hypoxia and low pH have been shown to activate BNIP3 and initiate cell death pathways in cardiac cells (21). Taken together, the above data suggest that, during TKA, altered signaling via the Akt-FOXO3a pathway up regulates MAFbx and MuRF1, which stimulate ubiquitin-proteasomal-dependent protein degradation and may play a role in mitochondrial function via the BNIP3 pro-cell death pathway (Figure 4). A newly published paper by Jawhar and colleagues (16) shows that compared to TKA performed without tourniquet, proteolytic activity was elevated in vastus medialis muscle tissue after 60 minutes of continuous tourniquet application. To our knowledge, besides the studies we have conducted, there is a dearth of studies similar to Jawhar *et al*, (16) looking at the acute effects of tourniquet-induced anoxia on markers of catabolism and stress-related signaling at the tissue level. Either way, their data (16) corroborate our view that cell catabolism is uniquely altered during TKA under anoxic (tourniquet) conditions.

ER Stress Response during TKA-induced IR Injury

The endoplasmic reticulum (ER) can be divided into rough and smooth; rough equating to the co-localization of ribosomes within the membrane and smooth representing ER membrane without ribosomes. In skeletal muscle, the smooth ER and the sarcoplasmic reticulum (SR) are the same and are juxtaposed with mitochondria. The SR functions as a calcium reservoir; with calcium ATPase pump continuously hydrolyzing ATP to transport cytosolic calcium back into the SR against its electro-chemical gradient. The rough ER membrane is contiguous with the nuclear envelope and facilitates the folding of intra-organellar, secretory and membrane bound proteins. The ER maintains high Ca^{2+}

concentrations necessary for proper protein folding and protein chaperones to perform properly (26). Disruption in Ca^{2+} concentrations, due to either sarcolemmal disruption and/or the inability to maintain high calcium levels in the ER, leads to misfolding of proteins, which accumulate in the ER lumen. Alterations in redox state and glucose levels may also cause disruptions in protein folding; in particular glucose is necessary for protein glycosylation during posttranslational processing/modification. Lack of oxygen will also directly inhibit the enzyme protein-disulfide isomerase, which requires adequate oxygen to function properly, and alter protein folding. The signaling cascade initiated by ER stress is known as the unfolded protein response (UPR).

Unfolded (misfolded) proteins are sensed in the lumen of the ER by the chaperone protein, binding Ig protein (BIP), also known as glucose regulated protein of molecular weight 78 (GRP78). BIP has a high affinity for hydrophobic segments of the unfolded protein. When the protein is properly folded, these hydrophobic regions are found within the interior. Chaperones other than BIP, along with Ca^{2+} , play a role in sequestering these hydrophobic regions during the folding process. However, if misfolding occurs or Ca^{2+} homeostasis is not maintained, these hydrophobic regions are exposed and lure BIP away from the ER membrane. Detachment of BIP allows the release of transmembrane proteins such as PKR-like ER kinase/pancreatic eIF2 kinase (PERK/PEK), activating transcription factor 6 (ATF6), and inositol requiring 1 α (IRE1). (See figure 2)

Upon release from BIP, PERK is released from the ER membrane and binds to and phosphorylates eukaryotic initiation factor 2- α (eIF2 α) at Ser51. eIF2 α phosphorylation at Ser51 inhibits cap-dependent translation initiation. In addition to the critical role 4E-BP1 plays in inhibiting the binding of the 5' cap of mRNA to the ribosomal complex (described above), translation initiation involves translocation of the initiator methionyl tRNA (met-tRNA_i) to the AUG start codon of the mRNA. eIF2 plays a key role in this process when phosphorylated by blocking the GTP-exchange for eIF2-GDP, effectively binding eIF2 α with the guanine exchange factor, eIF2B, inhibiting the formation of a ternary complex consisting of methionine-tRNA and eIF2 coupled to GTP. Thus, eIF2 α phosphorylation at Ser51 is a primary mechanism for down regulating global rates of protein synthesis; we have shown that eIF2 α phosphorylation is increased during TKA (14). eIF2 α phosphorylation also selectively stimulates the expression of ATF4 and growth and arrest and DNA damage 34 (GADD34). We have measured an increase in eIF2 α phosphorylation during TKA (14) as well as increases in ATF4 and GADD34 proteins (37). Each of these proteins (ATF4 and GADD34) is up regulated by eIF2 α activity in response to skeletal muscle cell stress. We have also measured increased expression of GADD45A transcripts (mRNA), which is a downstream target of ATF4. GADD34 works to feed back on eIF2 α and inhibit its activity. The increase in GADD34 protein may indicate that, during TKA-induced IR injury, appropriate feedback mechanisms are activated to attenuate initial inhibition on protein synthesis during ischemia and reperfusion. Finally, we have measured an increase in glycogen synthase kinase 3 β (GSK3 β) protein levels during TKA (1). GSK3 β can be activated when signaling through Akt is down regulated. GSK3 β has been shown to be a negative regulator of eIF2B, which regulates eIF2 α phosphorylation and inhibits formation of a ternary complex. GSK3 β is also a known regulator of the transcription factor p53, which is activated by cell stress and especially damaged DNA. Collectively, the above

results suggest that PERK activation and phosphorylation of eIF2 α and down regulation of Akt activity have parallel implications for inhibiting translation initiation at multiple control points during TKA-induced IR injury in response to ER and cell stressors.

Upon release from BIP, ATF6 translocates to the Golgi where it is cleaved, and then translocates again to the nucleus, where it up regulates a process involving a set of genes controlling selective protein catabolism referred to as ERAD. After it is released from BIP, IRE1 α targets XBP1 mRNA via a ribonuclease domain that splices out a 26nt hairpin from the XBP1 transcript, thus changing its reading frame. This change in reading frame leads to translation of a spliced XBP1 (XBP1s) protein with a C-terminal end that is an active bZIP transcription factor and a stimulator of UPR target genes. Activated IRE1 α also stimulates ER membrane associated initiator caspases that activate terminal, apoptotic caspases (30), in particular, caspase 3 (CASP3). Increase in CASP3 activity is significant under these conditions during TKA because it has been shown to play a role in muscle protein degradation via the ubiquitin-proteasome system by targeting actin and myosin, proteins important for muscle contraction. Together these three branches of the UPR act to alleviate ER stress by down regulating protein synthesis and up regulating chaperones and folding proteins in the ER (7) (Figure 4).

Cell Swelling and Transcriptional changes during TKA-induced IR Injury

A recent finding from our lab is that muscle cells tend to swell during ischemia (not significantly so, but a trend, nonetheless) and are significantly swollen after 2 to 3 hours. The most obvious cause is the imbibing of water into the cell in response to osmotic changes resulting from shifts in bulk ion movement into the cytosol as the cell attempts to buffer the drop in pH resulting from anaerobic glycolysis. Briefly, the cell exports protons via Na⁺-H⁺ exchange, which leads to Na⁺ accumulation inside the cell. Accumulation of Na⁺ may also occur if ATP is depleted or is unavailable for use by the Na⁺/K⁺ ATPase pump, although it is not clear if the pump begins to decline before ATP levels begin to drop, and there are some data suggesting that acute post-translational modifications of the pump occur during the ischemic phase (13). As the Na⁺ ions accumulate, negatively charged chloride ions (Cl⁻) traverse the cell membrane in an attempt to balance the electrical gradient, which has the effect of driving out potassium ions, while increasing the net ion concentration inside the cell, thus drawing in more water. Finally, as the intracellular concentration of Na⁺ increases, the gradient used by the Ca²⁺-Na⁺ exchanger is rendered null and Ca²⁺ accumulates. Similar to Na⁺, either a decline in ATP levels or posttranslational modifications to the Ca²⁺ATPase result in inhibition of its pumping ability, and a net increase in Ca²⁺ occurs. The source of the Ca²⁺ is the SR/ER membrane, and as the cytosolic concentration increases it enters the mitochondrial matrix. Entry of Ca²⁺ into the cytosol and mitochondria has negative consequences. First, cytosolic Ca²⁺ can stimulate the calcium-activated calpain system. Calpains are non-lysosomal cysteine proteases that are activated in the presence of cytosolic Ca²⁺. The calpain proteases exist in an inactive state under basal conditions, but when activated can have interactions with the 26S proteasome and Akt, and specifically target myofibrillar proteins such as actin and myosin. The rise in cytosolic Ca²⁺ can result in apoptosis and/or necrosis. Second, the accumulation of cytosolic Ca²⁺ causes an inward flux into the mitochondrial matrix, which is a potential mechanism for initiating mitochondrial-

mediated apoptosis, potentially via the mitochondrial permeability transition pore (mPTP). Either way, muscle swelling occurs about 2.3 hours after TKA (33), and we were able to measure an increase in cross-sectional area of all three fiber types (Type I, IIa, and IIx) (Figure 5).

To further elucidate the effects of IR injury following TKA with tourniquet on genomic changes, we performed transcriptional profiling experiments using QuantSeq 3' mRNA (Lexogen, Greenland, NH, USA) sequencing. Nearly half of the genes we found to be differentially expressed play a role in cell stress response, including genes involved in MAPK, JNK, Akt, NFkB, and JAK-STAT signaling. In order to visualize these results, we mapped our set of differentially expressed genes onto gene regulatory network diagrams we constructed using established, annotated pathways (Biocarta and Kegg) or, for less well-characterized genes, relied on literature searches to determine putative interactions (Figure 6). By constructing these gene regulatory network maps, it became clear that TKA with tourniquet leads to a significant cell stress response. For example, one of the most significantly up regulated genes in this study, PIM-1, is hypoxia-inducible, synergizes with MYC, a transcription factor involved in a variety of cell stress responses, is regulated by the JAK/STAT pathway, and regulates FOXO3a activity as well as MAPK activity. Moreover, Akt and JNK interact with FOXO3a, and are regulated by inflammatory cytokines CXCL2, CXCL1, and CCL2, which we show in our transcriptional profiling, are upregulated. As well, MuRF1, a key regulator of the proteasomal pathway, is regulated by NFkB (and by FOXO3a), which is regulated by several molecules in our dataset including NFKBIZ, CYR61, KDM6B, MT1M, TNFRSF1A, HES1, and BCL3 (33). Therefore, our gene expression analysis shows that TKA with tourniquet induces expression of the molecular components of muscle atrophy. Therefore, PIM-1 is clearly a critical “node” in the gene regulatory network controlling cell stress response. From a clinical standpoint, this is an important observation because it suggests that older individuals retain the capacity to mount a significant cellular defense response following TKA and tourniquet application (33).

SUMMARY

In addition to the insidious loss of muscle mass in older healthy adults of 1% per year, life events, such as major knee surgery (9, 28, 37, 40), accelerate sarcopenia. The clinical view that tourniquet use is benign in older adults having TKA must be reevaluated in light of our recent data showing reductions in anabolic signaling (37) and up regulation of the catabolic FOXO (1) and UPR (14) pathways that precede, by a few hours, muscle cell swelling and gene clusters associated with cell stress (33). Muscle atrophy after TKA occurs at a rate of 1% per day (14% loss over 2 weeks), and slows significantly between 2 and 6 weeks post-TKA (i.e., -14% loss at 2 weeks vs. -18% at 6 weeks) (9). Our interpretation is that muscle atrophy following TKA is an active process involving muscle catabolism, rather than purely a reduced rate of muscle protein synthesis at rest and in response to meal (protein) ingestion (35). Moreover, in light of the few benefits of using tourniquet, such as providing a clear surgical field and proper bone implant cementing, the potential positives of not using a tourniquet, such as less pain (4, 11), better range of motion (4, 11, 38), less limb swelling (4), greater strength (8), and faster recovery (11), may benefit from further study at the molecular and cellular level in order to determine the acute affects of anoxia on muscle

metabolism over the first 14 days post-surgery and how they may impact long-term physiologic and functional outcomes.

By 2030, the number of older adults having primary TKA in the U.S. is projected to increase by 673% to more than 3.4 million surgeries performed annually (22). The increase in the number of older adults having TKA surgery presents a significant financial challenge to our healthcare system, which in 2000 spent \$18.5 billion on sarcopenia-related care (15). Thus, devising ways to mitigate the accelerated sarcopenia that occurs primarily within the first 2 weeks after surgery (9) is of clinical and fiscal importance. Our approach has been to conduct preliminary proof-of-principle clinical studies to determine if essential amino acid (EAA) supplementation offsets atrophy in older adults having TKA, compared to placebo. Initial results were positive (9), although some muscle atrophy still occurred. We have also examined the potential mechanisms of action that cause the effects of IR injury on muscle metabolism acutely, and on functional mobility during rehabilitation. Our investigations have documented effects of tourniquet-induced IR injury on skeletal muscle cell metabolism and signaling, and demonstrate the need to reevaluate current clinical understanding that ischemia/anoxia has no lasting negative effects on muscle cells in older adults following TKA.

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SUMMARY

Tourniquet use during knee replacement surgery in older adults is poorly understood at the cellular level and may contribute to muscle atrophy in this clinical population.

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Figure 1. Muscle Atrophy

Percent change from baseline in muscle volume at 2 and 6 weeks post-TKA. Data derived from (11). Approximately 80% of muscle atrophy measured at 6 weeks post-TKA occurred within the first 14 days after surgery. This is equivalent to a rate of atrophy of 1% per day.

Figure 2. MRI of Edema of the Mid-thigh Region

Images from mid-thigh region using MRI obtained before (A and C) and 2 weeks after TKA (B and D). Top image (A and B) shows results for a 66-year-old male patient. Bottom images (C and D) are from a 62-year-old female patient. Note the edema in the muscle tissue and surrounding subcutaneous fat region in images B and D, obtained 2 weeks after TKA.

Figure 3. Theoretical Model

We hypothesize that IR-injury contributes to muscle atrophy following TKA.

Figure 4. Signaling Pathways Altered by IR-injury during TKA

Data indicating altered cell metabolism during TKA derived from (2, 18, 41). Proteins in bold represent change in phosphorylation, protein, or transcript levels. Up arrows represent increased phosphorylation. Down arrows represent decreased phosphorylation. Net effect on processes is shown. Myosin and eIF3f are examples of proteins targeted for degradation. Hashed lines indicate translocation or indirect pathways. \$ Represents the fact that BNIP3 levels in the nucleus decreased significantly during TKA, but a reciprocal increase in the cytoplasmic fraction was not measured. * Represents change in both protein and transcript levels; # Represents change in transcript levels; § Represents increase in total protein; † Represents cleaved caspase; ‡ Represents trend, $p < 0.1$.

Figure 5. Muscle Cell Swelling

Rightward shift in cross-sectional area frequency distribution of Type I, Type IIa, and Type IIx fibers. The shift represents cell swelling. Fibers from left (baseline, white boxes) to right (2.3 hours post-TKA, black boxes; range 84–181 minutes). The average tourniquet time was 39 ± 4 min (mean $\pm$ SD); range 33–44 minutes. Change from baseline to post-TKA CSA is significant for each fiber type ($p < 0.05$). Data from Muyskens et al, (33).

Figure 6. Gene Network Up Regulated after TKA

The image represents a subset of the entire network from Muyskens *et al.* (33). PIM-1 highly up regulated is a central node. Green nodes are genes from our dataset; the grey nodes are key signaling pathways discussed. The size of the green nodes relates to the degree of up regulation. The diagram was made using Cytoscape.