Title: Acute resistance exercise increases the expression of chemotactic factors within skeletal muscle.

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Abstract

Intense resistance exercise causes mechanical loading of skeletal muscle, followed by muscle adaptation. Chemotactic factors likely play an important role in these processes. **Purpose:** We investigated the time course of changes in the expression and tissue localization of several key chemotactic factors in skeletal muscle during the early phase of recovery following resistance exercise. **Methods:** Muscle biopsy samples were obtained from *vastus lateralis* of eight untrained men (22±0.5 yrs) before and 2, 4 and 24 h after three sets of leg press, squat and leg extension at 80% 1 RM. **Results:** Monocyte chemotactic protein-1 (95×), interleukin-8 (2,300×), IL-6 (317×), urokinase-type plasminogen activator (15×), vascular endothelial growth factor (2×) and fractalkine (2.5×) mRNA was significantly elevated 2 h post-exercise. Interleukin-8 (38×) and interleukin-6 (58×) protein was also significantly elevated 2 h post-exercise, while monocyte chemotactic protein-1 protein was significantly elevated at 2 h (22×) and 4 h (21×) post-exercise. Monocyte chemotactic protein-1 and interleukin-8 were expressed by cells residing in the interstitial space between muscle fibers and, in some cases, were co-localized with CD68+ macrophages, PAX7+ satellite cells and blood vessels. However, the patterns of staining were inconclusive and not consistent. **Conclusion:** In conclusion, resistance exercise stimulated a marked increase in the mRNA and protein expression of various chemotactic factors in skeletal muscle. Myofibers were not the dominant source of these factors. These findings suggest that chemotactic factors regulate remodeling/adaptation of skeletal muscle during the early phase of recovery following resistance exercise.

Key words: resistance exercise, adaptation, myokines, skeletal muscle

Abbreviations:

FKN: fractalkine

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

IL-6: interleukin-6

IL-8: interleukin-8

MDC: macrophage-derived chemokine

MCP-1: monocyte chemotactic protein-1

RM: repetition maximum

RT-PCR: reverse transcription polymerase chain reaction

TNF-α: tumor necrosis factor-α

uPA: urokinase-type plasminogen activator

VEGF: vascular endothelial growth factor

INTRODUCTION

Skeletal muscle shows a remarkable capacity for rapid and extensive adaptation following mechanical loading. This adaptation involves complex and coordinated interactions between various cell types (e.g., macrophages and satellite cells) and proteins expressed and secreted by these cells (Charge and Rudnicki 2004). Proteins such as monocyte chemotactic protein (MCP)-1, tumor necrosis factor (TNF)-α, interleukin (IL)-8, vascular endothelial growth factor (VEGF), macrophage-derived chemokine, leukemia inhibitory factor, fractalkine and urokinase-type plasminogen activator mediate muscle adaptation by acting as chemotactic factors for satellite cells, neutrophils and macrophages (Lu et al. 2011a; Peterson et al. 2006; Peterson and Pizza 2009; Bryer et al. 2008; Chazaud et al. 2003). In turn, these cells clear cellular debris and release factors that regulate satellite cell activity and myogenesis (Arnold et al. 2007; Tidball and Wehling-Henricks 2007; Lu et al. 2011b; Bryer et al. 2008; Chazaud et al. 2003). Some secreted proteins such as MCP-1, IL-6 and TNF-α also directly stimulate myoblast proliferation and differentiation (Chen et al. 2007; Kurek et al. 1997; Serrano et al. 2008; Yahiaoui et al. 2008).

The role of chemotactic factors as mediators of muscle adaptation is well established following severe and prolonged muscle injury and degeneration in mice (Bryer et al. 2008; Chen et al. 2005; Lu et al. 2011b; Lu et al. 2011a; Warren et al. 2002; Warren et al. 2004). By contrast, resistance exercise does not cause such severe muscle damage (Malm and Yu 2012), yet it induces many of the hallmark characteristics of muscle regeneration and hypertrophy, including satellite cell activation, leucocyte infiltration and angiogenesis (Beaton et al. 2002; Moore et al. 2005; Psilander et al. 2003; Vella et al. 2012; Trenerry et al.

2007). Various studies have examined changes in the gene expression of various chemotactic factors, such as MCP-1, IL-8, TNF-α and VEGF within skeletal muscle following resistance exercise (Buford et al. 2009; Louis et al. 2007; McKay et al. 2009; Nieman et al. 2004; Hyldahl et al. 2011; Trenerry et al. 2007; Vella et al. 2012; Hubal et al. 2008). However, there were several important limitations in some of the studies.

First, some of these studies only collected muscle samples at one time point after exercise (Buford et al. 2009; Nieman et al. 2004; Hyldahl et al. 2011; Hubal et al. 2008), which does not necessarily capture adaptation/remodeling of skeletal muscle during the early phases of recovery after exercise. Second, only a few of these studies examined the protein expression and cellular localization of chemotactic factors in skeletal muscle after exercise (McKay et al. 2009; Hyldahl et al. 2011; Hubal et al. 2008). Due to post-transcriptional modifications, cytokine/chemokine mRNA may not necessarily be translated to corresponding proteins in skeletal muscle after resistance exercise (Anderson 2008). Furthermore, measuring cytokine/chemokine mRNA expression in muscle homogenates to assess cytokine gene expression (Buford et al. 2009; Louis et al. 2007; Nieman et al. 2004; Trenerry et al. 2007; Vella et al. 2012) does not indicate which resident cell types in skeletal muscle secrete cytokines and chemokines after resistance exercise.

Considering these analytical issues and the critical role of chemotactic factors as mediators of muscle adaptation, the aims of the present study were two-fold: (1) to explore the time course of changes in the expression of MCP-1, IL-6, IL-8, TNF-α, VEGF, fractalkine and urokinase-type plasminogen activator following resistance exercise and (2) to assess the cellular localization of MCP-1 and IL-8 in cross-sections of muscle tissue. The findings from

this study demonstrate that mRNA and protein expression of many of these chemotactic factors increased in the first few hours after resistance exercise, and returned to normal after 24 h. Immunofluorescent staining for MCP-1 and IL-8 also increased after exercise, but it was unclear which cell types were the dominant sources of these chemokines in skeletal muscle after resistance exercise.

MATERIALS AND METHODS

Ethics statement

Before participating in the study, the nature, purpose and risks of the study were explained to the subjects. They then provided informed written consent. All experimental procedures involved in this study adhered to the principles of the Declaration of Helsinki and were formally approved by the Deakin University Human Research Ethics Committee.

Subjects

Thirteen untrained, but recreationally active individuals volunteered to take part in this study. Eight males participated in the exercise component of this study (mean $\pm$ SD age 22.0 ± 0.5 yrs, height 1.79 ± 0.05 m, body mass 83.3 ± 19.1 kg and BMI 24.1 ± 0.1 $kg.m^{-2}$). The other individuals comprised a non-exercising control group (23.0 ± 0.9 yrs, 1.76 ± 0.05 m, 74.0 ± 4.8 kg and 23.8 ± 2.0 $kg.m^{-2}$). Exclusion criteria included resistance training within the past six months, any medications, or a previous history of a diagnosed condition or illness that would present a health risk during strenuous resistance exercise. We also restricted this

study to males because of the well known effects of estrogen on muscle inflammation and regeneration (Kendall and Eston 2002), which was not the main focus of this study.

Experimental design

Each subject in the exercise group completed a familiarization session prior to the experimental protocol to learn how to perform each exercise, and to determine their individual 1 repetition maximum (1 RM). During the session, a 5 RM test was performed for each subject for the leg press, squat (assisted by the Smith machine) and leg extension. 1 RM was then calculated using the Brzycki equation (1 RM=weight lifted (kg)/1.0278–[reps to fatigue x 0.0278]). The familiarization session took place at least 7 days prior to allow ample recovery time before the experimental trial commenced.

For the 24 h preceding exercise, and the day of the trial, the subjects consumed a standard diet (20% fat, 14% protein and 66% carbohydrate) and abstained from alcohol, caffeine, tobacco and additional exercise. On the morning of the trial, subjects presented to the laboratory in a fasted state. Following 30 min of supine resting, a muscle sample was collected from the *vastus lateralis* under local anesthesia (Xylocaine 1%) by percutaneous needle biopsy technique modified to include suction. Excised muscle tissue from each biopsy was immediately frozen and stored in liquid nitrogen for later analysis. Subjects then completed a single bout of resistance exercise, specifically targeting muscles of the leg. Following 5 min of light cycling for warm-up, subjects completed two sets of eight to twelve repetitions of bilateral leg press, squat and leg extension at 80% 1-RM. This was followed by a third set to voluntary fatigue, also at 80% 1-RM. Subjects were allowed 1 min rest between exercises and 3 min rest between sets. The entire exercise protocol lasted 20–25

min. This intensity of this exercise protocol was similar to other studies on resistance exercise (Buford et al. 2009; Louis et al. 2007; Vella et al. 2012), but lower than other studies involving purely eccentric exercise that caused severe muscle damage (McKay et al. 2009; Hubal et al. 2008).

Additional muscle samples were collected at 2 h and 4 h after the exercise. Subjects were provided with their evening meal on the day preceding the trial as well as lunch and their evening meal for the days of the trial. The following morning, subjects again reported to the laboratory in a fasted state for the collection of a final muscle sample 24 h post-exercise. To minimize the potential for inflammation arising from the biopsy procedure itself, biopsies were collected from separate incisions in the same leg, at least 2 cm distal from previous biopsy sites. We collected venous blood samples before exercise, and again at 24 and 48 hours after exercise. Blood samples were collected in tubes containing lithium heparin and centrifuged for 10 min at 2,500 rpm to separate the plasma. Plasma was stored in aliquots at −80°C prior to analysis.

We included a non-exercising control group to examine whether the muscle biopsy procedure itself caused any inflammatory responses in skeletal muscle. Five individuals in the control group reported to the laboratory in a fasted state, having abstained from alcohol, caffeine, tobacco and additional exercise in the previous 24 h. Muscle biopsies were collected (as described above) at rest (0 h) and at 2 and 4 h following the initial muscle sampling. Between the collection of the biopsies, the control subjects rested with minimal movement.

RNA extraction and RT-PCR

Total cellular RNA was extracted using a modification of the phenol/chloroform extraction and isopropanol precipitation protocol, using the TōTALLY RNA™ Kit (Ambion Inc., Austin, TX). RNA quality and concentration were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). First-strand cDNA was generated from 0.5 μg total RNA using the AMV RT kit (Promega, Madison, WI). RT-PCR was performed using the GenAmp 7500 sequence detection system (Applied Biosystems, Foster City, CA). PCR was performed in duplicate with reaction volumes of 20 μl, containing SYBR Green 1 (Applied Biosystems), forward and reverse primers and cDNA template (diluted 1:20). Data were analyzed using a comparative critical threshold (Ct) method where the amount of target normalized to the amount of endogenous control relative to control value is given by $2^{-\Delta\Delta Ct}$ (Applied Biosystems, Foster City, CA). GAPDH was selected as an endogenous control because expression of this gene was unchanged in response to the exercise intervention or sampling time (data not shown). Primers for MCP-1, IL-8, TNF-α, IL-6, macrophage-derived chemokine (MDC), VEGF, fractalkine (FKN), urokinase-type plasminogen activator (uPA) and myogenin were designed using Primer Express software package version 3.0 (Applied Biosystems) from gene sequences obtained from GenBank (see Table 1 for details).

Multiplex analysis

A bio-plex assay (Bio-Rad Labarotories, Hercules, CA) was used to analyze the protein expression of cytokines within skeletal muscle tissue. In the present study, kits were designed for the simultaneous analysis of IL-6, IL-8, MCP-1 and TNF-α. The assay was conducted following the manufacturer's instructions (Bio-Rad Labarotories, Hercules, CA) and using reagents from the Cytokine reagent kit (Bio-Rad). Tissue samples (10 mg) were

homogenized in lysis buffer (20 mM Tris-HCl, 5 mM EDTA, 10 mM Na-pyrophosphate, 100 mM NaF, 2 mM Na_3VO_4, 1% Igepal CA-630, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin, 3 mM Benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF) using a hand-held homogenizer. The homogenate was rotated at 4°C for 1 h, centrifuged at 13,000 rpm at 4°C for 10 min, and the supernatant was collected. This supernatant was then diluted to 500 μg/ml. The supplied standards were diluted according to the manufacturer's instructions for running the plate with the high sensitivity range (High PMT), which equated to 0.3–4,182pg/ml for IL-6, 0.1–2,353 pg/ml for IL-8, 0.2–2,718 pg/ml for MCP-1, and 0.6–9176 pg/ml for TNF-α. The plate was then read on the Bio-Plex Suspension Array System (V.5.0, Bio-Rad). All samples were run in triplicate, using the High PMT function. Average intra-assay CV% was as follows: IL-6: 10.6%, IL-8: 5.7%, MCP-1: 5.6% and TNF-α 12.1%.

Immunohistochemistry

A small section from each biopsy was dissected free of any connective tissue, blotted dry and then mounted in Tissue Tek (ProSciTech, Australia) and frozen in isopentane cooled in liquid nitrogen. Tissue sections (10 μm) were fixed with 4% (w/v) paraformaldehyde in PBS for 10 min, permeated with 5% (v/v) Triton X-100 (TX100) for 5 min and blocked in 3% (w/v) bovine serum albumin in PBS overnight at 4˚C. Primary antibodies were diluted in blocking reagent as outlined in Table 2. All mouse primary antibodies were co-incubated with rabbit anti-Laminin (1:50 Sigma-Aldrich, St. Louis, MO), which was used to stain the sarcolemma. Tissue sections were incubated overnight at 4°C with combinations of these primary antibodies. After five washes with PBS, sections were incubated at room temperature for 2 h with appropriate secondary antibodies coupled to fluorophores (Alexa Fluor goat anti-mouse 488 or donkey anti-rabbit 594 [Molecular Probes, Invitrogen, Australia]). Nuclei were

stained by incubating with the DNA binding dye, Bisbenzamide Hoechst 33285 (Sigma) for 10 min. Following another series of washes, immuno-stained sections were visualized using an Olympus IX70 fluorescent microscope (Olympus, Australia), and digital images were collected using Spot RT slider camera and Magnifire Software (Olympus, Australia).

Blood analysis

We analyzed serum creatine kinase activity in pre- and post-exercise blood samples using an enzymatic assay (CK-NAC kit, CDT14010, Thermo-Fisher Scientific Clinical Diagnostics, Sydney, Australia) and an automated clinical analyser (Cobas Mira, Roche Diagnostics, Germany). We also analysed serum myoblobin concentration in these samples using an immunoassay (Roche Diagnostics, Germany) and an automated clinical analyzer (Cobas E411, Roche Diagnostics, Germany). The intra-assay coefficient of variation was 10.4% for creatine kinase and 1.7% for myoglobin.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.1 (GraphPad Software, San Diego, CA). Means were compared using one-way repeated measures analysis of variance (ANOVA) and any significant differences analyzed using a Newman-Keuls Multiple Comparison Test. Data are presented as mean±SEM. A probability level of <0.05 was adopted throughout to determine statistical significance.

RESULTS

Compared with pre-exercise values, the mRNA abundance of MCP-1 (95×) (Figure 1A), FKN (2.5×) (Figure 1B), uPA (15×) (Figure 1C), VEGF (2×) (Figure 1D), IL-6 (317×) (Figure 1E) and IL-8 (2,300×) (Figure 1F) was significantly elevated at 2 h following exercise. uPA (11×) and VEGF (2×) mRNA was also significantly elevated beyond 2 h post-exercise. TNF-α mRNA did not change significantly following exercise (Figure 1G), while MDC mRNA expression was not detectable at any time point (data not shown). Our group has previously reported no changes in mRNA expression of these chemotactic factors as a result of the biopsy procedure itself (Vella et al. 2012).

The bio-plex suspension assay system was used to assess the protein content of MCP-1, IL-8, IL-6 and TNF-α within muscle in both the exercise and non-exercising control groups. Following exercise, the expression of MCP-1 (Figure 2A), IL-6 (Figure 2B) and IL-8 (Figure 2C) protein followed a very similar pattern to the mRNA expression, with the largest increase evident 2 h following exercise (MCP-1 22×, IL-6 38× and IL-8 58×). Although all of these factors remained elevated at later time points, only MCP-1 expression at 4 h post-exercise was significantly greater than pre-exercise values. TNF-α expression was below the range of the standards at each time point. Data from the non-exercising control group indicated no significant changes in the protein expression of MCP-1, IL-6 or IL-8 in response to the muscle biopsy procedure itself (Figure 2).

At rest, both IL-8 (Figure 3A) and MCP-1 (Figure 3C) were not visible in skeletal muscle sections. Following exercise, however, staining for both IL-8 (Figure 3B) and MCP-1 (Figure 3D) was clearly visible 2 h after exercise. MCP-1 (Figure 4A) and IL-8 (Figure 4B) was

localized within the endomysium between the muscle fibres and not within the muscle fibers themselves. To investigate which cells within the endomysium were associated with MCP-1 and IL-8 expression, serial sections of muscle tissue were stained, firstly, with MCP-1 and IL-8, and then with antibodies for CD68 for macrophages, PAX7 for satellite cells and Collagen IV for blood vessels. Within the subject cohort, we found several examples of MCP-1 and IL-8 co-staining with or in close proximity to macrophages (Figure 5) and small blood vessels (Figures 6 and 7), and of MCP-1 co-staining with satellite cells (Figure 6). However, the staining was not conclusive or consistent, and did not account for the widespread staining and large increase in the protein abundance of MCP-1 and IL-8 after following exercise. IL-8 staining also appeared turquoise in many of the images, suggesting that IL-8 (shown in green) may be predominantly expressed by the nuclei of interstitial cells (shown in blue) (Figure 4). Large blood vessels were also evident in some sections and showed strong expression of MCP-1 (Figure 8).

There were no significant changes in plasma creatine kinase activity ($p=0.36$) and plasma myoglobin concentration ($p=0.56$) after exercise (Figures 9A and 9B).

DISCUSSION

In this study, we have profiled changes in the gene and protein expression of various chemotactic factors in skeletal muscle at several time points during the early phases of recovery from resistance exercise. We have also demonstrated that MCP-1 and IL-8 expression was not restricted to mature myofibers after resistance exercise. This study

highlights the complex interactions between various cell types and secreted proteins in the skeletal muscle microenvironment during early adaptation of skeletal muscle after exercise.

Some exercise studies have only investigated changes in the gene expression of MCP-1, IL-6 IL-8 and VEGF at a single time point after resistance exercise: immediately (Nieman et al. 2004), 3 h (Hyldahl et al. 2011; Buford et al. 2009) or 6 h (Hubal et al. 2008) after exercise. We present new evidence that in addition to major chemotactic factors such as MCP-1 and IL-8, the gene expression of urokinase-type plasminogen activator and fractalkine also increased after exercise. Specifically, urokinase-type plasminogen activator and fractalkine was elevated at 2 h after exercise, while urokinase-type plasminogen activator mRNA expression remained elevated at 4 h post-exercise. Urokinase-type plasminogen activator is a chemoattractant for monocytes (Chazaud et al. 2003), which accounts for its role in muscle regeneration (Bryer et al. 2008). Fractalkine also likely promotes muscle adaptation after exercise by attracting monocytes (Chazaud et al. 2003) and stimulating angiogenesis (Volin et al. 2001). In contrast with muscle-damaging eccentric exercise, we propose that the increased gene expression of these chemokines after intense resistance exercise likely reflects their role in muscle adaptation/remodeling, rather than muscle repair. This notion is supported by our finding exercise did not cause any substantial muscle damage, as indicated by the lack of any significant changes in plasma creatine kinase activity and plasma myoglobin concentration at 24 and 48 h after exercise (see Figure 9).

We discovered for the first time that in addition to IL-8 and MCP-1 mRNA, the protein abundance of these chemokines also increased markedly after resistance exercise. IL-8 mRNA and protein expression peaked after 2 h. MCP-1 mRNA expression also peaked after 2

h, while MCP-1 protein expression remained elevated 4 h after exercise. The importance of MCP-1 and its receptor CCR2 in muscle adaptation is well established (Lu et al. 2011b; Shireman et al. 2007; Warren et al. 2005; Warren et al. 2004; Yahiaoui et al. 2008). One of the key mechanisms by which MCP-1/CCR2 regulate muscle adaptation is by recruiting monocytes to the site of muscle injury, where these cells are converted to macrophages (Chazaud et al. 2003; Lu et al. 2011b). MCP-1 may also assist muscle adaptation by stimulating the proliferation of myoblasts (Yahiaoui et al. 2008), vascular smooth muscle cells (Selzman et al. 2002) and endothelial cells (Weber et al. 1999). By contrast, the role of IL-8 in muscle adaptation is not so well defined. IL-8 may promote muscle adaptation by attracting neutrophils (Peterson and Pizza 2009) and upregulating expression of VEGF receptors (Petreaca et al. 2007).

The large increase in the protein expression of MCP-1 and IL-8 (as measured using the bio-plex assay) strongly suggests that there was also a substantial increase in the number and/or activity of cells secreting these chemokines in skeletal muscle. Relatively few studies have attempted to identify the cells types that secrete cytokines and chemokines in skeletal muscle after resistance exercise (Hubal et al. 2008; McKay et al. 2009). Muscle cells secrete MCP-1 and IL-8 *in vitro* in response to cyclic strain (Nedachi et al. 2009; Peterson and Pizza 2009) and stimulation with other cytokines (Sugiura et al. 2000; De Rossi et al. 2000; Marino et al. 2008). However, in healthy individuals, and patients with inflammatory myopathies, MCP-1 and IL-8 are not expressed within myofibers. Rather, they are expressed within blood vessels and regions of inflammation (De Paepe et al. 2007). It was therefore not surprising that we could not detect MCP-1 and IL-8 within myofibers before exercise. After exercise, there was little change in MCP-1 and IL-8 staining within myofibers. Instead, these factors

were expressed within the endomysium between the myofibers. We attempted to identify the cells within this region that were secreting MCP-1 and IL-8 after exercise by staining tissue sections with CD68 for macrophages, PAX7 for satellite cells and collagen IV for blood vessels. There were no consistent patterns of staining associated with these cell types. In a few samples, MCP-1 staining was co-localized with satellite cells (Figure 5). By contrast, in other samples MCP-1 and IL-8 staining was co-localized with macrophages and/or within large blood vessels (Figures 4, 5 and 6).

These findings highlight two important issues. First, muscle cells themselves do not appear to be a major source of MCP-1 and IL-8 after resistance exercise. Second, despite the marked increase in the gross protein abundance of MCP-1 and IL-8 in skeletal muscle, the patterns of tissue staining for the chemokines show substantial variation. Further work using more advanced imaging technology (e.g., confocal microscopy) is warranted to investigate which types of cells secrete chemotactic factors in skeletal muscle following exercise, and whether this secretion is regulated in an autocrine or paracrine manner.

Consistent with the changes in MCP-1 and IL-8 expression, we identified that expression of IL-6 mRNA and protein also increased dramatically in skeletal muscle after resistance exercise. IL-6 regulates muscle adaptation after exercise by stimulating satellite proliferation (Toth et al. 2011). The lack of any change in TNF-α expression following exercise in the present study was somewhat surprising. TNF-α is a chemotactic agent *in vitro* (Peterson et al. 2006), and plays an important role in muscle regeneration following myotoxin injury (Chen et al. 2005) and freeze injury (Warren et al. 2002). Other research has reported an increase in TNF-α mRNA expression in skeletal muscle after resistance exercise (Buford et al.

2009; Louis et al. 2007; Nieman et al. 2004). Possible reasons for the disparity between our findings and other studies may include differences in the timing of muscle biopsies, training status of the subjects and/or methods of molecular analysis. Only a few studies have detected TNF-α protein expression in human skeletal muscle using enzyme-linked immunoassay (Barreiro et al. 2008; Greiwe et al. 2001), Western blotting (Plomgaard et al. 2007; Kim et al. 2011) and immunohistochemistry (Plomgaard et al. 2007; Plomgaard et al. 2005). The bio-plex assay that we used in the current study may not have been sensitive enough to detect TNF-α protein. Plomgaard et al (Plomgaard et al. 2005) reported that only type II muscle fibers express TNF-α protein. In the present study, we collected muscle samples from *vastus lateralis*, which is comprised of equal proportions of type I and II fibers (Plomgaard et al. 2005). TNF-α protein expression may therefore be greater after exercise in muscle comprised of a higher proportion of type II fibers.

In conclusion, this study demonstrates that the early phase of recovery from intense resistance exercise is characterized by a large increase in the gene and protein expression of several important chemotactic factors. Although somewhat inconclusive, the immunohistochemistry analysis in this study is suggestive of complex communication and secretory actions between myofibers, macrophages, satellite cells and other stromal cells, and the major sources of these factors within skeletal muscle are cells residing within the endomysium. Importantly, the marked increased in the protein expression of MCP-1, IL-8 and IL-6 in the first few hours after resistance exercise follows a similar time course to myogenesis and protein synthesis in skeletal muscle. The chemotactic function of chemokines is an essential aspect of muscle adaptation to mechanical loading. However, the current data indicate that chemokine expression in skeletal muscle after exercise peaks

before monocytes typically begin to invade muscle fibers. More research is therefore required to determine the sequence of events leading to chemokine production in skeletal muscle, in addition to the targets and functions of chemokines in muscle fiber adaptation following exercise.

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Figure Legends

Figure 1 – The effect of a single bout of resistance exercise on skeletal muscle mRNA expression of MCP-1 **(A)**, fractalkine **(B)**, urokinase-type plasminogen activator **(C)**, VEGF **(D)**, IL-6 **(E)** IL-8 **(F)** and TNF-α **(G)**. Values are arbitrary units normalized to the expression levels of the housekeeping gene GAPDH representing the mean ± SEM for eight individuals. * Significantly different from resting levels ($p<0.05$).

Figure 2 – Protein abundance of MCP-1, IL-8 and IL-6 within skeletal muscle homogenate in response to resistance exercise and in a biopsy-only control group. Multiplex analysis using a Bio-plex assay was used to quantify expression of MCP-1 **(A)**, IL-8 **(B)**, IL-6 **(C)** in biopsy-only controls (white bars) and in response to resistance exercise (black bars). Values are mean ± SEM protein abundance normalized to total protein loaded per well for eight individuals. TNF-α was below the range of measurement at all time points. * Significantly different from resting levels ($p<0.05$).

Figure 3 – IL-8 **(A,B)** and MCP-1 **(C,D)** localization within skeletal muscle before and 2 h after a single bout of resistance exercise from a representative subject. At rest, neither IL-8 **(A, green)** nor MCP-1 **(C, green)** staining was visible. Following exercise, widespread staining for both in IL-8 **(B, green)** and MCP-1 **(D, green)** was visible. Sections were also double stained with an antibody against laminin (red, sarcolemma) and bisbenzamide (blue, nuclei). Scale bar = 100 μm.

Figure 4 – High magnification (40x) images of MCP-1 (A) and IL-8 (B) localization within skeletal muscle before and 2 h after a single bout of resistance exercise from a

representative subject. Images show expression of both MCP-1 and IL-8 was confined to cells outside the sarcolemma and within the endomysium. Sections were also double stained with an antibody against laminin (red, sarcolemma) and bisbenzamide (blue, nuclei). Scale bar = 100 μm.

Figure 5 – Representative serial sections indicating co-localization of MCP-1 and IL-8 with macrophages (CD68) 2 h following resistance exercise. Sections were stained with either anti-mouse CD68 **(A)**, MCP-1 **(B**) or IL-8 **(C)**, all of which appear green. Sections were also double stained with an antibody against laminin (red, sarcolemma) and bisbenzamide (blue, nuclei). Scale bar = 100 μm.

Figure 6 - Representative serial sections indicating co-localization of MCP-1 with satellite cells (PAX7) and showing MCP-1 in close proximity to blood vessels (Collagen IV) 2 h following resistance exercise. Sections were stained with either anti-mouse MCP-1 **(A)**, PAX7 **(B)** or Collagen IV **(C)**, all of which appear green. Sections were also double stained with an antibody against laminin (red, sarcolemma) and bisbenzamide (blue, nuclei). Scale bar = 100 μm.

Figure 7 - Representative serial sections indicating co-localization of IL-8 with blood vessels (Collagen IV) 2 h following resistance exercise. Sections were stained with either anti-mouse IL-8 **(A)** or Collagen IV **(B)**, both of which appear green. Sections were also double stained with an antibody against laminin (red, sarcolemma) and bisbenzamide (blue, nuclei). Scale bar = 100 μm.

Figure 8 – Large blood vessels within skeletal muscle cross sections expressed MCP-1 (green) 2h (A) and 24h (B) following resistance exercise. Sections were also double stained with an antibody against laminin (red, sarcolemma) and bisbenzamide (blue, nuclei). Scale bar = 100 μm.

Figure 9 – Plasma creatine kinase (CK) activity **(A)** and myoglobin concentration **(B)**. Values are mean ± SEM.

Tables

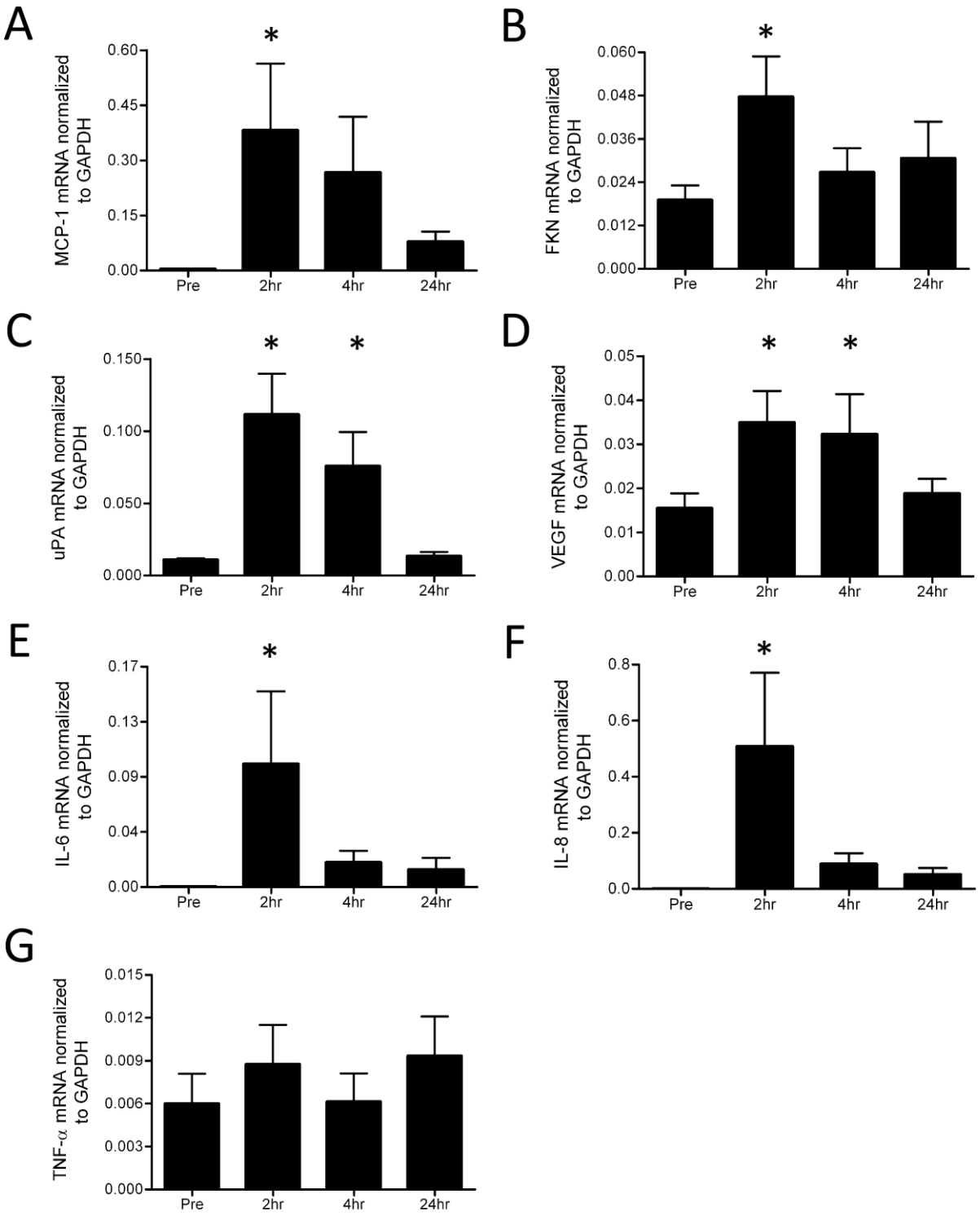
Table 1 – List of Primers used for RT-PCR.

Gene	GenBank Accession no.	Forward (5’ – 3’)	Reverse (5’ – 3’)
MCP-1	NM_002982	CGCCTCCAGCATGAAAGTCT	GGAATGAAGGTGGCTGCTATG
MDC	NM_002990.3	GCGCGTGGTGAAACACTTC	ACTCTGGGATCGGCACAGAT
VEGF	AYO41581	GCGCAAGAAATCCCGGTATA	GCTTTCTCCGCTCTGAGCAA
FKN	NM_002996.3	CGAAAGATGGCAGGAGAGATG	GGGCACCAGGACATATGAATTAC
uPA	NM_002658.3	GGAAAACCTCATCCTACACAAGGA	GGATCTTCAGCAAGGCAATGTC
IL-8	NM_000584	CTGGCCGTGGCTCTCTTG	TTAGCACTCCTTGGCAAAACTG
TNF-α	NM_000594	GGAGAAGGGTGACCGACTCA	TGCCCAGACTCGGCAAAG
IL-6	NM_000600	GTGACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
GAPDH	NM_002046	CATCCATGACAACTTTGGTATCGT	CAGTCTTCTGGGTGGCAGTGA

Table 2: Details of primary antibodies used for immunofluorescence.

Antigen	Antibody	Clone	Dilution	Source
MCP-1	Mouse monoclonal IgG	5J	1:20	Santa Cruz (Santa Cruz, CA)
IL-8	Mouse monoclonal IgG	NYR-HIL8	1:40	Santa Cruz
CD68	Mouse monoclonal IgG	E-11	1:100	Santa Cruz
PAX7	Mouse monoclonal IgG	PAX7	1:20	Developmental Studies Hybridoma Bank (Iowa city, IA)
Collagen IV	Mouse monoclonal IgG	M3F7	1:100	Developmental Studies Hybridoma Bank

Figure 1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MCP-1 mRNA normalized to GAPDH
0.60
0.45
0.30
0.15
0.00
Pre
2hr
4hr
24hr
*
B
FKN mRNA normalized to GAPDH
0.060
0.048
0.036
0.024
0.012
0.000
C
uPA mRNA normalized to GAPDH
0.150
0.100
0.050
0.000
D
VEGF mRNA normalized to GAPDH
0.05
0.04
0.03
0.02
0.01
0.00
E
IL-6 mRNA normalized to GAPDH
0.17
0.13
0.09
0.04
0.00
F
IL-8 mRNA normalized to GAPDH
0.8
0.6
0.4
0.2
0.0
G
TNF-α mRNA normalized to GAPDH
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0.012
0.009
0.006
0.003
0.000

Figure 2

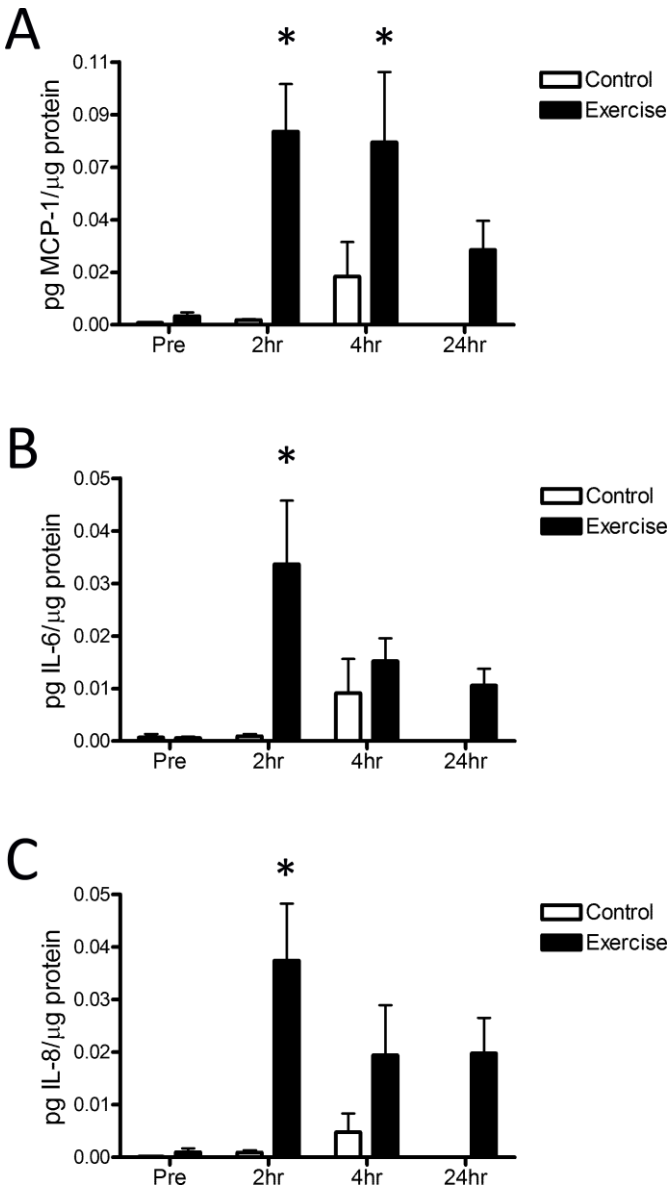
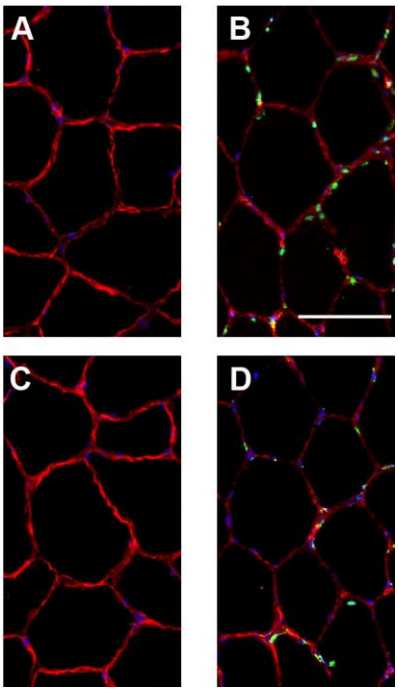

A
pg MCP-1/μg protein
0.11
0.09
0.07
0.04
0.02
0.00
Pre
2hr
4hr
24hr
*
*
Control
Exercise
B
pg IL-6/μg protein
0.05
0.04
0.03
0.02
0.01
0.00
Pre
2hr
4hr
24hr
*
Control
Exercise
C
pg IL-8/μg protein
0.05
0.04
0.03
0.02
0.01
0.00
Pre
2hr
4hr
24hr
*
Control
Exercise

Figure 3



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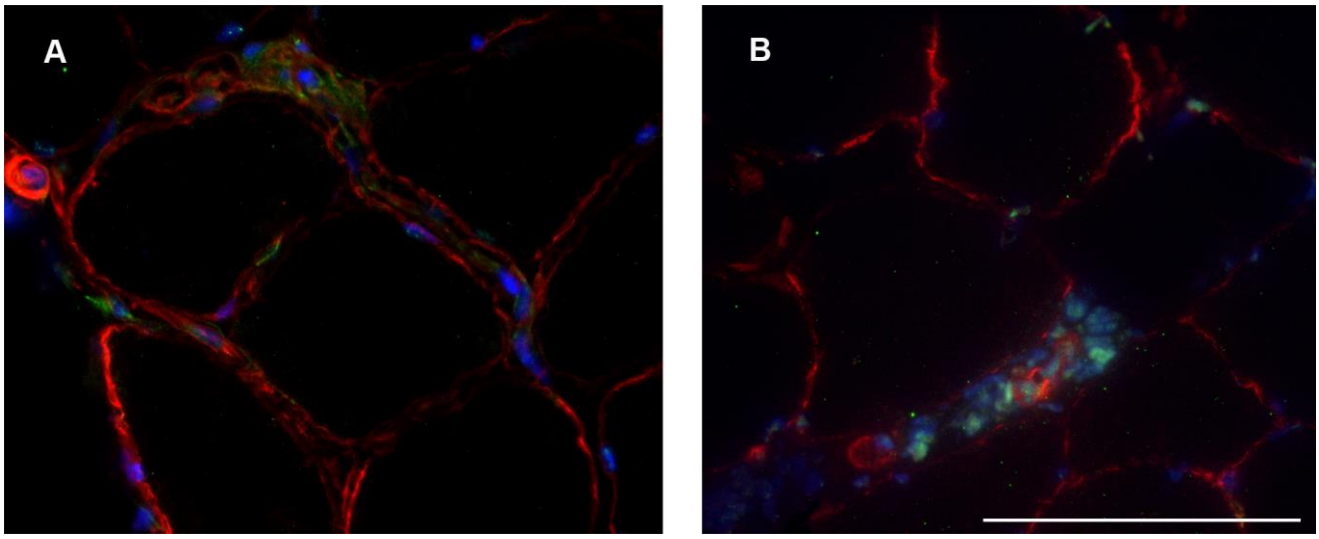
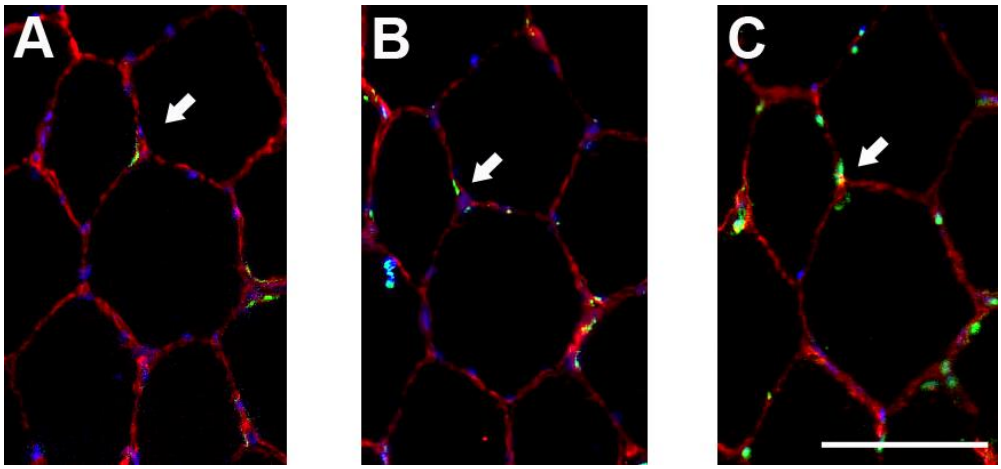


Figure 5



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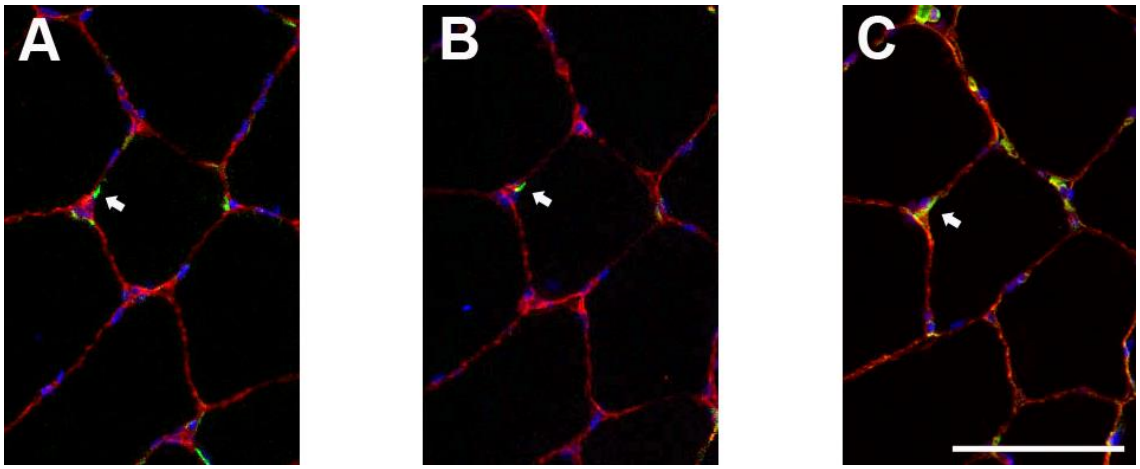

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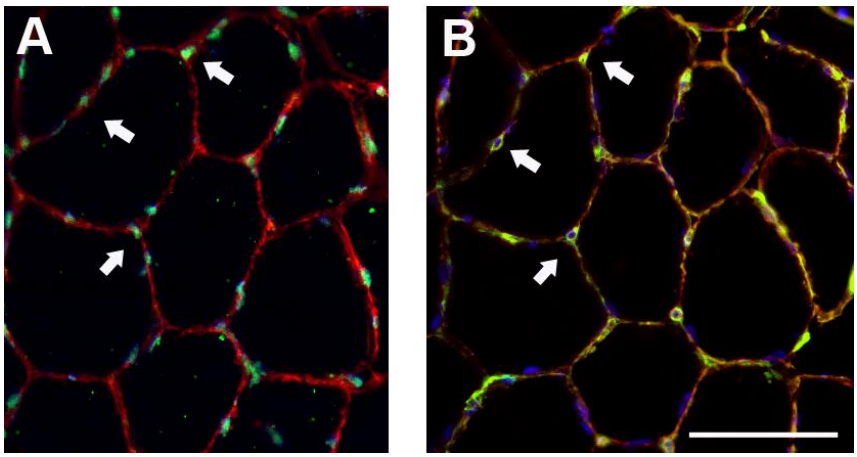


Figure 8

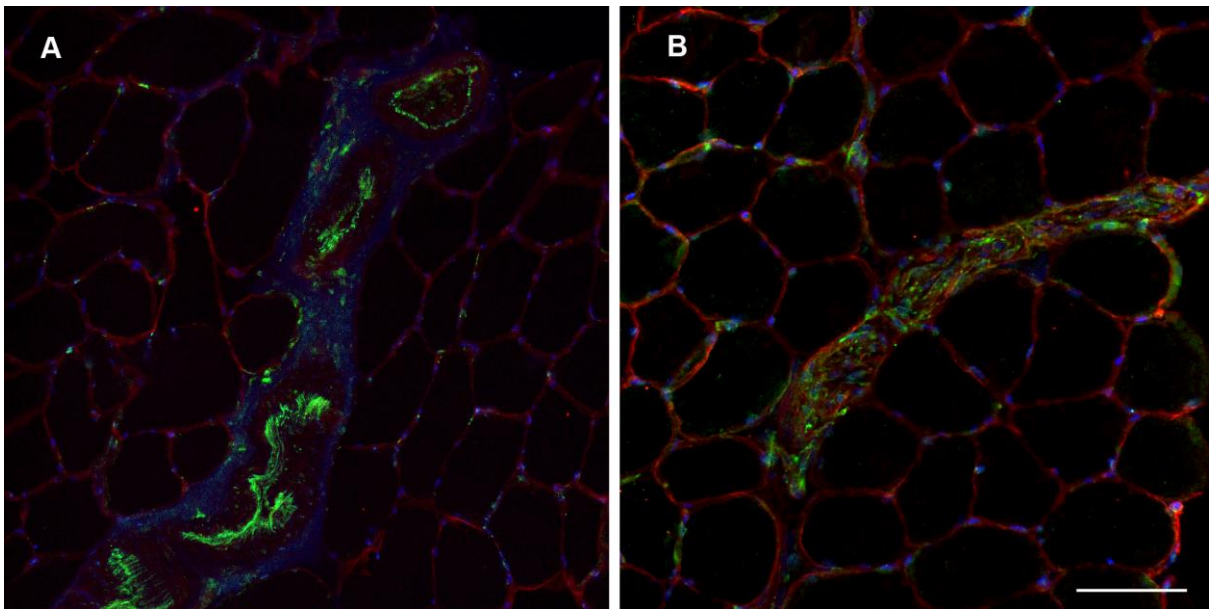
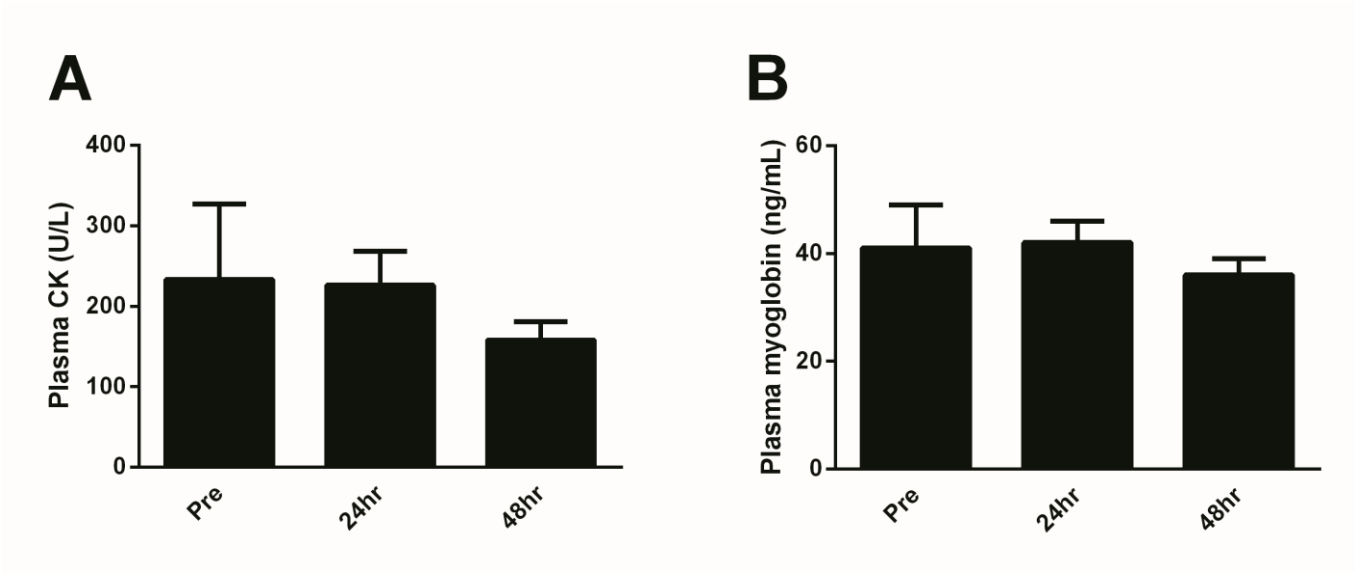


Figure 9


A
Plasma CK (U/L)
400
300
200
100
0
Pre
24hr
48hr
B
Plasma myoglobin (ng/mL)
60
40
20
0
Pre
24hr
48hr