Influence of fiber-type composition on recovery from tourniquet-induced skeletal muscle ischemia–reperfusion injury

Thomas J. Walters, John F. Kragh, and David G. Baer

Abstract: This study was designed to determine if previously reported differences in the functional impairment of muscles composed of predominantly different fiber types occurs following extended periods of ischemia. We hypothesized that the soleus (Sol) muscle, a predominantly slow-twitch muscle, would be less vulnerable to tourniquet-induced ischemia–reperfusion than the plantaris (Plant), a predominantly fast-twitch muscle, as determined by the assessment of isometric contractile function. Male Sprague–Dawley rats were assigned to one of the following groups to undergo tourniquet application (TKA) \( n = 6 \) (group): 2 h TKA, 2 d recovery; 4 h TKA, 2 d recovery; 2 h TKA, 14 d recovery; or 4 h TKA, 14 d recovery. In situ isometric contractile properties were assessed in the predominantly slow-twitch Sol and the predominantly fast-twitch Plant; the contralateral muscle served as the internal control. At 2 d, muscle contraction could not be elicited via neural stimulation, but muscles did contract with direct stimulation, which indicates neural injury. This condition was resolved by day 14. At this time point, tetanic tension (Po) in the Plant was reduced by 45% and 69% in the 2 and 4 h groups, respectively. Po for the Sol was unaffected in the 2 h group, but was reduced by 30% in the 4 h group. The fatigue resistance of the Plant was increased 2 fold in the 4 h group and was unchanged in all other groups. These results demonstrate that vulnerability to tourniquet-induced ischemia–reperfusion injury is dramatically different with respect to muscle fiber-type composition.

Key words: atrophy, contractile properties, edema, rats, regeneration.

Introduction

Ischemia–reperfusion (IR) injury in skeletal muscle can occur as a result of vascular trauma, direct muscle trauma, disease, and tourniquet application (TKA) (Blaisdell 2002). Vascular trauma and TKA, although uncommon in civilian medicine, are extremely common in battlefield trauma (Beckley et al. in press). Under well-controlled surgical conditions tourniquets (TKs) are safely used for an estimated...
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**ABSTRACT**

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Materials and methods

Animal care

Male Sprague–Dawley rats weighing 448 ± 9 g were obtained from Harlan (Indianapolis, Ind.). Rats were assigned to one of the following treatment groups: (i) 2 h TKA, 2 d recovery (2 h/2 d); (ii) 4 h TKA, 2 d recovery (4 h/2 d); (iii) 2 h TKA, 14 d recovery (2 h/14 d); or (iv) 4 h TKA, 14 d recovery (4 h/14 d); n = 6/group. Animals were individually housed and cared for in accordance with the Guide to the Care and Use of Laboratory Animals in a vivarium accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. Animals were provided with food and water ad libitum before and after all procedures. The United States Army Institute of Surgical Research Institutional Animal Care and Use Committee approved all animal procedures and protocols.

General surgical procedures

All procedures (TKA and in situ contractile measurements) were performed under 1.5%–2.5% isoflurane anesthesia in oxygen, administered via nosecone and adjusted to maintain a surgical plane. Body temperature was monitored continuously with an electronic rectal temperature probe (Physiotemp Instrument, Inc.) inserted 5 cm beyond the anal sphincter, and was maintained at 37 ± 1 °C with a temperature-regulated bed (Euthanex Corp.; EZ-212). Post-treatment analgesia (buprenorphine, 0.1 mg/kg; i.m.) was administered 30 min prior to recovery from anesthesia and 12 h post-treatment.

Tourniquet application

Both hindlimbs were shaved, and one was randomly selected by coin toss. A pneumatic digital tourniquet (D.E. Hokanson, Inc., Bellevue, Wash., model DC 1.6) was placed (but not inflated) as proximal as possible around the thigh of the selected leg. Exsanguination of the leg was performed by elevation for 5 min prior to inflation. The cuff was then inflated and maintained at 250 mmHg using a cuff inflator (D.E. Hokanson, Inc., model E20) and air source (D.E. Hokanson, Inc., model AG101) and left in place for 2 or 4 h. Following the ischemic period, anesthesia was withdrawn and the animals were allowed to recover for 2 or 14 d. Orthogonal spectral imaging using a Cytoscan Video Microscope (Cytometrics, Bridleway, England) was used in preliminary studies to determine affective tourniquet pressure. A pressure threshold of 230 mmHg was required to completely halt blood flow to the muscles of the lower leg. We therefore used 250 mmHg, which included a safety factor of 20 mmHg.

In situ contractile properties

The order in which the contractile properties were determined was randomized for the treated and untreated contralateral limb. It was not technically feasible to randomize the order of the muscles within the limb. Animals were again anesthetized with isoflurane, and then placed in a prone position on a heated surgical bed. To immobilize the limb of interest, two parallel pins were placed through the femur using a surgical drill; the first was immediately proximal to the knee, the second was approximately 1 cm proximal to the first. The pins were then fixed in a custom jig. An incision was made through the skin from the calcaneus to a point midway between the greater trochanter and the base of the tail. The skin was retracted and the sciatic nerve
was exposed by blunt dissection of the biceps femoris muscles starting at the popliteal space. A nerve cuff was placed around the nerve and secured in place with 4–0 sutures as previously described (Walters et al. 1990, 1991). The nerve was cut proximal to the cuff to prevent antidromic stimulation; nerve branches including the peroneal nerve and those branches of the tibial nerve innervating the medial (MG) and lateral gastrocnemius (LG) were cut to prevent movement artifact. The two heads of the gastrocnemius muscle were separated and tenotomized (distal tendon) to expose the Plant. The MG was then excised; the distal half to two-thirds of the LG and Plant were separated, taking care to maintain blood supply. If necessary, electric cautery was used to control bleeding. The distal tendon of the Plant was isolated, cut, and threaded through a hole in the lever arm of the muscle lever and secured with 4–0 suture. The distal third of the underlying Sol was then gently separated from the Plant. The skin was then pulled back over the proximal portion of the incision and closed with surgical staples to a point corresponding to the proximal half to two-thirds of the exposed Plant. The entire Plant was covered with mineral oil; the distal exposed portion of the Plant was then covered with a piece of clear polyethylene film. To avoid damaging the Plant, the LG was used as a surrogate for monitoring temperature. The temperature of the LG was monitored with a needle thermistor (Physiotemp Instrument, Inc., Clifton, N.J.). Muscle temperature was maintained at 35 ± 1 °C using a fiberoptic light focused on the lower leg. Following the determination of contractile properties of the Plant, the muscle was detached from the lever and the procedure was repeated for the Sol. Muscle length was measured using a digital micrometer and recorded prior to detachment.

Contractile properties were measured using a dual-mode servo muscle lever system (Aurora Scientific, Aurora, Ont., models 305b and 305b-LR for the Sol and Plant, respectively). A PC loaded with a Labview®-based program (National Instruments, Inc., Austin, Tex.) controlled the muscle lever and stimulator, recorded all signals (2000 Hz), and performed real-time analysis of the force and length signals. The nerve was stimulated at 2× the voltage required to elicit maximal twitch tension (P₁) at a pulse width of 50 μs. In selected experiments, muscles were also tetanized by direct muscle stimulation using platinum electrodes inserted into the muscle near the myotendinous junction at the proximal and distal ends of the muscle; the stimulus voltage was set at 50 V with a pulse width of 500 μs. All measurements were made with the muscles set at optimal length (Lₒ), defined as the peak P₁ established from a series of twitches at increasing muscle lengths performed at 0.5 mm increments; each twitch was separated by 30 s. Stimulus frequency for peak tetanic tension (Pₒ) was set at 100 Hz for the Sol and 150 Hz for the Plant. Following establishment of Lₒ, Pₒ was determined from the average of 3 unpotentiated twitches (2 min between each twitch); Pₒ was determined from the average of 3 tetani separated by 2 min. Five minutes after the last tetani the fatigue index (FI) was determined using the Burke fatigue test (Burke et al. 1973). The fatigue test comprised 4 min of 40 Hz stimulation (330 ms burst width) delivered at a rate of 1 Hz. The FI was defined as the the mean force of the peak force of the last 5 bursts, divided by the initial peak force.

Following the completion of each experiment, rats were euthanized (sodium pentobarbital, 150 mg/kg i.p.; 21 gauge needle) muscles were excised, cleaned of fascia and connective tissue, then weighed. The Plant and Sol were then placed in a holder that maintained them at Lₒ and were snap frozen in isopentane cooled in liquid N₂.

**Histology**

Routine histology was performed on formalin-fixed, paraffin-embedded sections stained with hematoxylin and eosin (H&E) and on snap frozen muscle samples, cryostat sectioned and stained for myosin ATPase (pH 4.53) to reveal fiber types (Guth and Samaha 1969). Samples used for histology were obtained from a subset of animals (2/group) that did not undergo contractile measurements. These samples served only as representative samples — no attempt was made to perform systematic histological or morphological analysis.

**Statistical analysis**

To test the hypothesis that the Sol, a predominantly slow-twitch muscle, would be less affected by TK-induced IR than would the Plant, a predominantly fast-twitch muscle, the two were compared. The untreated contralateral limb served as the control for each rat. The ratios of TK to contralateral limb muscle weight and contractile measurements were calculated for each muscle by dividing TK limb values by the corresponding contralateral muscle values. Statistical comparisons were made between this ratio for the Sol and Plant within animals using Student’s t test. Differences were considered significant at p < 0.05. All data are presented as mean ± standard error of the mean (SE).

**Results**

**Body mass**

The average body mass at the time of sacrifice was 444 ± 4 g, 450 ± 22 g, 443 ± 15 g, and 454 ± 7 g for the 4 groups (2 h/2 d, 4 h/2 d, 2 h/14 d, and 4 h/14 d, respectively). There were no significant differences between groups.

**Muscle wet mass**

Muscle wet mass was not significantly changed in any of the muscles from 2 h/2 d. However, there were significant increases in the wet mass of all TK muscles from 4 h/2 d (Fig. 1). The increase in wet mass of the predominantly fast-twitch Plant was approximately 44%, compared with a 30% increase in the slow-twitch Sol. The wet weight for the untreated Sol was 223 ± 12 mg and 221 ± 19 mg for 2 h/2 d and 4 h/2 d, respectively. The wet weight for the untreated Plant was 519 ± 32 mg and 537 ± 36 mg for 2 h/2 d and 4 h/2 d, respectively.

At 14 d post-TKA this edema had resolved, and the wet mass was not different from the contralateral limb following 2 h TKA for any muscle. Significant reductions in mass occurred in both muscles following 4 h TKA after 14 d (Fig. 2). The decline in wet mass was less in the Sol (18% decline) than in the Plant (28% decline). These reductions were not statistically different. The wet mass for the un-
treated Sol was 204 ± 12 mg and 245 ± 8 mg for 2 h/14 d and 4 h/14 d, respectively. The wet mass for the untreated Plant was 528 ± 37 mg and 620 ± 25 mg for 2 h/14 d and 4 h/14 d, respectively.

**Contractile properties**

Stimulation of either the Sol or Plant via the motor nerve did not elicit detectable contraction at day 2 (Fig. 3A). Direct stimulation of the muscle successfully elicited contraction, indicating that a portion of the lesion involved a neural component.

Neural stimulation was possible by day 14 (Fig. 3B). For reference purposes, a summary of the mean values for the contractile properties for each muscle are presented in Table 1.

No significant differences were seen between the relative changes in the Plant and Sol in $P_o$, specific $P_o$, time-to-peak tension (TPT), and half relaxation time (1/2 RT) for either 2 or 4 h TK (Table 1). Specific forces were expressed relative to muscle mass, i.e., N/g.

In contrast to the twitch properties, significant differences between the Sol and the Plant were seen in $P_o$ and specific $P_o$ in both groups following 14 d (Table 2; Fig. 4A, 4B). Compared with the untreated contralateral Plant, the $P_o/P_o$ increased by 93% and 124% following 2 and 4 h of TK, respectively. The FI was not changed in the Sol in 2 h/14 d and 4 h/14 d (1.01 ± 0.07 and 1.13 ± 0.22, respectively), but increased significantly in the Plant for 2 h/14 d and 4 h/14 d (1.92 ± 0.21 and 2.07 ± 0.29, respectively).

**Histochemistry**

Examination of histological sections revealed a dramatic difference in the response of the Sol and Plant to severe (4 h) ischemia (Fig. 5). Sections stained with H&E reveal significant damage in both the Plant and Sol 2 d following...
ischemia; both characterized by interstitial edema, inflammatory cells, and red blood cells. The majority of the myocytes in the Plant show signs of necrosis with some intercellular edema. In contrast, the Sol is characterized by intercellular edema in virtually all intact myocytes, with only a few fibers showing signs of necrosis. At day 14, both the Plant and Sol reveal extensive atrophy when compared with the untreated control muscles. Evidence of widespread regeneration is seen in the Plant, with the central nuclei present in the majority of the cells. The Sol contains a few myocytes with central nuclei, but the majority of the cells appear normal, with the exception of size.

Routine staining for myosin-ATPase (pH 4.43) (Fig. 6) shows the existence of possible fiber-type grouping in the Plant (Fig. 6C), indicating possible reinnervation following neural injury. The Sol (Fig. 6D) appears essentially normal.

Discussion
The most important finding of this study is that there is a greater functional loss in predominantly fast-twitch muscle than in predominantly slow-twitch muscle in response to TK-induced IR. Although this has been shown by others for ischemic durations of less than 4 h, this is the first study to extend these observations through 4 h of ischemia. (Carvalho et al. 1995). Prior to this investigation, it was unknown whether the differences in the sensitivity of the two major fiber types, as determined by functional measurements, would be maintained when the ischemic insult was more severe, i.e., lasting longer than 2 h. It had been hypothesized that longer ischemic periods would cause severe enough injury to overwhelm any differences in susceptibility due to fiber type.

Most studies that have examined muscle recovery following IR with reference to fiber type have compared the Sol with the tibialis anterior (TA) or extensor digitorum longus (EDL). The TA and EDL are contained in the anterior compartment of the lower leg, and are vulnerable to compartment syndrome following acute ischemia (Awerbuck et al. 1994; Guth and Samaha 1969). As we are interested in the effect of TK-induced ischemia on muscle, we sought to remove the possibility of additional ischemic time resulting from compartment syndrome. The Sol and Plant are contained in the more compliant posterior compartment, and
are therefore not vulnerable to compartment syndrome. Thus, in the current study, differences in muscles of predominantly different fiber types can more accurately be attributed to true fiber-type differences.

It cannot be determined from the present investigation what caused the loss of specific $P_o$ in the Plant, although a number of possible explanations can be suggested. Although not directly examined in the present study, fibrosis has been shown to be a major component of many forms of muscle injury (Best and Hunter 2000; Stauber 2004). It is likely that an increase in the contribution of connective tissue to the total mass of the muscle contributed to the loss $P_o$. Histological sections of representative muscles contained numerous small fibers, particularly in the Plant, a finding that lends support to the hypothesis that fibrosis contributed to the loss of $P_o$. Histological evidence of fibrotic changes with more severe ischemic injury is consistent with previous reports (Awerbuck et al. 1994; Carvalho et al. 1995). These small fibers are presumably regenerating fibers that may not be innervated and (or) have not developed muscle–tendon junctions. Regenerating fibers are composed of developmental forms of myosin heavy chains (MHCs) (d’Albis et al. 2008 NRC Canada).
which produce significantly less force per cross-sectional area than do fibers composed of adult MHCs (Zhan et al. 1998). This is consistent with a recent report by Esposito et al. (2007), which attributed the loss of specific tension following bupivacaine to both the presence of a number of regenerating fibers expressing neonatal and embryonic MHC and the alterations in excitation–contraction coupling. All of these factors may contribute to the observed loss in absolute and specific force production.

Fatigability

The increase in fatigue resistance in the Plant following TKA was striking. Using a different fatigue test, Woitaske and McCarter (1998) reported a large increase in fatigue resistance in the EDL in mouse muscle 14 d after 3 h TKA. Although fiber-type composition was not determined following treatment, the authors speculated that an increase in slow-twitch fibers during regeneration was responsible for the increase in fatigue resistance. Awerbuck et al. (1994) reported a small but significant increase in the type I fiber area of the Plant 6 weeks after 4 h of ischemia, which likely reflects a physical loss of type II fibers. In the Sprague–Dawley rat, the Plant is composed of 6% slow-twitch fibers (Armstrong and Phelps 1984); it is therefore likely that the fatigue index may reflect the disproportionate contribution of the less-damaged slow-twitch fibers. At 14 d following TKA may reflect a number of factors, including (i) different sensitivity to ischemic injury, (ii) different sensitivity to reperfusion injury, (iii) different levels of compression-induced neural injury, (iv) differences in repair and regeneration, and (v) differences in activation during recovery.

Acute injury

The majority of the studies that have examined acute IR with reference to fiber type have demonstrated that predominantly slow-twitch muscle is less sensitive to ischemic and reperfusion injury (Gardner et al. 1984; Idstrom et al. 1990; Carvalho et al. 1997; Chan et al. 2004). However, the mechanisms for these differences remain speculative. During ischemia, the rate of adenine nucleotide degradation is less, and the rate of recovery is greater during reperfusion in slow-twitch than in fast-twitch muscle (Idstrom et al. 1990). Other factors including capillary density (Hudlicka 1985; Reichmann et al. 1985), antioxidant levels (Ji et al. 1992; Powers et al. 1994), and heat shock proteins (i.e., HSP70, HSP32) (Locke et al. 1991, 1994; Locke and Tanguay 1996) that are more prevalent in slow-twitch muscle than in fast-twitch muscle are likely to play a role in providing greater tolerance to IR in predominantly slow-twitch muscle.

Neural injury

We observed little to no force production via neural stimulation in either the Sol or Plant 2 d after TKA (Fig. 1). However, force production could be elicited using direct application.
muscle stimulation, although it was significantly less than that produced by the contralateral control muscle. This suggests that a portion of the lesion was neural in nature and probably caused by compression injury to the motor nerve underlying the TK. Nerve injury following TKA has been documented in similar animal models (Pedowitz et al. 1990; 1991b; Eastlack et al. 2004) and is a common clinical complication of TKA. In the present study, neural stimulation was possible by 14 d; however, it is unknown to what extent this functional denervation interacted with IR to produce the results observed at day 14. In selected experiments, direct and indirect stimulation were compared at the end of the session. In these experiments, \( P_0 \) was essentially the same regardless of method of stimulation. However, a more systematic comparison is required to conclusively determine whether neural function was completely restored. The extent of TK-induced compression injury is a function of neuronal size, i.e., larger neurons are affected more than small neurons (Ochoa et al. 1972). The diameter of motor nerves innervating slow-twitch motor units are smaller those innervating fast-twitch motor units (Ulthake and Kellerth 1982). Although not determined in our investigation, it is possible that the magnitude of the compression injury was less in the Sol than in the Plant. This is consistent with reports that contraction in the Sol can be elicited via motor nerve stimulation sooner than in the Plant following TKA (Carvalho et al. 1995).

**Activation patterns**

Activation patterns following TKA may play a role in the rate of recovery from the injury. In the present investigation, the rats maintained normal cage activity in the days following treatment. Because during normal cage activity the Sol and Plant have different patterns of activation, i.e., the Sol is tonically active, whereas the Plant is activated only during locomotion or rearing (Lomo et al. 1974), cage activity may have provided a greater stimulus for regeneration for the Sol, than in the Plant. Thus, the recovery of predominantly fast- and slow-twitch muscles to ischemia and reperfusion is a function of neuronal size, i.e., larger neurons are affected more than small neurons (Ochoa et al. 1972). Although not determined in our investigation, it is possible that the magnitude of the compression injury was less in the Sol than in the Plant. This is consistent with reports that contraction in the Sol can be elicited via motor nerve stimulation sooner than in the Plant following TKA (Carvalho et al. 1995).

In conclusion, following TK injury and 2 weeks of recovery, muscle function is significantly better in the predominantly slow-twitch Sol than in the predominantly fast-twitch Plant, even after 4 h of ischemia. Initially, this appears to be because the Sol is less sensitive to the actual mechanism of injury. Following injury, these differences may be amplified due to faster rate of regeneration. Other factors, such as activation patterns and/or earlier functional innervation, may have also played a role. Further studies examining the recovery of predominantly fast- and slow-twitch muscles for longer than 2 weeks are required to fully describe the role of fiber-type differences in the recovery of muscle from TK-induced IR and to determine if there is an actual difference in the regenerative capacity of different fiber types. A greater understanding of the reason for these differences is critical for developing therapies for treating injured muscles, as well as reducing the magnitude of injury.

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