Inflammatory gene changes associated with the repeated-bout effect

Monica J. Hubal, Trevor C. Chen, Paul D. Thompson, and Priscilla M. Clarkson

1Department of Kinesiology, University of Massachusetts, Amherst, Massachusetts; 2Department of Physical Education, National Chiayi University, Taiwan, Republic of China; and 3Department of Preventative Cardiology, Hartford Hospital, Hartford, Connecticut

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Hubal MJ, Chen TC, Thompson PD, Clarkson PM. Inflammatory gene changes associated with the repeated-bout effect. Am J Physiol Regul Integr Comp Physiol 294: R1628–R1637, 2008.—This study proposed that attenuated expression of inflammatory factors is an underlying mechanism driving the repeated-bout effect (rapid adaptation to eccentric exercise). We investigated changes in mRNA levels and protein localization of inflammatory genes after two bouts of muscle-lengthening exercise. Seven male subjects performed two bouts of lower body exercise (separated by 4 wk) in which one leg performed 300 eccentric-concentric actions, and the contralateral leg performed 300 concentric actions only. Vastus lateralis biopsies were collected at 6 h, and strength was assessed at baseline and at 0, 3, and 5 days after exercise. mRNA levels were measured via semiquantitative RT-PCR for the following genes: CYR61, HSP40, HSP70, IL1R1, TCF8, ZFP36, CEBPD, and MCP1. Muscle functional adaptation was demonstrated via attenuated strength loss (16% less, P = 0.04) at 5 days after bout 2 compared with bout 1 in the eccentrically exercised leg. mRNA expression of three of the eight genes tested was significantly elevated in the eccentrically exercised leg from bout 1 to bout 2 (+3.9-fold for ZFP36, +2.3-fold for CEBPD, and +2.6-fold for MCP1), while all eight mRNA levels were unaffected by bout in the concentrically exercised leg. Immunohistochemistry further localized the protein of one of the elevated factors [monocyte chemoattractant protein-1 (MCP1)] within the tissue. MCP1 colocalized with resident macrophage and satellite cell populations, suggesting that alterations in cytokine signaling between these cell populations may play a role in muscle adaptation to exercise. Contrary to our hypothesis, several inflammatory genes were transcriptionally upregulated (rather than attenuated) after a repeated exercise bout, potentially indicating a role for these genes in the adaptation process.

muscle damage; immunohistochemistry; repair

ECCENTRIC (muscle-lengthening) actions typically cause transient muscle damage followed by a period of muscle regeneration (for reviews see Refs. 1, 7, and 22). One bout of eccentric exercise also results in adaptation within the muscle, so that it is less vulnerable to further muscle damage, a phenomenon known as the “repeated-bout effect” (6, 19). This effect is typically indicated by faster recovery of muscle function, reduced muscle soreness, blunted appearance of muscle proteins in the blood, and decreased levels of inflammatory cells in the circulation (for review see Refs. 17 and 18). Although the repeated-bout effect has been demonstrated in many studies, the molecular mechanisms driving this phenomenon remain unclear.

An initial eccentric exercise bout results in damage to fibers caused by a combination of physical stress and mechanical strain of the contractions (10, 15). This physical damage results in secondary damage, which is thought to be caused by sarcolemmal disruption and altered cell calcium homeostasis in the days following exercise (4, 37). Inflammatory processes activated by the initial damage stimuli are also thought to contribute to secondary damage to myofibers via release of chemotactic factors, which can lead to degradation of muscle tissue proteins (for review see Ref. 33). These processes are vital to muscle recovery from damage, but they can also add to cellular damage by degrading nearby intact proteins.

Optimal degradation of damaged elements and rebuilding rely on a balance between pro- and anti-inflammatory signals and precise signaling between inflammatory factors and other muscle elements (e.g., satellite cells and structural elements). Alterations have been found in the inflammatory response after repeated bouts of exercise (11, 14, 27) and may be responsible, at least in part, for the lower indexes of damage and/or faster recovery from a repeated bout of eccentric exercise. Several studies have demonstrated attenuated inflammatory factors in the circulation after a repeated eccentric exercise bout. Pizza et al. (20, 21) demonstrated a blunted neutrophilia and decreased expression of leukocyte receptors after a second eccentric bout. Smith et al. (26) found a 50% decrease in IL-6 and a 10% reduction in circulating monocyte chemoattractant protein-1 (MCP1) after repeated downhill running. Smith et al. also demonstrated a 95% increase in the anti-inflammatory factor IL-10, and Hirose et al. (11) found an increase in IL-10 after a repeated bout of eccentric exercise in the elbow flexors.

Although several studies have investigated inflammatory factors in the circulation after repeated exercise bouts, fewer studies have examined inflammatory factors within skeletal muscle tissue. Koh et al. (13) demonstrated that passive stretches reduced damage and dysfunction after a subsequent lengthening bout of exercise, but they only demonstrated attenuated inflammatory cells with passive stretch training in older mice. Willoughby et al. (36) documented increases in the glucocorticoid receptor after a second bout of eccentric exercise, and glucocorticoids have potent anti-inflammatory functions within tissues. Expression of two inflammatory genes [heat shock proteins (HSP) 27 and 70] in the elbow flexors at 48 h after two bouts of eccentric exercise was examined by Thompson et al. (32); although levels of HSP27 and HSP70 expression were not different between bouts at 48 h after exercise, preexercise levels of HSP expression were lower before the second exercise bout, potentially altering the inflammatory response to exercise.
The studies of Willoughby (36) and Thompson et al. (32) provide significant but limited information about the inflammatory processes in muscle with repeated bouts of exercise. Myriad other inflammatory pathway components, including pro- and anti-inflammatory elements, could mediate the repeated-bout effect. In a previous study, we described the gene expression profile for eccentrically exercised muscle in the hours after exercise (5). In that study, we examined the effect of eccentric exercise on the transcriptome of skeletal muscle in human volunteers who performed 300 concentric contractions with one leg and 300 eccentric contractions with the opposite leg. Functional clustering detected robust changes in several genes involved in inflammation, as well as genes associated with tissue structure, energy metabolism, sarcomere organization, membrane stability, apoptosis, and DNA damage.

The purpose of the present study was to examine, after two bouts of eccentric exercise, mRNA expression of the inflammatory genes identified in our previous study (5) as being robustly upregulated after a single eccentric exercise bout. On the basis of the attenuation of damage markers with the repeated-bout effect, we hypothesized that inflammatory gene expression would be significantly altered after a second bout of eccentric exercise, so as to attenuate inflammation. Our most important finding was a transcriptional increase in MCP1 mRNA after bout 1 that was further increased after bout 2. On the basis of this further increase, we hypothesized that MCP1 was involved in the promotion of muscle repair via enhanced signaling between macrophages and satellite cells. Thus we decided to determine whether MCP1 was localized to these cell subpopulations to elucidate the potential role of MCP1 in the repeated-bout effect.

MATERIALS AND METHODS

Subjects

Seven young adult men [22.3 ± 3.4 (mean ± SD) yr old, 176.7 ± 6.4 cm height, 82.9 ± 8.6 kg body wt, and 26.6 ± 2.4 kg/m² body mass index] completed the study. Sample size was estimated a priori (α = 0.05, β = 0.8) using strength loss and mRNA levels as separate dependent variables on the basis of results from Chen et al. (5) and Thompson et al. (32). All subjects signed informed consent documents approved by the University of Massachusetts and Hartford Hospital Institutional Review Boards and completed a medical screening form.

Subjects were healthy, with no diagnosed cardiovascular, pulmonary, metabolic, or chronic diseases. Subjects had not resistance trained (especially in the lower body) in the past 6 mo and did not perform any recreational activities (e.g., snowboarding, hill running, skiing, and volleyball) that include large eccentric components.

Testing Schedule

The testing protocol consisted of 14 visits (Fig. 1). The first seven time points (visits 1–7) encompassed bout 1 of exercise, followed by a 4-wk rest period and then seven more time points (visits 8–14) encompassing bout 2 of exercise. On visit 1, subjects read and signed the informed consent document and completed the medical screening questionnaire. They then performed a baseline knee-extension maximal voluntary contraction (MVC) torque test. On visits 2 and 3, they repeated baseline MVC evaluations. After baseline testing on visit 3, subjects performed an eccentric/concentric lower body exercise, as described below. MVC assessment was repeated immediately after exercise. Subjects remained on site between visits 3 and 4 and were fed a standardized meal 3 h after exercise (3 h before biopsy). At visit 4, subjects were transported to Hartford Hospital, where they underwent bilateral needle biopsies at 6 h after exercise. On visit 5 (1 day after exercise), incision sites were visually inspected to ensure healing. On visits 6 and 7 (3 and 5 days after exercise, respectively), MVC was reassessed.

Bout 2 was performed 4 wk after bout 1, which was shown in pilot work to provide sufficient time for recovery from biopsy procedures within the time period associated with the repeated-bout effect. Testing protocols, including strength testing, exercising, and obtaining biopsies at the same time of day, were identical to bout 1. Also, the same standardized meal was consumed by each subject at 3 h after exercise.

Strength Testing

MVC isometric torque of the knee extensors was assessed at 90° of knee flexion on an isokinetic dynamometer (System 3, Biodex, Shirley, NY). The subject internally rotated each foot during each contraction to ensure sufficient activation of the vastus lateralis. Each subject performed three 3-s contractions, with 1 min of rest between contractions, for each leg. The order of leg testing was randomized across trials and subjects. Postexercise MVC testing was initiated immediately after exercise. Data were sampled at 100 Hz, and a three-point moving average was used to smooth the data for analysis. MVC was defined as the highest average torque within the smoothed data for each trial.
Exercise

Subjects performed a series of movements in which one (randomized during bout 1 and repeated in bout 2) leg underwent 300 concentric contractions (rising from a chair) and the opposite leg underwent 300 concentric and 300 eccentric contractions (rising from and lowering to a seated position). The total exercise time was 20 min, with one cycle (sit to stand to sit) every 4 s. This exercise protocol has been shown to result in damage (assessed via magnetic resonance imaging analysis) to the vastus lateralis muscle of the concentric + eccentric leg (30) and a unique gene expression profile in which the inflammatory genes tested in the present study demonstrated exaggerated upregulations (5). This protocol was adapted slightly from our previously published study (5), with the only difference being the adjustment of seat height to standardize vertical excursion between subjects. To accomplish this, the height of the chair was set so that each subject had a hip displacement of 20 cm, which demonstrated the largest damage response (postexercise strength loss of 60% MVC) in pilot testing (i.e., highest single-subject MVC loss among 5 pilot subjects).

The height of the hip was measured by marking with ink the greater trochanter of each leg while the subject was standing. An anthropometric tape measure was used to record the height from the ground to the mark. Then the height of the hip was again measured while the subject was seated. The chair height was adjusted until the difference between standing hip height and seated hip height was 20 cm.

Biopsy

Muscle biopsies were obtained 6 h after each exercise bout from the left and right vastus lateralis muscles (≈0.3-femur length distal from the hip) using Bergstrom 5-mm biopsy needles. The 6-h time point was selected to capture a representational transcriptional response to the stimulus, allowing time for the muscle to recover from any temporary muscle fatigue while capturing early-response genes and those with a more moderate transcription rate. The 6-h time point was also selected to correspond with responses observed in our previous study (5).

The leg that performed eccentric exercise was always biopsied first. All biopsy procedures were done at Hartford Hospital by an experienced physician. The biopsy site was shaved with a disposable razor and then cleaned with povidone-iodine (Betadine). The skin was anesthetized with 2% lidocaine hydrochloride solution, a small incision was made through the skin and fascia with a scalpel blade, and the biopsy needle was inserted. Approximately 200 mg of tissue were removed from each site and rapidly frozen in liquid nitrogen. Samples selected for immunohistochemistry were frozen in isopentane and then in liquid nitrogen. Tissue was frozen at −80°C until further analyses. Incision sites for the second series of biopsies were immediately proximal to those for the first series.

Semi-quantitative Real-Time PCR

Total RNA was isolated from 20–25 mg of muscle tissue using the TRIzol (Invitrogen, Carlsbad, CA) method and quantified via spectrophotometry (Nanodrop, Wilmington, DE). Total RNA integrity was validated by the ratio of absorbance at 260 nm to absorbance at 280 nm (all samples had 260-280 ratios of 1.9–2.0). Equal amounts (0.2 μg) of total RNA were reverse transcribed using a first-strand cDNA synthesis kit (Fermentas, Hanover, MD). The following genes were tested: heat shock 70-kDa protein 1B (HSP1B); DnaJ (Hsp40) homolog, subfamily B, member 4 (DNAJB4); cysteine-rich angiogenic inducer 61 (CYR61); transcription factor 8 (TCF8); interleukin 1 receptor type I (IL1R1); zinc finger protein 36 (ZFP36), chemokine (C-C motif) ligand 2 (CCL2), also known as monocytic chemotactic protein-1 (MCP1); and CCAAT/enhancer-binding protein-α (CEBP). These genes represent the subset of genes that were upregulated at least fivefold in the eccentrically vs. the concentrically exercised leg in our previous study (5) that are ascribed inflammatory functions by various bioinformatics databases (i.e., National Center for Biotechnology Information). GAPDH was used as an internal control. Forward and reverse primers were designed for each gene using National Center for Biotechnology Information gene sequences, SciTools software (Integrated DNA Technologies, Coralville, IA), and Netprimer software (Premier Biosoft International, Palo Alto, CA) (Table 1). Final primer concentration used in amplification reactions was 70 μM.

cDNA was added to SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with ROX dye and the appropriate primer sequences for each gene. All samples were run in triplicate on 96-well plates on a real-time PCR system (model MX3000p, Stratagene, La Jolla, CA). The thermal cycling conditions consisted of 95°C for 15 min followed by 40 cycles of amplification at 95°C for 15 s for denaturing and then at 60°C for 30 s and 72°C for 30 s min for annealing and extension, respectively, and, finally, a standard 80-cycle melting curve to ensure quality of samples. All PCR products were confirmed using 2% agarose gel electrophoresis with ethidium bromide staining.

Gene expression was quantified as the relative incorporation of SYBR green dye during the PCR in the experimental vs. control conditions according to the ΔΔCT method. Estimation of amplified

Table 1. Primer sequences for targeted inflammatory genes and housekeeping gene

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession No.</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>Homo sapiens heat shock 70 kDa (HSP70)</td>
<td>NM_005346</td>
<td>5′-TGGAGCCTCAGGCTTGAACATGTA-3′</td>
<td>5′-TCTCTTCTTGCTCAGGTAACCTGCT-3′</td>
</tr>
<tr>
<td>Homo sapiens DnaJ (Hsp40) homolog</td>
<td>NM_007034</td>
<td>5′-AGACGAAATGGGTTGTGAAGAT-3′</td>
<td>5′-AATGGAGGATCTTCTTCTTCTGAGG-3′</td>
</tr>
<tr>
<td>Homo sapiens cysteine-rich angiogenic inducer 61 (CYR61)</td>
<td>NM_001554</td>
<td>5′-AGCCCTGAGATCTTACAAACACCTT-3′</td>
<td>5′-GTTCCTCTCTCAACAGGCGGCTAC-3′</td>
</tr>
<tr>
<td>Homo sapiens transcription factor 8 (TCF8)</td>
<td>NM_030751</td>
<td>5′-AGCTCATGCGGCAATTCACCCAAAT-3′</td>
<td>5′-TTGGAGGGTGTTGTGGAGGAGTC-3′</td>
</tr>
<tr>
<td>Homo sapiens IL-1 receptor type I (IL1R1)</td>
<td>NM_000877</td>
<td>5′-AATGCTACACCAAGCATAAGGAAGA-3′</td>
<td>5′-TGGAGAAGTGAGGAGATCATGAA-3′</td>
</tr>
<tr>
<td>Homo sapiens zinc finger protein 36, C3H type, homolog (ZFP36)</td>
<td>NM_003407</td>
<td>5′-ATCCGAGACCTCAGGATCTAACCGAG-3′</td>
<td>5′-GGGACATTCTTCTGACTCAGA-3′</td>
</tr>
<tr>
<td>Homo sapiens chemokine (C-C motif) ligand 2 (CCL2, MCP1)</td>
<td>NM_002982</td>
<td>5′-AAAGATCACGACAGCAGCTTCG-3′</td>
<td>5′-AGGTGGTTGTTGGTCCTGCGG-3′</td>
</tr>
<tr>
<td>Homo sapiens CCAAT/enhancer-binding protein (C/EBP)-δ (CEBPδ)</td>
<td>NM_005195</td>
<td>5′-CTACAGCCCTTGACTGTCTAAGGCTG-3′</td>
<td>5′-TTCAGTGGGAATCGGATTTTGCC-3′</td>
</tr>
</tbody>
</table>

Housekeeping gene

GAPDH | NM_002046 | 5′-CATGGGCGCTCAAGGACCACATTGGT-3′ | 5′-TCTCTTCTGCTCTCTCTGGG-3′
gene products was normalized to GAPDH (housekeeping gene) to compensate for variations in quantity and differences in RT efficiency. Briefly, the “threshold cycle” (Ct value) was noted as the thermal cycle in which signal exceeded an automatically set threshold. The “ΔΔCt” was calculated by subtracting the Ct for GAPDH from the Ct for the gene of interest for each sample.

**Immunohistochemistry**

Because of limited amounts of tissue, we were able to accomplish either Western blotting or immunohistochemistry. We decided on immunohistochemistry, because MCP1 is a chemokine that can easily travel between circulation and tissue; therefore, absolute protein levels in the tissue samples likely would not reflect the total amount of protein being produced. On the basis of the increase in MCP1, along with a faster recovery of strength with the repeated bout, we proposed that MCP1 was involved in signaling between macrophages and satellite cells (which promotes repair), so it was important to demonstrate that MCP1 was localized to these cell subpopulations.

Serial 8- to 10-μm-thick frozen muscle sections were cut with a cryostat (MICROM HM 505E, Richard Allan Scientific) at −30°C, mounted to Superfrost Plus Slides (Fisher Scientific), and air-dried overnight. All samples from a given subject were processed concurrently with appropriate controls, including null-primary antibody sections. Sections were rehydrated for 5 min with 1× PBS (pH 7.4) with 0.01% NaN3, blocked for 15 min in 5% goat serum (Jackson Immunoresearch Laboratories, West Grove, PA), and incubated in primary antibody overnight at 4°C. The following primary antibodies were used: MCP1 (polyclonal, 1:10 dilution; Abcam, Cambridge, MA), MAC387 for macrophages (monoclonal, 1:100 dilution; Abcam), and PAX7 for satellite cells (monoclonal, 1:20 dilution; Developmental Studies Hybridoma Bank, Iowa City, IA). PAX7 is expressed in quiescent and newly activated muscle satellite cells and is a commonly used marker for satellite cell presence (3). MAC387 reacts with mature macrophages via the L1 antigen site.

Sections were washed with 1× PBS and 5% goat serum and then incubated for 30 min at 37°C with secondary fluorochrome-labeled antibodies against primary antibodies [Alexa green, Alexa red, goat anti-rabbit, or goat anti-mouse (Molecular Probes; Eugene, OR); 1:300 dilution] with appropriate controls. After secondary antibody incubation, sections were washed in PBS and mounted using 10 μl of Vectashield mounting medium (Vector Laboratories, Burlingame, CA) with 4,6-diamidino-2-phenylindole (DAPI).

A phase-contrast microscope (Eclipse E600, Nikon) was equipped with a Spot Insight QE camera and EclipseNET software (version 1.16). IMAGE for Windows (Scion, Fredrick, MD) was used for image analysis. The same investigator performed all image analyses, which were repeated at least twice for reliability. The investigator was blinded with regard to condition and bout.

For immunohistochemistry quantification of macrophages and MCP1, ~100 myofibers (excluding those around the perimeter of the section) were randomly selected for analysis of the presence of fluorescent signal in the adjacent extracellular space. For satellite cell quantification, 100–200 randomly selected (DAPI-positive) nuclei were assessed for overlap of PAX7 staining, and the average percentage of PAX7-positive nuclei was assessed. For colocalization of MCP1 with macrophages or satellite cells, 20–50 MAC387 or PAX7 targets were assessed for overlap with MCP1 fluorescence, and the percentage of overlap was calculated. Each of these analyses was repeated at least twice, and a third analysis was done if the first two analyses generated results with a difference of >10%.

**Data Analysis**

Strength measures were assessed via three-factor ANOVA (leg) with repeated measures over time and bout (significance at α ≤ 0.05). Tukey’s post hoc tests were used to assess planned comparisons.

Paired t-tests were used to assess differences in mRNA expression between bouts. To determine fold changes between legs within a time point (i.e., exercise bout), ΔΔCt was calculated by subtraction of ΔCt eccentric from ΔCt concentric. To determine fold changes within leg between time points (i.e., comparing eccentric leg from bout 1 with that from bout 2), ΔΔCt was calculated by subtraction of ΔCt bout2 from ΔCt bout1.

Quantification of immunohistochemistry results was assessed via two-factor (bout × condition) ANOVA.

**RESULTS**

**Strength Loss**

Knee-extension isometric strength loss following exercise is depicted in Fig. 2. A three-factor ANOVA (leg) with repeated measures over time and bout detected a significant overall interaction term between all factors (P < 0.001). The faster recovery of strength in the eccentrically exercised leg at 5 days after exercise supports the repeated-bout effect.

Strength losses were significantly greater in the eccentrically than the concentrically exercised leg at all time points. The concentrically exercised leg experienced an average strength loss of 12% following bout 1 and 8% following bout 2 (P = 0.69), demonstrating no repeated-bout effect. These minor strength losses were eliminated by 5 days after exercise, and recovery was also not different between bouts. The eccentrically exercised leg demonstrated a significant effect of exercise bout (P = 0.047). Post hoc testing showed that 6% and 11% attenuations in strength loss between bouts 1 and 2 at 0 and 3 days after exercise were not statistically significant (P = 0.49 and 0.18, respectively). However, a significant 16% difference between bouts (23% loss after bout 1 and 7% loss after bout 2, P = 0.04) at 5 days after exercise was found.

**Gene Expression**

All genes were tested in triplicate, and 98% of all values for the triplicate Ct measures were within ±5%. We also tested mRNA expression of MCP1 on two separate occasions to assess reliability of results from day to day. No significant differences between these two sets of results were found for Ct values for MCP1 or GAPDH. Fold changes for the eccentric leg between bouts 1 and 2 for MCP1 were 2.6 for each reliability experiment. Fold changes for the concentrically
exercised leg between bouts 1 and 2 for MCP1 were 0.7 and 0.65, respectively, for the reliability experiments. Thus the measurements were consistent over days. Furthermore, GAPDH expression was not different between bouts ($P = 0.96$) or between legs ($P = 0.63$); therefore, it was a valid housekeeping gene for normalization purposes.

**Single exercise bout effects.** Seven of the eight genes tested by semiquantitative real-time PCR were expressed to a greater degree in the eccentrically than in the concentrically exercised leg in the present study; the only exception was CEBPD (1.1-fold change between legs; Table 2). All fold changes from the concentrically and the eccentrically exercised leg can be found in Table 2, which also compares the fold changes from the present study with those detected by RT-PCR in our previous study (5). For the four genes for which we have single-bout RT-PCR results from our present and previous studies, fold changes were more moderate in the present study; this could have resulted from a difference in RT-PCR methodology (i.e., Taqman RT-PCR in the previous study vs. the SYBR platform in the present study).

**Repeated-bout effects.** Fold changes within each leg from bout 1 to bout 2 were also tested by semiquantitative real-time PCR. None of the eight genes were differentially expressed in normalization purposes. SYBR platform in the present study).

None of the eight genes tested demonstrated a repeated-bout effect, ever, in the eccentrically exercised leg, we found that three of the genes tested demonstrated a repeated-bout effect, expressing more mRNA after bout 2 than after bout 1. These three genes (MCP1, CEBPD, and ZFP36) were also increased over bouts to a greater extent in the eccentrically than in the concentrically exercised leg ($P < 0.05$; Table 3).

**Protein Localization**

MCP1 was targeted for further protein localization studies because of its documented alterations with muscle injury and regeneration (28, 34, 35). MCP1 localization, rather than total protein levels, was targeted, because MCP1 is a highly mobile cytokine that easily moves between tissue and circulation. MCP1 is also an important factor in macrophage chemotaxis, and macrophage-deficient muscle demonstrates impaired recovery from injury (35). Location of MCP1 protein expression within the skeletal muscle biopsy samples was assessed by immunohistochemistry.

Double staining of MCP1 with PAX7 (satellite cells) or MAC387 (macrophages) revealed expression of MCP1 by satellite cells and macrophages (Figs. 3 and 4) after both bouts of exercise. Macrophage expression was detected in the interstitial space between myofibers. No macrophages were detected within myofibers, and no gross deformities of muscle fiber structure were detected. MCP1 expression was also detected in the interstitial space between myofibers, and MCP1 was often expressed by the same structures that expressed MAC387 (macrophages). Although some background staining of MCP1 was seen throughout the myofibers, most expression of MCP1 was outside fiber boundaries.

PAX7 (satellite cell) expression was detected after both exercise bouts on the border of myofibers, consistent with a location between the sarcolemma and basal lamina of most satellite cells. No PAX7 expression was detected within myofibers, demonstrating specificity of this antibody to satellite cells. MCP1 expression was also detected in the area around the satellite cells. Similar to the macrophage-MCP1 double-staining experiments, some background staining of MCP1 was observed throughout the myofibers, while most of the expression of MCP1 surrounded fiber boundaries.

**Immunohistochemistry quantification.** Quantification of macrophage and MCP1 expression and the extent of protein colocalization are presented in Table 4. Similar increases in MCP1 colocalization between bouts was found in both legs ($P = 0.28$). Colocalization of MCP1 with macrophages was also similarly elevated from bout 1 to bout 2 in both conditions ($P = 0.58$).

The percentage of PAX7-positive nuclei and the extent of colocalization with MCP1 are also presented in Table 4. Approximately 10–12% of DAPI-positive nuclei were also PAX7-positive nuclei. This percentage was not significantly different between conditions or bouts, and there was no significant interaction between condition and bout ($P = 0.14$). Colocalization of MCP1 with satellite cells was also similarly elevated from bout 1 to bout 2 in both conditions, although there was a trend for greater increases in MCP1 colocalization with satellite cells in the eccentrically exercised leg ($P = 0.06$).

**DISCUSSION**

The repeated-bout effect describes the protective effect of an initial bout of eccentric exercise on muscle damage indexes after repeated bouts of exercise performed up to 6 mo later. One of the mechanisms proposed to be driving this adaptation to eccentric exercise is change in inflammation (17, 18). Inflammation following damaging exercise is both beneficial and detrimental, creating secondary tissue damage while, at the same time, clearing out damaged tissue and paving the way for muscle regeneration (33). The present study characterized the

Table 2. Gene expression fold differences between the Ecc and Con leg after bout 1

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Present Study (n = 7)</th>
<th>Chen et al. (5) (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYR61</td>
<td>14.3 ± 0.65</td>
<td>20.9 ± 0.27</td>
</tr>
<tr>
<td>Hsp40 homolog</td>
<td>6.5 ± 0.9</td>
<td>20.0 ± 0.01</td>
</tr>
<tr>
<td>HSP70</td>
<td>10.9 ± 2.47</td>
<td>1.2 ± 0.01</td>
</tr>
<tr>
<td>IL1R1</td>
<td>5.0 ± 0.5</td>
<td>24.8 ± 2.75</td>
</tr>
<tr>
<td>TCF8</td>
<td>1.7 ± 0.4</td>
<td>18.4 ± 0.94</td>
</tr>
<tr>
<td>ZFP36</td>
<td>3.4 ± 0.53</td>
<td>18.4 ± 0.94</td>
</tr>
<tr>
<td>MCP1</td>
<td>9.2 ± 13.5</td>
<td>27.5 ± 1.2</td>
</tr>
<tr>
<td>CEBPD</td>
<td>1.1 ± 1.4</td>
<td>0.04 ± 0.01</td>
</tr>
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</table>

See Table 1 for gene description and GenBank accession number. Con, concentric; Ecc, eccentric. *Statistically significant difference.
expression of a subset of genes involved in inflammation (HSP70, HSP40, CYR61, TCF8, IL1R1, ZFP36, MCP1, and CEBPD) after two bouts of eccentric exercise to determine whether specific factors within the inflammatory process are downregulated with the repeated-bout effect. The repeated-bout effect in the present study was demonstrated by faster recovery of strength at 5 days after exercise. Several studies have demonstrated that the repeated-bout effect is associated more with recovery of strength in the days (6, 19) after exercise than with immediate strength losses (which reflect fatigue and damage-related dysfunction).

Different mRNA Expression Between Bouts: Eccentric Leg

MCP1, a secreted chemokine that signals monocytes and basophils, was significantly elevated in the leg that performed eccentric contractions after bout 1 (9.2-fold change) and was subsequently significantly elevated even more in the eccentric leg between bouts 1 and 2 (2.6-fold change from bout 1 to bout 2). Increased MCP1 expression has been widely associated with diseases with underlying inflammatory pathologies (for review see Ref. 16), and studies have demonstrated that the primary role of MCP1 is recruitment of macrophages to sites of injury or infection (16).

Table 3. Gene expression fold differences in bout 2 vs. bout 1 for Ecc and Con legs

| Gene Name | Ecc Leg Fold Change | SD | P   | Ecc vs. Con P  | Con Leg Fold Change | SD | P   | Ecc vs. Con P  |
|-----------|---------------------|----|-----|---------------|---------------------|----|-----|---------------|===============|
| CYR61     | 1.7                 | 8.0| 0.45|               | 0.9                 | 0.7| 0.32|               | 0.39           |
| HSP40     | 1.1                 | 1.3| 0.88|               | 0.5                 | 2.4| 0.25|               | 0.26           |
| HSP70     | 0.8                 | 2.5| 0.68|               | 0.9                 | 0.5| 0.31|               | 0.92           |
| IL1R1     | 1.9                 | 1.2| 0.27|               | 1.1                 | 2.7| 0.66|               | 0.33           |
| TCF8      | 1.0                 | 0.7| 0.89|               | 1.0                 | 4.2| 0.97|               | 0.93           |
| ZFP36     | 3.9*                | 13.1| 0.02|               | 1.0                 | 0.8| 0.51|               | 0.02†          |
| MCP1      | 2.6*                | 5.0| 0.04|               | 0.7                 | 1.9| 0.76|               | 0.02†          |
| CEBPD     | 2.3*                | 1.8| 0.01|               | 1.0                 | 0.6| 0.82|               | 0.02†          |

See Table 1 for gene description and GenBank accession number. *Significantly greater than Ecc. †Ecc significantly greater than Con.

Fig. 3. Monocyte chemoattractant protein-1 (MCP1) and macrophage localization within skeletal muscle tissue from a representative subject. Nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI) and appear blue. Macrophages appear red, and MCP1 appears green. C1 and C2, concentric, bouts 1 and 2; E1 and E2, eccentric + concentric, bouts 1 and 2. A: merged images of MCP1, macrophages, and nuclei. Co-localization of MCP1 and macrophages appears yellow (arrows). B: single images of MCP1 stain. C: single images of macrophages stained with MAC387. D: single images of DAPI-stained nuclei.
protein with activated macrophages and activated muscle precursor cells. These data support previous studies that demonstrated the ability of muscle precursor cells (also known as satellite cells) to produce MCP1 in cell culture (3, 8). The most recent study by Warren et al. (34) in the mouse model suggests that the MCP1 response involves macrophages and satellite cells, both of which play important roles in muscle regeneration after injury.

Two additional genes, CEBPD and ZFP36, demonstrated differential transcriptional regulation with the repeated-bout effect in the damaged leg, and each of these two genes directly or indirectly interacts with MCP1 (Fig. 5). CEBPD is a transcriptional activator in the immune and inflammatory responses (23). In smooth muscle cells, Sekine et al. (25) demonstrated that increased MCP1 after insulin treatment was mediated by CEBPD, suggesting that binding elements for this transcription factor are present on the MCP1 gene. Furthermore, with use of biostatistical software (TFSEARCH: Searching Transcription Factor Binding Sites, 2006, Y. Akiyama, Kyoto University, Kyoto, Japan), two potential binding sites were located on the MCP1 gene for this transcription factor, suggesting that CEBPD directly stimulates transcription of MCP1.

Although upregulation of CEBPD likely has a stimulatory effect on MCP1, ZFP36 upregulation would theoretically downregulate MCP1 expression via two potential mechanisms: 1) destabilization of the mRNA of multiple inflammatory genes, including TNFA (31), would attenuate TNFA induction of MCP1 expression, and 2) ZFP36 has been shown, under in vitro LPS-stimulation conditions, to reduce MCP1 expression. Therefore, the increase in ZFP36 along with an increase in MCP1 with repeated exercise bouts means that in vivo exercise-induced inflammation does not induce a similar destabilization effect of ZFP36 on MCP1 mRNA or that the increase in ZFP36 serves to keep MCP1 upregulations “in check”, preventing an excessive (and potentially damaging) inflammatory response. These data suggest a complex regulation of certain inflammatory processes at the level of transcription that modulate the skeletal muscle’s response to damaging exercise stimuli.

**MCP1 Protein Localization**

To further elucidate which cell populations (i.e., myofiber, satellite cell, and macrophage) were associated with the observed differential transcriptional expression, we used immunohistochemistry to determine whether the MCP1 protein colocalized with macrophage and satellite cell subpopulations within the tissue. We chose not to examine CEBPD and ZFP36 protein localizations, because each is a generalized transcription factor with multiple targets, and both are known to be expressed in macrophage and skeletal muscle nuclei. Given the multiple targets associated with each of these transcription factors, we assumed that each would be expressed in most, if not all, nuclei within the samples.
were no significant interactions (H11022 P) at the same time. Colocalization at this time point to the transcriptional changes in satellite cells at 6 h after exercise and try to relate the extent of could colocalize MCP1 protein to resident macrophages and satellite cells at 6 h after exercise, inasmuch as the majority of macrophage infiltration from circulation and satellite cell proliferation/differentiation occurs at later time points. However, we demonstrate a greater level of macrophage and satellite cell infiltration 2 b ou ts 1 (54%) than after bout 1 (29%) in eccentrically exercised leg (P = 0.06), suggesting that these two cell populations to affect one another. Human cocultures of macrophages with satellite cells enhanced satellite cell growth and promoted proliferation (3). Chazaud et al. (3) also demonstrated amplified monocyte chemotaxis in cocultured macrophages and satellite cells, suggesting a symbiotic relationship of these two cell populations in creating chemotaxis. Altered macrophage infiltration or activity, altered satellite cell activity, or altered communication between these two subpopulations of cells (via MCP1 or other factors) could significantly contribute to the enhanced recovery observed with the repeated-bout effect. Our data suggest that more satellite cells express MCP1 in subjects demonstrating faster recovery from freeze injury, suggesting that macrophage activity is integral to muscle recovery. It is thought that the positive effects of macrophages on skeletal muscle repair are mediated through communications between the macrophages and chemokine signaling targets. Satellite cell proliferation and differentiation are also vital to muscle regeneration (for review see Refs. 2, 9, and 12). Elimination of satellite cell activity via irradiation or depletion significantly impairs the ability of muscle to regenerate after eccentric exercise (24).

There is mounting evidence that chemokines released by macrophages and satellite cells communicate with surrounding cell populations and that this communication is essential for muscle repair. MCP1, in particular, seems to be an important element of this signaling. In vitro studies have demonstrated the ability of these two cell populations to affect one another. Human cocultures of macrophages with satellite cells enhanced satellite cell growth and promoted proliferation (3). Chazaud et al. (3) also demonstrated amplified monocyte chemotaxis in cocultured macrophages and satellite cells, suggesting a symbiotic relationship of these two cell populations in creating chemotaxis. Altered macrophage infiltration or activity, altered satellite cell activity, or altered communication between these two subpopulations of cells (via MCP1 or other factors) could significantly contribute to the enhanced recovery observed with the repeated-bout effect. Our data suggest that more satellite cells express MCP1 in subjects demonstrating faster recovery from eccentric exercise (24).

Table 4. Quantification of immunohistochemistry results

<table>
<thead>
<tr>
<th></th>
<th>Fibers or Nuclei With Positively Stained Areas</th>
<th>Colocalization With MCP1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>SD</td>
</tr>
<tr>
<td><strong>Macrophages</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con1</td>
<td>16.3</td>
<td>7.9</td>
</tr>
<tr>
<td>Con2</td>
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</tr>
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<td>Ecc1</td>
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</tr>
<tr>
<td>Ecc2</td>
<td>58.6</td>
<td>22.0</td>
</tr>
<tr>
<td><strong>Satellite cells</strong></td>
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<td></td>
</tr>
<tr>
<td>PAX7</td>
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<td></td>
</tr>
<tr>
<td>Con1</td>
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</tr>
<tr>
<td>Con2</td>
<td>10.7</td>
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<td>16.0</td>
</tr>
</tbody>
</table>

Con1 and Con2, bouts 1 and 2 Con; Ecc1 and Ecc2, bouts 1 and 2 Ecc. There were no significant interactions (P > 0.05) between condition and bout. *Trend for greater increases in MCP1 colocalization with satellite cells in Ecc (P = 0.06).

We observed colocalization of the MCP1 protein with macrophages and satellite cells in our samples. On the basis of the increase in MCP1 mRNA expression specifically in the eccentrically exercised leg, we hypothesized that this leg would also demonstrate a greater level of macrophage and satellite cell colocalization. It is important to note that we did not expect a significant increase in the number of macrophages or satellite cells 6 h after exercise, inasmuch as the majority of macrophage infiltration from circulation and satellite cell proliferation/differentiation occurs at later time points. However, we could colocalize MCP1 protein to resident macrophages and satellite cells 6 h after exercise and try to relate the extent of colocalization at this time point to the transcriptional changes at the same time.

Overall, at 6 h after exercise, similar changes from bout 1 to bout 2 were seen in each condition (leg) for macrophage, MCP1, satellite cell, and colocalization levels. However, there was a trend toward a greater percentage of satellite cells expressing MCP1 after bout 2 (54%) than after bout 1 (29%) in the eccentrically exercised leg (P = 0.06), suggesting that satellite cells may be either “primed” to make MCP1 protein or more MCP1 protein is attracted to the satellite cells via chemotaxis.

Role of MCP1 in Adaptation?

These data demonstrate an upregulation of specific inflammatory components after the repeated bout. MCP1 has been demonstrated to be overexpressed at the mRNA and protein levels in animal studies and to be important for recovery. In the present study, we demonstrated a significant upregulation in MCP1 mRNA after a single exercise bout and an even greater upregulation after a second bout, with a trend toward greater numbers of satellite cells expressing MCP1 protein.

Macrophages communicate with other cell populations via chemokine (including MCP1) secretion. These other populations include satellite cells. Macrophage signaling has also been suggested to be important for muscle regeneration. Summan et al. (29) recently demonstrated that mice depleted of peripheral monocytes via liposomal clodronate treatment experienced decreased MCP1 expression and impaired recovery from freeze injury, suggesting that macrophage activity is integral to muscle recovery. It is thought that the positive effects of macrophages on skeletal muscle repair are mediated through communications between the macrophages and chemokine signaling targets. Satellite cell proliferation and differentiation are also vital to muscle regeneration (for review see Refs. 2, 9, and 12). Elimination of satellite cell activity via irradiation or depletion significantly impairs the ability of muscle to regenerate after eccentric exercise (24).

Although many studies have measured overall inflammation (i.e., swelling and loss of range of motion) and particular inflammatory components (i.e., inflammatory cell infiltration)
after eccentric exercise, muscle inflammation at the molecular level after eccentric exercise is not well characterized. The data from the present study point to a complex regulation of specific inflammatory factors as the muscle “adapts” to eccentric stimuli. The transcriptional upregulation of MCP1 after repeated exercise bouts and its localization within cells associated with muscle repair suggest that alterations in macrophage/satellite cell signaling may, in part, drive muscle adaptation. The specific function of MCP1 and other inflammatory factors in adapting skeletal muscle remains to be determined.

Summary

This study assessed inflammatory gene expression after two bouts of eccentric exercise in humans to delineate mechanisms of skeletal muscle adaptation. Faster strength recovery demonstrated the repeated-bout effect. Although seven of the eight genes tested were transcriptionally upregulated after bout 1 in the damaged vs. concentrically exercised control leg, only three (MCP1, CEBPD, and ZFP36) of the eight genes demonstrated differential transcriptional regulation after bout 2. These genes represent pro- and anti-inflammatory roles, and these data suggest that a targeted inflammatory response (i.e., upregulation of genes associated with repair), rather than a general up- or downregulation of overall inflammation, may underlie the repeated-bout effect. Especially important for adaptation may be an increase in MCP1 that colocalized with macrophages and satellite cells, which could play a role in promoting recovery.

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