Exercise-Induced Myokine Response Following 75-km Cycling

by

Brandon Shane Tolbert

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Kevin Zwetsloot, Ph.D., Thesis Director

Nathan Mowa, Ph.D., Second Reader

Ted Zerucha, Ph.D., Interim Director, The Honors College

Abstract

Myokine is a term referring to a variety of cytokines or chemokines that are produced and released by myocytes in response to skeletal muscle contractions. The myokine response to prolonged exercise is not well known. Therefore, the purpose of this study was to investigate the myokine response to prolonged exercise by measuring fifteen different myokines in skeletal muscle biopsies. Trained cyclists (N=14) participated in a 75-km cycling time trial, with muscle samples from the vastus lateralis taken before and after the exercise bout. The skeletal muscle biopsy samples were analyzed for 15 myokines including: APLN, CX3CL1, BDNF, EPO, SPARC, LIF, IL15, GDF8, FABP3, Irisin, FSTL1, OSM, IL6, FGF21, and OSTN. These myokines were analyzed with a magnetic bead-based multiplex assay. The 75km cycling exercise bout significantly increased skeletal muscle Interleukin-15, Oncostatin M, and Fractalkine protein levels from, 0.043±0.008 to 0.055±0.021, 0.0014±0.002 to 0.0059±0.004 and 4.45±0.46 to 4.60±0.54 pg/µg, respectively (all $p<0.05$). Apelin protein levels exhibited a decrease from 3.95±1.18 to 3.33±1.21 pg/µg ($p<0.05$). No statistically significant difference was observed for FSTL1, IL6, SPARC, BDNF, FGF21, LIF, or OSTN. Erythropoietin, Irisin, and Myostatin were all below detectable limits for the assay, and Fatty Acid-Binding Protein 3 exhibited values above the detection limit. Over the last couple decades, our knowledge of cytokine proteins has increased considerably as has our understanding of their functions and regulatory mechanisms. However, with few exceptions, our knowledge is still relatively limited as to the mechanism and regulation of cytokine production during exercise, especially in the muscle tissue. Consequently, research is still ongoing for many of the tested proteins to determine their function when it comes to skeletal muscle adaptation.

Introduction

Cytokines are small proteins produced by cells with functions typically specific to the immune system. The immune response is generally accomplished through chemotaxis of inflammatory cytokines. A subgroup of cytokines, called chemokines, function to attract cells to the sites of inflammation or infection. More specifically, chemokines typically direct the migration of leukocytes to an area of infection[20] or damaged tissue. Cytokine release during exercise has a role in many physiological processes including immune cell recruitment, inflammation, lipolysis stimulation, and tissue repair.

Myokine is a term referring to a variety of cytokines or chemokines that are produced and released by myocytes in response to skeletal muscle contractions (i.e. exercise). The discovery of specific myokines has only recently been described in the literature. Of what little published research is available, it suggests that the myokine response varies between individuals, and may differ depending on a variety of factors that may include mode, type, intensity, or duration of exercise, gender, medications, and fitness condition.[5] The myokine response to prolonged exercise is not well known. Therefore, the purpose of this study was to investigate the myokine response to prolonged exercise by measuring fifteen different myokines in skeletal muscle biopsies of trained cyclists before and after 75 km of cycling.

Overview of specific myokines measured

Apelin

Apelin is encoded by the APLN gene and is expressed in vital organs such as the heart, lungs, kidneys, and liver. It is also present in fatty tissue, the GI tract, and blood plasma. Apelin

may exist in a variety of amino acid lengths, as the APLN gene encodes an original protein of 77 amino acids.[21] After various cleavage mechanisms, the initial proprotein may give rise to several active fragments. One type of expression of Apelin is in the vasculature as it participates in the control of blood pressure as angiogenesis is stimulated by its activation. This results in the lowering of mean arterial pressure through the means of a nitric oxide dependent mechanism, which acts as a powerful vasodilator.[27] In the cardiac system, Apelin has been described as one of the most potent stimulators of cardiac contractility.[21] Perhaps acting as a compensatory measure, Apelin levels have been shown to be elevated in obese individuals and patients with chronic heart failure.[21, 23] Several functions are exhibited in the digestive system as well. In the pancreas, increased levels of Apelin result in the inhibition of insulin secretion, revealing an interdependence between insulin production and the introduction of Apelin through a signaling system. Acid secretion of parietal cells is also repressed by the inhibition of histamine release within the stomach lining.[23,27] There is little known about the role of Apelin in the skeletal muscle, but recent research suggests that it plays a role in glucose uptake in skeletal muscle cells.

Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF) is a growth factor protein encoded by the BDNF gene. BDNF is especially useful in the central and peripheral nervous system, as it encourages the growth of new neurons while promoting the survival of existing ones.[1] It is particularly active in the hippocampus, cortex, and basal forebrain. Its role in neurogenesis suggests that BDNF is important in basic neural development and even may play a role in the

formation of long term memory in the hippocampus.[1] Production of BDNF is exclusive to the endoplasmic reticulum and an absence of adequate levels results in severe consequences, often times proving lethal. The effect of physical activity on BDNF synthesis has shown a significant positive correlation. In fact, certain aerobic exercise has been shown to consistently raise BDNF levels in association with the level of physical activity.[4] In response to skeletal muscle contraction, BDNF has been found to enhance fat oxidation via activation of AMP-activated protein kinase.[14]

Erythropoietin

Erythropoietin (EPO) is one of the more well known cytokines that is responsible for erythropoiesis with exercise. A primary role of EPO is to serve as a signaling molecule for red blood cell that have not reached maturity in the bone marrow. EPO is predominately produced in the kidneys by fibroblasts during adulthood and in the liver by peridinusoidal cells in the perinatal period.[22] Without EPO, erythropoiesis does not take place as it is an essential hormone for the process. Erythrocyte production and survival is promoted by EPO as it protects these immature cells from apoptosis.[22] In a medical sense, EPO has commonly been used as a therapy for certain types of anemia in an attempt to combat low red blood cell counts. EPO production is vastly increased under hypoxic conditions, as erythrocytes are the body's mechanism for transporting oxygen.[22] During exercise, when the body is in a state of hypoxia (low blood oxygen levels), EPO production may increase exponentially in an attempt to get adequate oxygen for biological processes.

Fatty Acid-Binding Protein 3

Fatty acid-binding protein 3 (FABP3) is a member of the FABP family that is located primarily in the muscle and the heart. FABP3 is a relatively small protein that is originally released from cardiac myocytes. It is involved in fatty acid metabolism and transports these fatty acids into the mitochondria for oxidation.[19] A potential diagnostic use of FABP3 is in the prevention of myocardial infarctions, as it can be detected in the blood in the hours leading up to the event. This results from detectable levels of FABP3 being released into the circulation in the event of myocardium injury.[19] When measured pre and post exercise, levels of FABP3 were found to increase in plasma samples in response to aerobic exercise.[6]

Fibroblast Growth Factor 21

Fibroblast Growth Factor 21 (FGF21) is a member of the fibroblast growth factor family that are involved in a variety of biological processes such as embryonic development and tissue repair.[13] Expression of the protein is found in the liver, adipose tissues, and the pancreas. Glucose uptake stimulation in adipocytes is a function of FGF21 and treatment of animals with the protein resulted in increased energy expenditure and fat excretion.[13] Because of its role in glucose uptake, it is claimed to have a great therapeutic potential for those with type 2 diabetes. Although typically not expressed in skeletal muscle, there is evidence to support that serum FGF21 expression is increased after acute periods of exercise in animal and human trials.[13]

Follistatin-related Protein 1

Follistatin-related protein 1 (FSTL1) is a protein that plays a role in development and is encoded by the FSTL1 gene. FSTL1 plays a part in lung, ureter, central nervous system, and skeletal development.[3] Inflammatory disorders such as arthritis also seem to be correlated with an increased presence of FSTL1. Levels of FSTL1 in the serum seem to be associated with the up-regulation of proinflammatory mediators that have significance in rheumatoid arthritis.[15] Research has shown that the FSTL1 myokine is secreted by myotubes in humans and that plasma levels of FSTL1 increase in response to exercise as well.[3]

Fractalkine

Fractalkine, or CX3CL1, is a large cytokine protein consisting of 373 amino acids. It is most commonly found throughout the brain in neural cells with its receptor found on microglial cells.[16] Fractalkine is an essential component for microglial cell migration. Existing in two forms, Fractalkine is believed to greatly enhance cell adhesion function in its membrane anchored form and plays a role in attracting and activating natural killer (NK) cells, T cells, and monocytes, which participate in the immune response and act against a variety of infected cells.[28] For this reason, the immune recruitment function is being closely studied to determine its viability as a treatment in pathological conditions. Upregulation of Fractalkine takes place in the hippocampus briefly following spatial learning, and neuroprotective effects have also been observed in the inhibition of microglial activation.[16] Fractalkine increases the expression of pro-inflammatory factors in human myoblasts and myotubes in skeletal muscle. According to recent

research, Fractalkine mRNA and protein level in the skeletal muscle has been shown to increase after one bout of exercise.[28]

Interleukin 6

Interleukin 6 (IL6) is another protein that acts as both a pro-inflammatory and anti-inflammatory cytokine.[7] Interleukin 6 is encoded by the IL6 gene and is secreted by T cells to enact an immune response, often resulting in inflammation. IL6 also participates in osteoclast formation when excreted by osteoblasts, and when produced by blood vessels, it behaves as a pro-inflammatory cytokine.[7] Other capabilities include crossing the blood-brain barrier to initiate the synthesis of prostaglandin and stimulating protein synthesis[7], in addition to its function as a myokine. As a myokine, IL6 levels are elevated in response to skeletal muscle contraction, and was the first myokine to be reported to be secreted into the blood stream in response to exercise[7]. Studies have shown that the magnitude of IL6 response is dependent on duration and intensity of exercise, while the method of exercise exhibited little effect.

Interleukin 15

Interleukin 15 (IL15) is a cytokine belonging to a superfamily of similarly structured proteins differentiated by their number. IL15 is expressed in a variety of different cells including macrophages, fibroblasts, and nerve cells.[24] Upon discovery, it was determined that IL15 plays an important role in immunity as a T cell growth factor. Immune regulation is mainly characterized by activation and propagation of NK and T cells by inducing apoptosis inhibitor.[24] During muscle contraction, IL15 is produced in an attempt to mediate the effects of exercise on

muscle metabolism. It is regarded as having an anti-inflammatory effect in the muscle tissue in an attempt to maintain muscle homeostasis.

Irisin

Fibronectin type III domain-containing protein 5 is the precursor of Irisin and is encoded by the FNDC5 gene. Irisin production is stimulated by the increased expression of PGC-1alpha, which is believed to aid in adaptation to exercise.[12] Since Irisin production is involved in a mechanism that begins with a muscular contraction, it is classified as a myokine. It has been recently debated, however, whether or not Irisin is circulated in detectable amounts in the blood, but the most recent research seems to indicate that it is secreted by skeletal muscle and plays an important role in the homeostasis of energy balance, indicating that Irisin does indeed circulate at nanogram levels and increases with exercise.[12] After participating in resistance exercise, circulating Irisin levels have been shown to increase in both mouse models and human studies.[12]

Leukemia Inhibitory Factor

Leukemia inhibitory factor (LIF) has a function, quite literally, that leads to the prevention of leukemic cell growth. It accomplishes this by inducing terminal differentiation of these cells, thus ending their proliferation.[2] In addition to its primary function, it is also responsible for certain types of cell differentiation and cell growth. Bone metabolism, inflammation, and neural development are other areas where LIF exhibits promotion of growth.[2] During exercise, there is an up-regulation of LIF in skeletal muscle; however, LIF levels

in the circulation do not increase, indicating that LIF may have an autocrine effect within the muscle tissue. Based on its cell proliferation function, there is evidence to support that LIF may be produced by the skeletal muscle in response to exercise to contribute to muscle adaptation.[2]

Musclin

Musclin, also known as Osteocrin (OSTN) is a human myokine that is expressed in skeletal tissue, primarily in osteoblasts, which suggests a role in bone formation. Through a single fiber analysis, it was also found that Musclin is produced by type IIb muscle fibers.[26] Musclin has been considered an exercise-responsive myokine vital to skeletal muscle adaptation during exercise. Through the use of Musclin-encoding gene knockout mice, it was found that elimination of OSTN significantly lowered maximum aerobic capacity and mitochondrial protein content.[26] This suggests that Musclin enhances physical endurance through the promotion of mitochondrial biogenesis.

Myostatin

Myostatin (GDF8) is a relatively well known myokine whose function is to inhibit myogenesis (muscle growth). Acting as a key signaling molecule, Myostatin signals the dormancy of satellite cells, therefore inhibiting growth and differentiation of myoblasts. It is encoded by the MSTN gene and is produced and released by myocytes.[9, 18] Myostatin is found almost exclusively in the skeletal muscle, with a small amount being located in the blood.[18] Myostatin is active before and after birth, playing a part in muscle development and preventing excess muscle growth.[9] Lack of the MTSN gene or the blocking of Myostatin activity results in

the formation of significantly more muscle mass. This is a genetic abnormality that may occur in animals but also may be able to serve a therapeutic purpose as a treatment of muscle wasting diseases, although further research is needed. In response to aerobic exercise, it was found that Myostatin levels in both muscle and plasma samples decrease, compared to rest.[9]

Oncostatin M

Oncostatin M (OSM) is a cytokine encoded by the OSM gene that belongs to the Interleukin family of cytokines. The function of OSM splits into two schools of thought: as a pro-inflammatory protein and as an anti-inflammatory protein.[17, 29] The pro-inflammatory role is suggested by the effect of OSM on endothelial cells, as it may induce an inflammatory response.[17] Anti-inflammatory properties have also been observed as OSM effectively suppressed inflammation and destruction of tissue in animal models of chronic inflammatory diseases.[29] Serum and muscle samples taken after an exercise bout appeared to exhibit an increase in OSM levels, with significant upregulation taking place.[17] Typically, OSM is undetectable in vivo, however, detectable levels may be induced upon exercise or muscle injury.[17]

Osteonectin

Osteonectin, also known as secreted protein acidic and rich in cysteine (SPARC), is a glycoprotein located in the bone that binds calcium and is encoded by the SPARC gene.[11] Several biological functions have been attributed to osteonectin such as bone and cartilage mineralization, as well as collagen binding. Elevated levels of osteonectin are also beneficial to

tumor growth, and overexpression has been reported in several types of cancer as it aids in cell attachment and proliferation.[11] There have been numerous studies indicating that the presence of Osteonectin in various tissues indicate a regenerative function. Osteonectin has often been expressed during repair periods including expression in response to muscle damage. Osteonectin has also been found to be present in neonatal muscle cells.[11] This, in addition to its regenerative properties indicates a role in early muscle development as well as reformation, as the cytokine is found in satellite cells as well as the muscle fibers themselves.[11]

Materials and Methods

Subjects and Baseline Testing

The purpose of this study was to investigate the myokine response to prolonged exercise. The samples analyzed in this pilot investigation were obtained from a previously performed study (IRB #14-0173), whereby skeletal muscle biopsy samples were collected from trained cyclists before and immediately after performing a 75-km cycling exercise bout. The participants were 14 male cyclists (age 38.5 ± 9.3 years) who regularly raced and were familiar with long distance cycling bouts.

One week before the 75-km cycling session, each cyclist underwent orientation and baseline testing. The orientation consisted of questionnaires to determine demographic information and training history. A graded exercise test was used to determine maximal power, heart rate, ventilation, and maximum oxygen consumption. This test began at 150 watts and a 25 watt increase occurred every two minutes. The test was performed using the Cosmed Quark CPET metabolic cart (Rome, Italy) and the LODE cycle ergometer (Lode Excaliber Sport, Lode

B.V., Groningen, Netherlands). Body composition was also measured using the Bod Pod body composition analyzer (Life Measurement, Concord, CA).

75-km Cycling Time Trial

A week after the initial baseline testing, subjects participated in the 75-km cycling exercise bout using their own bicycles which were mounted on CompuTrainer Pro Model 8001 trainers (Racermate, Seattle, WA). Using the software, a mountainous 75-km course of moderate difficulty was selected and programmed into the system. Both heart rate and rating of perceived exertion (RPE) were recording after the first 15 minutes, and for every 60 minutes following. Workload in watts was also monitored continuously using the CompuTrainer MultiRider software system (version 3.0). Ventilation and oxygen consumption were measured after 16 km and 55km using the metabolic cart. Subjects were only allowed to consume water during the trial.

Muscle Biopsy Analysis

Pre- and post- exercise muscle biopsy samples were acquired from the vastus lateralis of the same leg at locations roughly 2 cm apart. A local anesthetic (1% xylocaine, Hospira, Inc., Lake Forest, IL) was injected subcutaneously. The muscle biopsy sample was obtained using the suction-modified percutaneous needle biopsy procedure.[25] The muscle samples were trimmed of fat and connective tissue and immediately frozen in liquid nitrogen and stored at -80°C until analysis took place.

The skeletal muscle biopsy samples were analyzed for 15 myokines including: APLN, CX3CL1, BDNF, EPO, SPARC, LIF, IL15, GDF8, FABP3, Irisin, FSTL1, OSM, IL6, FGF21, and OSTN.

These myokines were analyzed with a magnetic bead-based multiplex assay (MILLIPLEX® map Human Myokine Panel) using the MAGPIX instrument and MILLIPLEX® Analyst Software (version 5.1). Briefly, ~30 mg of each skeletal muscle tissue biopsy sample was homogenized in lysis buffer (Millipore, Billerica, MA; #43-040) with AEBSF protease inhibitor added (Millipore; #101500). Next, 81.25 µg of muscle protein was added to each well (in duplicate) and the concentration of each myokine was measured using the MILLIPLEX® map assay kit (Millipore, HMYOMAG-56K) following the manufacturer's specifications. The lower limit of detection for this panel of analytes was: APLN = 11.11 pg/mL; BDNF = 0.040 pg/mL; EPO = 123.81 pg/mL; FABP3 = 2.51 pg/mL; FGF21 = 1.22 pg/mL; FSTL1 = 0.045 ng/mL; CX3CL1 = 20.36 pg/mL; IL-6 = 0.087 pg/mL; IL-15 = 0.12 pg/mL; Irisin = 190.05 pg/mL; LIF = 0.75 pg/mL; OSTN = 8.93 pg/mL; GDF8 = 0.25 ng/mL; OSM = 0.010 pg/mL; SPARC = 84.03 pg/mL;

Statistical Analysis

Data are expressed as mean±SD. Change between pre- and post- exercise measurements were tested for change using a paired t-tests, with alpha level $P \leq 0.05$. Several transformations had to be performed in order for some of the data to be normalized. The log of the FGF21 results was taken and the square root of the log of OSTN was taken in order to obtain results. In addition, LIF data had to be analyzed sans subject 9, as this determined to be an outlier, based on the values being +2 SD from the mean. All statistical analysis was performed using SigmaPlot software (version 12.5, Systat Software, Inc.).

Results

The 75km cycling exercise bout significantly increased skeletal muscle Interleukin-15, Oncostatin M, and Fractalkine protein levels from, 0.043±0.008 to 0.055±0.021, 0.0014±0.002

to 0.0059±0.004 and 4.45±0.46 to 4.60±0.54 pg/µg, respectively (all $p<0.05$; Figures 3, 4, and 8). Apelin protein levels exhibited a decrease from 3.95±1.18 to 3.33±1.21 pg/µg ($p<0.05$; Figure 1). No statistically significant difference was observed for FSTL1, IL6, SPARC, BDNF, FGF21, LIF, or OSTN (Figures 2, 5, 6, 7, 9, 10, and 11). Erythropoietin, Irisin, and Myostatin were all below detectable limits for the assay, and Fatty Acid-Binding Protein 3 exhibited values above the detection limit.

Discussion

There seemed to be a large amount of variation between the different participants for many of the myokines tested. This is indicated by the large amount of overlap in the standard deviations between the pre- and post- measurements. Because of this large amount of variation, it is possible that the participants were at different levels of fitness for the testing period, even though they were all considered trained. This is especially true of subject number 9, whose data seems exaggerated when compared to most of the other athletes. For example, a mean decrease of 50 pg/mL Apelin protein was observed for the group, however, subject 9 actually exhibited an increase of 106 pg/mL. This large difference is also why subject 9 was excluded in the data analysis for Leukemia Inhibitory Factor.

Despite the variations, four myokines (Fractalkine, Oncostatin M, Interleukin 15, and Apelin) exhibited a significant difference between pre- and post-exercise samples. Of the remaining myokines, Interleukin-6 and Osteocrin were very close to exhibiting significant results, with P-values of 0.059 for both. Unfortunately, levels of Erythropoietin, Myostatin, and Irisin were below detectable levels, suggesting that these cytokines do not have a substantial

presence in the muscle tissue itself. This is unsurprising for Irisin, as it is still being actively debated whether or not there is any detectable amount in human skeletal tissue. In the case of GDF8, data has indicated that levels drop after aerobic exercise, making it unlikely that already low levels would rise into the detectable range post- exercise. EPO levels were all over the board with several being in the detectable range but most being below the readable limit. Erythropoietin is produced by the kidneys and secreted into the blood; so one possible explanation could be that there was blood contamination in some of the muscle biopsy samples.

Conclusion

Over the last couple decades, our knowledge of cytokine proteins has increased considerably as has our understanding of their functions and regulatory mechanisms. However, with few exceptions, our knowledge is still relatively limited as to the mechanism and regulation of cytokine production during exercise, especially in the muscle tissue. Consequently, research is still ongoing for many of the tested proteins to determine their function when it comes to skeletal muscle adaptation. Because of the sizeable variation between subjects and several of the proteins being outside of the detection limit, only four of the 15 cytokines exhibited a statistically significant difference from pre-to post-exercise. Of these four, CX3CL1, OSM, and IL15 showed an increase and APLN showed a decrease in protein concentration in response to a 75-km cycling exercise stimulus.

Figures

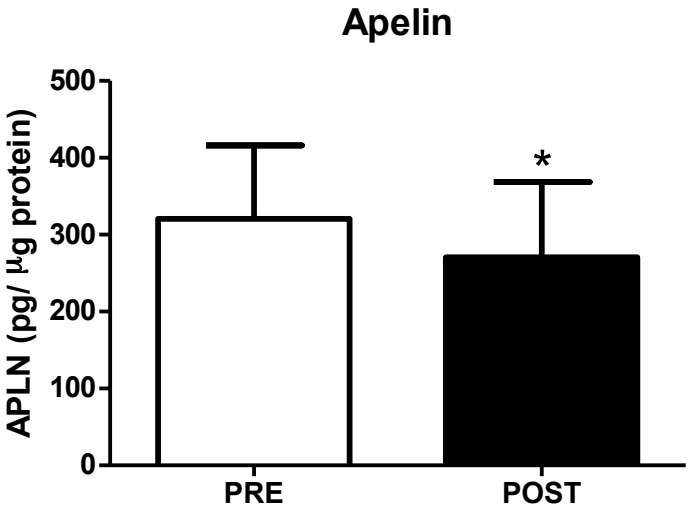

Figure 1: Concentration of APLN in the skeletal muscle before and after exercise (asterisk indicates statistically significant decrease)

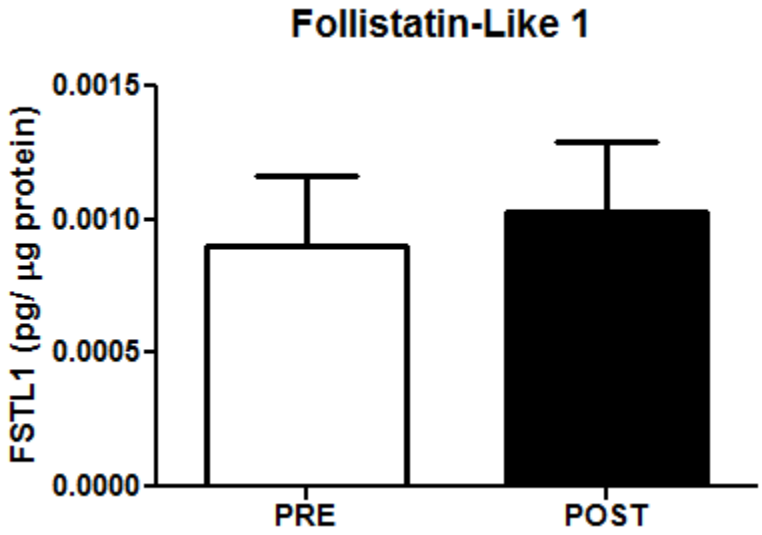

Figure 2: Concentration of FSTL1 in the skeletal muscle before and after exercise

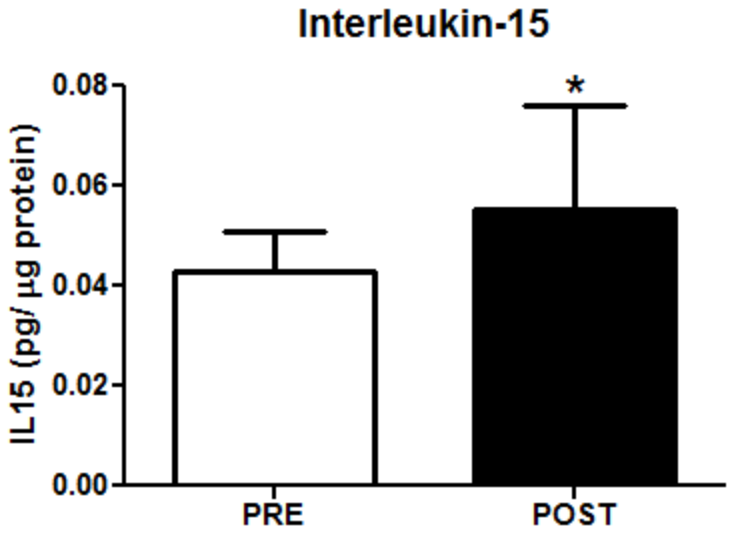

Figure 3: Concentration of IL15 in the skeletal muscle before and after exercise (asterisk indicates statistically significant increase)

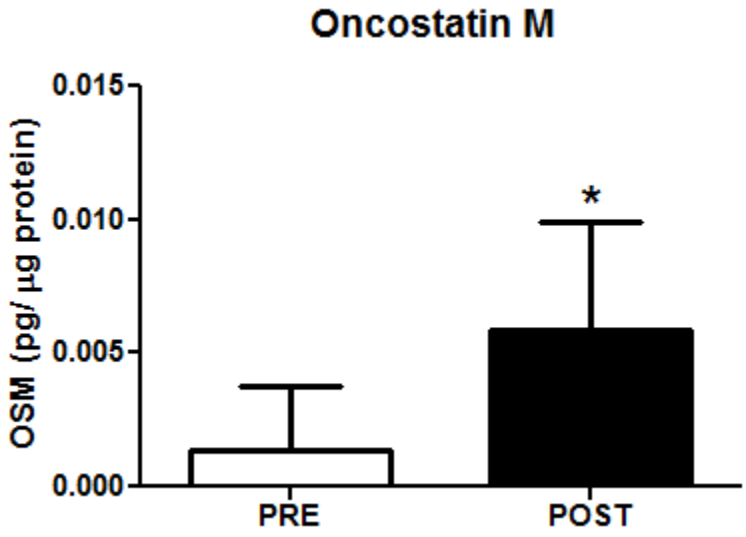

Figure 4: Concentration of OSM in the skeletal muscle before and after exercise (asterisk indicates statistically significant increase)

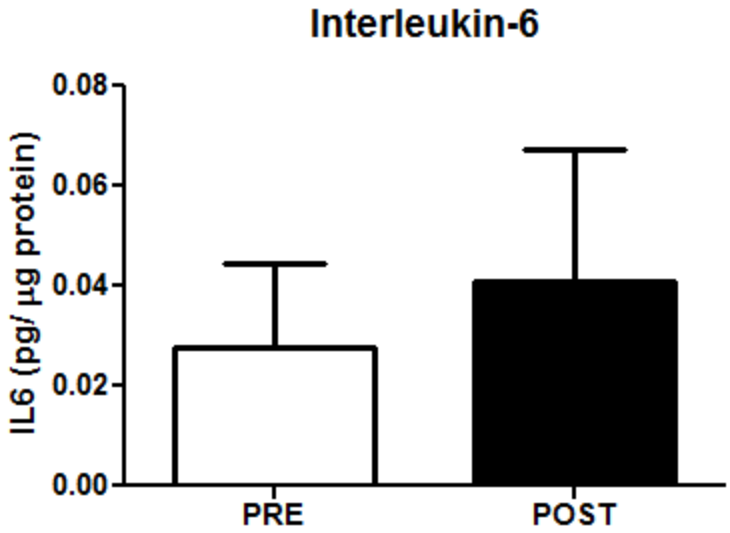

Figure 5: Concentration of IL6 in the skeletal muscle before and after exercise

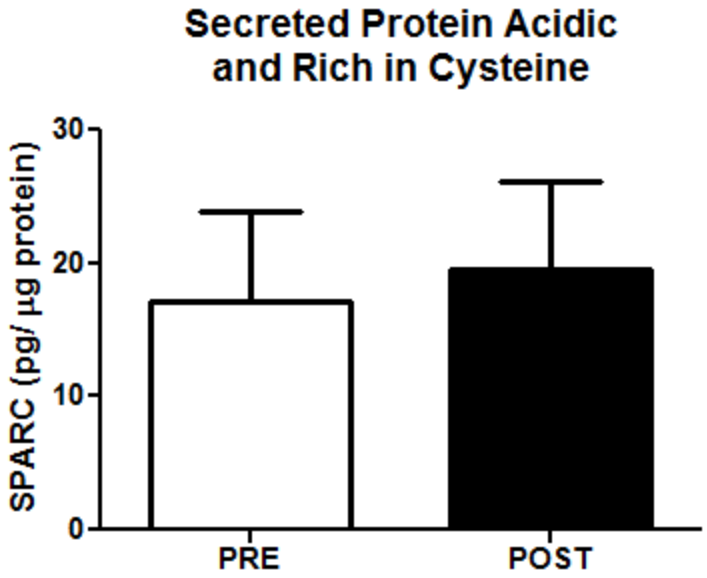

Figure 6: Concentration of SPARC in the skeletal muscle before and after exercise

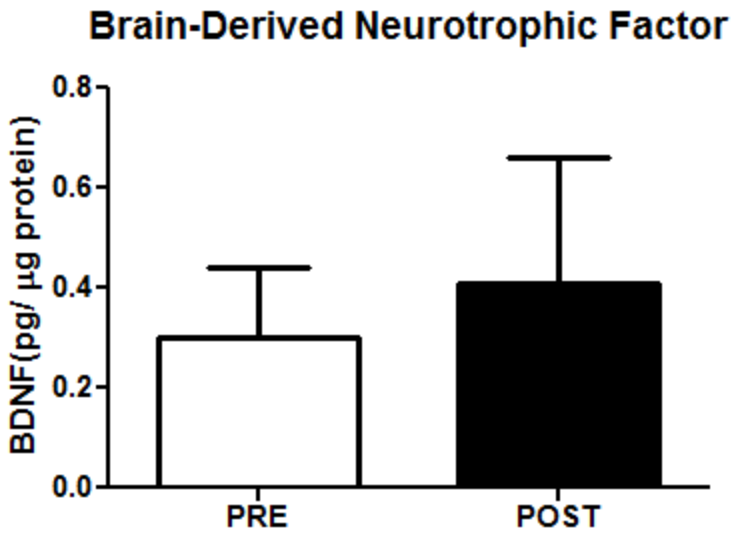

Figure 7: Concentration of BDNF in the skeletal muscle before and after exercise

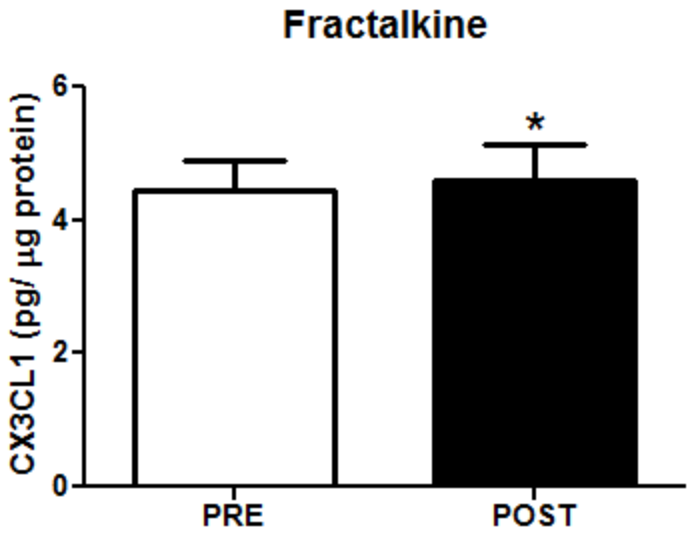

Figure 8: Concentration of CX3CL1 in the skeletal muscle before and after exercise (asterisk indicates statistically significant increase)

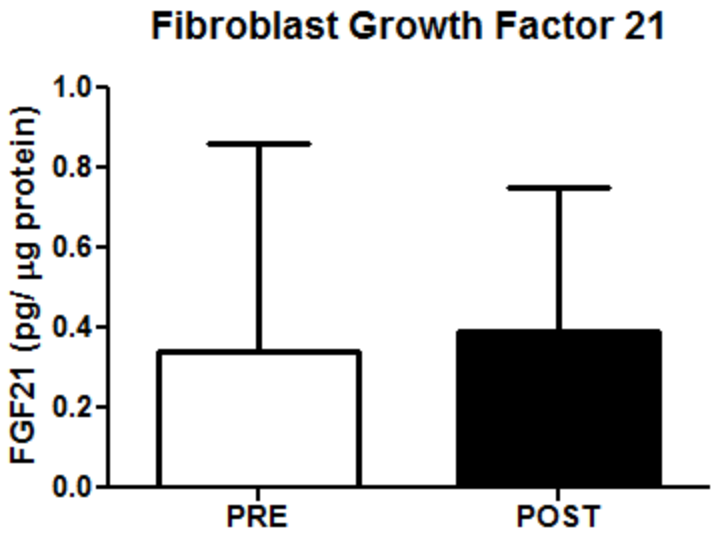

Figure 9: Concentration of FGF21 in the skeletal muscle before and after exercise

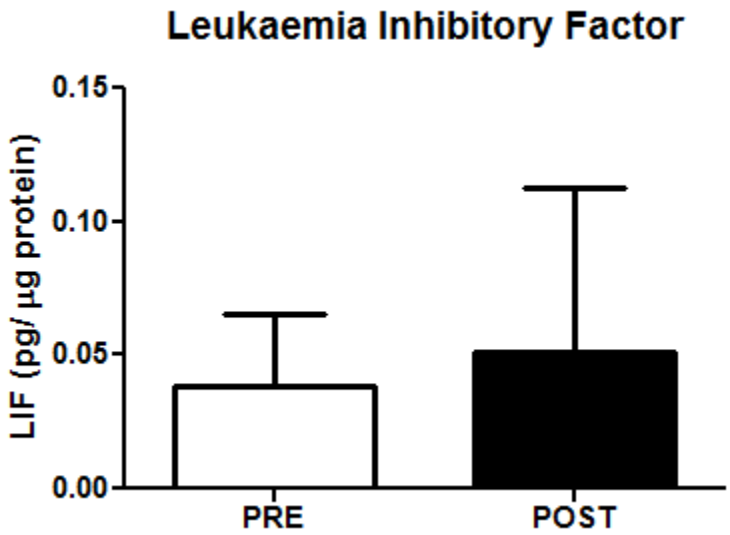

Figure 10: Concentration of LIF in the skeletal muscle before and after exercise

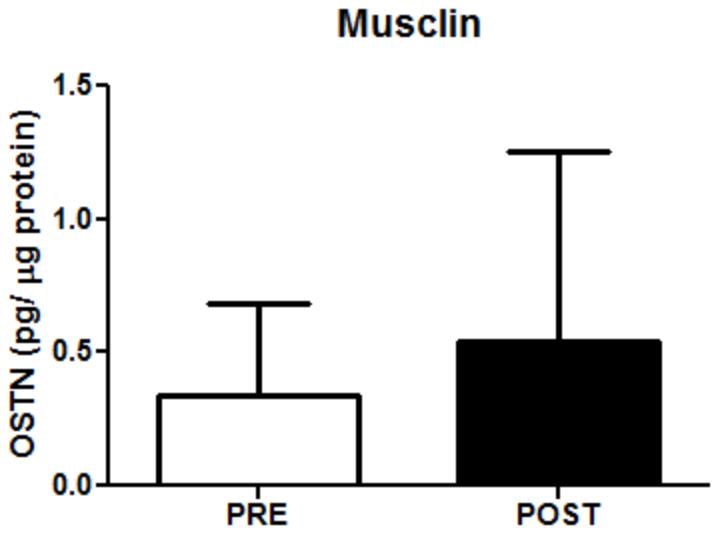

Figure 11: Concentration of OSTN in the skeletal muscle before and after exercise

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