Effects of eccentric exercise on toll-like receptor 4 signaling pathway in peripheral blood mononuclear cells

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The signaling pathways regulating the NF-κB are numerous. The NF-κB pathway is activated during exercise, and it is known that inflammation plays a critical role in the progression and recovery of muscle fiber injury after the initial mechanical insult (31). Several studies have investigated the pattern of different transcription factors involved in the inflammatory process following eccentric exercise. The factor that has received more attention is the nuclear transcription factor-κB (NF-κB), which is activated after an acute bout of eccentric exercise, both in human peripheral blood mononuclear cells (PBMC) (13, 16) and in rat skeletal muscle (24).

The signaling pathways regulating the NF-κB are numerous (2). Several of these pathways are regulated by the toll-like receptors (TLRs). TLRs are type I transmembrane glycoproteins mostly expressed on cells of the innate immune system, including macrophages, dendritic cells, B cells, and specific types of T cells, although they can also be expressed in nonimmune cells (e.g., fibroblast and epithelial cells) (1). They conciliate the recognition of pathogen-associated molecular patterns and coordinate inflammatory responses following pathogen incursion. Particularly, TLRs facilitate the recognition of pathogen subtypes, such as gram-negative and gram-positive bacteria, DNA and RNA viruses, fungi, and protozoa and, therefore, play an important role in the immune and inflammatory responses (14). One of these TLRs, TLR4, upon stimulation with lipopolysaccharide (LPS), in conjunction with CD14 (LPS receptor), mediates several processes in the inflammatory cascade, such as the production of tumor necrosis factor-α (TNF-α) and other inflammatory cytokines (1).

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established that eccentric exercise upregulates the activation of NF-κB in PBMC (13, 16).

Therefore, in an attempt to increase the knowledge on the inflammatory responses to exercise-induced muscle damage, this study was designed to pursue two objectives. First, we aimed to describe the PBMC TLR4 response to an acute bout of eccentric exercise in young male subjects. Since a similar bout of eccentric exercise has been shown to increase inflammatory mediators in human PBMC, like NF-κB, or IL-6 (13, 16), we hypothesized that acute eccentric exercise would provoke an upregulation of the TLR4 signaling pathway expression. Second, in an attempt to further understand the effects of eccentric training on inflammatory processes, we evaluated the influence of this training paradigm on the activity pattern of different molecules involved in the TLR4 signaling pathway after a repeated bout of acute eccentric exercise. Since a previous study from our laboratory already showed a reduced inflammatory profile, including decreased NF-κB activation, following eccentric training (13), it was hypothesized that expression of proteins involved in the TLR4 pathway would be reduced in PBMC after the acute bout that followed the eccentric training period.

MATERIALS AND METHODS

Subjects and procedures. Twenty sport science undergraduate male students volunteered for the study. To be eligible for the study, it was required that the subjects not be taking any medication known to affect their inflammation, hormonal, or metabolic response to exercise, be moderately active (3–5 h of exercise per week, mainly recreational team and ball sports), and not be involved in any kind of strength training for the last 6 mo. They had no previous muscle joint or bone injuries and were considered to be healthy individuals. Furthermore, subjects presenting symptoms of illnesses known to affect the inflammatory status at the onset of the study were excluded. The final 20 participants did not present any of the mentioned symptoms during the study period (assessed by individual interviews every 2–3 days). The study followed the principles of the Declaration of Helsinki, and all procedures were approved by the local ethics committee. Participants were informed of the purposes and possible risks involved in the study before giving their informed, written consent for participation. Subjects were randomly divided into two groups: a training group (TG, n = 12) and a control group (CG, n = 8). Descriptive characteristics before the first and the second acute eccentric bout are shown in Table 1. The study was completed in 11 wk, including pre- and posttraining descriptive and baseline data collection, training period, and pre- and posttraining testing. Each participant carried out two similar bouts of muscle-damaging eccentric exercise separated by 9 wk (including the 6-wk training period for the TG). During the performance of these acute eccentric bouts (pre- and posttraining, respectively), blood samples were collected for the inflammatory marker analysis.

Descriptive and baseline data collection. Descriptive and baseline data were collected during two laboratory sessions, carried out ~1 wk before the first and the second acute eccentric bouts. Height and weight were measured, followed by skinfold thickness assessment; body fat percentage was estimated using the six sites equations developed by Yuhasz (39) (Table 1). Next, the individuals carried out a standardized 8-min warm-up and then performed a maximal voluntary isometric contraction test (MVIC) in squat position, registered with a strain gauge (Globus Ergometer, Codogne, Italy), with 110° knee flexion, followed by a one repetition maximum (1RM) test, also performed in the squat movement. After 2–3 days, and in the second session, peak power was assessed in the squat movement. The peak power tests consisted of raising four loads, corresponding to 40 and 60% of the 1RM, and 40 and 60% of the MVIC, as fast as possible. Subjects performed a set of three repetitions with each load, and the best repetition was considered for further data analysis. Recovery between sets was 3 min. Loads were randomized for each subject. An encoder system (Globus Real Power, Codogne, Italy) placed next to the subject, with the sensor cable moving parallel to the movement of the subject, registered the time and the displacement of every repetition. Associated software (Globus Real Power version 3.11) was used to calculate peak power of every repetition. The tests carried out in the squat exercise were performed to assess any training effect in maximal strength or power performance, and to establish the loads that would be used during the eccentric damaging protocol and the eccentric training.

Eccentric-damaging protocol. This protocol was similar to the one described in García-López et al. (13). Briefly, it comprised 12 sets of 10 repetitions, using as eccentric movement the negative phase of the barbell squat. The load was equivalent to the 60% of the MVIC, and a 3-min rest period was permitted between sets. For safety reasons, a multipower apparatus (guided barbell squat exercise) was used (Salter, Barcelona, Spain). Thus, after each repetition, in which the subject only had to lower the imposed load from full extension (180°) to 90° knee flexion, two assistants raised the load up using a pulley system (11). Subjects were instructed to lower the load with the most comfortable velocity for them. Distance, time, and velocity of the vertical displacements of the barbell were registered through an encoder system (Globus Real Power). Emphasis was given by researchers such that the subjects could control the descent of the barbell, being able to stop the movement at the point equivalent to 90° knee flexion. The first and the second acute eccentric bouts were carried out 2 wk before the first training session and 1 wk after the last training session, respectively.

Muscular soreness. A visual analog scale (VAS) was used to assess soreness in the quadriceps muscle group. A 100-mm line with the legend “No pain” in the left border, and the legend “Unbearable pain” in the right limit was used. Subjects had to perform a squat movement without any additional weight and then draw a vertical line on the VAS representing their pain in the selected muscle group. Soreness scores were obtained before the two acute eccentric bouts and immediately, and 2, 24, 48, 72, and 96 h after the bouts. All subjects had been previously familiarized with the VAS.

Submaximal eccentric training. Subjects from TG attended 18 training sessions during 6 wk (3 sessions per week with a minimum of 48 h between sessions). Sessions started 2 wk after the first acute bout and...
finished 1 wk before the second acute eccentric bout. The same eccentric movement (eccentric barbell squat) and the same multipower device used during the damaging protocol were used during training sessions. The progression of the training was as follow: weeks 1 and 2: 3 × 10 and 5 × 10 at 40% MVIC, respectively; weeks 3 and 4: 3 × 10 and 5 × 10 at 45% MVIC, respectively; weeks 5 and 6: 3 × 10 and 5 × 10 at 50% MVIC, respectively. Knee flexion velocity was monitored during training sessions and used as real-time feedback to replicate mean velocity from the initial eccentric damaging exercise.

**Blood sample preparation.** Venous blood samples (30 ml) were taken using EDTA as an anticoagulant. Blood samples were obtained using Vacutainer (BD, Franklin Lakes, NJ) system from the brachiocephalic vein before, immediately after each eccentric bout, and at 2 h after cessation of exercise. The selection of these time points was based on a pilot study ($n = 5$), where peak mRNA expression of CD14 and TLR4 was found 2 h after an acute eccentric bout (CD14 mRNA abundance: 121% immediately, 139% 2 h, 85% 6 h, and 80% 24 h after the eccentric bout; TLR4 mRNA expression: 127% immediately, 147% 2 h, 89% 6 h, and 84% 24 h after the acute eccentric bout). PBMC were separated from the whole blood by density gradient centrifugation on Ficoll separating solution (Biochrom, Berlin, Germany) (9). For each sample, three 15-ml centrifuge tubes were used to layer 6 ml of blood onto 4 ml of Ficoll. The suspension was centrifuged for 30 min at 275 g at room temperature. The mononuclear cell layer was removed with manual pipetting, washed one time in Hank’s solution, and centrifuged for 10 min at room temperature and 450 g after the wash. Washed cells were resuspended in 1 ml of PBS. Analyses were performed on frozen cells, which had been stored at −80°C.

**Reverse transcription and quantitative real-time polymerase chain reaction.** Total RNA was isolated from PBMC using a RiboPure-Blood Kit (Ambion Life Technology, Grand Island, NY) and quantified by the fluorometric method Ribogreen RNA Quantitation Kit (Molecular Probes Life Technology, Grand Island, NY). Residual genomic DNA was removed by incubating RNA with DNase I RNase-free DNase (Ambion). First-strand cDNA was synthesized using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The negative control (no transcriptase control) was performed in parallel. cDNA was amplified using TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI 7000 (Applied Biosystems). TaqMan primers and probes for TLR4 (Genbank U98880.1 and Hs00370853_M1), CD14 (Genbank M86511.1 and Hs00169122_G1), TNF-α (Genbank M10998.1 and Hs00174128_m1), and the second acute eccentric bout, a two-way ANOVA with repeated measurements for group (TG and CG) and training (pretraining and posttraining). To compare differences within each group in the recovery after the eccentric bouts, data were analyzed using a two-way ANOVA with repeated measures for training (pre- and posttraining) and time (baseline, post, and 2 h after acute bouts for RT-PCR and Western blot data; and baseline, post, and 2, 24, 48, 72, and 96 h after acute bouts for soreness). To compare results between TG and CG after the first and the second acute eccentric bout, a two-way ANOVA with repeated measurements for group (TG and CG) and time (baseline, post, and 2 h after acute bouts for RT-PCR and Western blot data; and baseline, post, and 2, 24, 48, 72, and 96 h after acute bouts for soreness) was used. Bonferroni post hoc analysis was used where appropriate. A $P$ value of $<0.05$ was considered significant. SPSS version 17.0 statistical software (SPSS, Chicago, IL) was used.

**RESULTS**

**Maximal strength and peak power.** MVIC and 1RM ($P < 0.02$) and power ($P < 0.03$) (except at 60% of MVIC) increased in TG after the training program (Table 2). MVIC and power values (except power at 60% of 1RM) after the training period were higher in TG compared with CG ($P < 0.05$).

**Acute eccentric bouts and muscle soreness.** Mean distance values (cm) for the 120 repetitions during the first and second acute eccentric bouts were 27.4 ± 1.6 and 26.6 ± 1.4 cm, respectively, for TG, and 26.8 ± 1.3 and 27.3 ± 0.5 cm, respectively, for CG.

**Table 2.** Maximal dynamic and isometric strength and peak power at the four loads measured before and after training period for training group and control group

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<thead>
<tr>
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<th>Pre</th>
<th>Post</th>
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<tr>
<td></td>
<td>1RM, kg</td>
<td></td>
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<tr>
<td>TG</td>
<td>180 ± 5</td>
<td>194 ± 5#</td>
</tr>
<tr>
<td>CG</td>
<td>177 ± 7</td>
<td>181 ± 5</td>
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<tr>
<td>40% 1RM, W</td>
<td></td>
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<tr>
<td>TG</td>
<td>1,281 ± 47</td>
<td>1,459 ± 50#</td>
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<tr>
<td>CG</td>
<td>1,146 ± 26</td>
<td>1,168 ± 72</td>
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<tr>
<td>60% 1RM, W</td>
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<tr>
<td>TG</td>
<td>1,454 ± 54</td>
<td>1,590 ± 49#</td>
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<tr>
<td>CG</td>
<td>1,321 ± 57</td>
<td>1,387 ± 15</td>
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<tr>
<td>MVIC, N</td>
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<tr>
<td>TG</td>
<td>2,640 ± 91</td>
<td>2,849 ± 93#</td>
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<tr>
<td>CG</td>
<td>2,474 ± 164</td>
<td>2,364 ± 179</td>
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<tr>
<td>40% MVIC, W</td>
<td></td>
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<tr>
<td>TG</td>
<td>1,406 ± 49</td>
<td>1,589 ± 54#</td>
</tr>
<tr>
<td>CG</td>
<td>1,220 ± 52</td>
<td>1,343 ± 71</td>
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<tr>
<td>60% MVIC, W</td>
<td></td>
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<tr>
<td>TG</td>
<td>1,492 ± 61</td>
<td>1,628 ± 66*</td>
</tr>
<tr>
<td>CG</td>
<td>1,310 ± 32</td>
<td>1,318 ± 34</td>
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Values are means ± SD. Pre, before training period; Post, after training period. Maximal dynamic strength, 1 repetition maximum (RM); maximal isometric strength, maximal voluntary isometric contraction test (MVIC), *$P < 0.05$ vs. CG. $\#P < 0.05$ vs. Pre, value same group.
respectively, for CG. Mean velocity (m/s) was 0.172 ± 0.014 and 0.171 ± 0.026 m/s for the first and second acute eccentric bout, respectively, in TG, and 0.169 ± 0.012 and 0.165 ± 0.028 m/s in CG. No differences were evident between groups or within groups. Muscle soreness (Fig. 1) increased similarly in TG and CG after the first bout. CG presented significantly less muscle soreness at 24, 48, and 72 h after the second bout compared with the first one. TG showed a significant reduction in soreness in all time points after the second bout compared with the first bout and with CG.

**TLR4 signaling pathway.** CD14 and TLR4 mRNA levels (Fig. 2) increased \((P < 0.03)\) after both acute eccentric bouts for TG and CG, with no differences between bouts or groups. Protein levels of CD14 and TLR4 (Fig. 2) increased after the first acute bout in TG and CG \((P < 0.03)\). Although CG presented a significant decreased in TLR4 protein concentration 2 h after the second bout compared with the first one, this group still showed an upregulation of CD14 and TLR4 protein concentration after the second bout. Conversely, CD14 and TLR4 protein concentration was downregulated after the second bout in TG compared with the first eccentric bout and with CG \((P < 0.05)\).

MyD88, TRAF6, and TRIF protein expression (Fig. 3) increased after the first acute eccentric bout in both groups \((P < 0.02)\). After the second eccentric bout, CG showed an upregu-
tion of MyD88, TRAF6, and TRIF similar to that after the first bout. However, a significant downregulation of MyD88, TRAF6, and TRIF was observed in TG after the second acute eccentric bout compared with the first acute bout (except immediately after the bout for TRAF6) and with CG ($P < 0.05$).

pIKK and pIκB (Fig. 4) protein concentration increased immediately after the first acute eccentric bout and was maintained 2 h after in both groups ($P < 0.03$). After the second acute eccentric bout, an upregulation of pIKK and pIκB similar to the one described for the first bout was observed in CG. On the other hand, although pIKK was upregulated immediately after the second bout compared with basal values in TG, this group showed a clear downregulation of pIKK and pIκB after the second bout compared with CG and with the first acute eccentric bout ($P < 0.03$). The first acute eccentric bout triggered a significant ($P < 0.03$ immediately after and $P < 0.03$ 2 h after) upregulation of pIKK and pIκB in TG, while the second acute eccentric bout triggered a significant ($P < 0.03$ immediately after and $P < 0.03$ 2 h after) downregulation of pIKK and pIκB in TG.
0.01 2 h after) increase of p65 (Fig. 4) in TG and CG. After the second acute bout, CG still showed an upregulation of p65, while TG values were upregulated only 2 h after the second bout compared with basal values. Despite this upregulation, TG p65 levels were lower 2 h after the second bout \((P < 0.01)\) compared with the first bout and with CG.

The phospho-ERK-1/2 protein levels (Fig. 5) increased \((P < 0.03)\) in response to the first acute eccentric bout in both CG and TG. Similar results were observed for phospho-extracellular signal-regulated kinase (ERK)-1/2 after the second bout in CG. In contrast, phospho-ERK-1/2 was significantly reduced after the second bout in TG \((P < 0.01)\) compared with the first bout and with CG.

While TNF-\(\alpha\) mRNA levels (Fig. 6) increased after the first acute eccentric bout in both TG and CG \((P < 0.04)\), it increased only in CG \((P < 0.04)\) after the second bout. Furthermore, TG showed lower TNF-\(\alpha\) mRNA expression after the second acute eccentric bout compared with the first bout immediately and 2 h after, and with CG 2 h after the bout \((P < 0.05)\). TNF-\(\alpha\) protein concentration (Fig. 6) increased immediately \((P < 0.03)\) and 2 h \((P < 0.02)\) after the first acute eccentric bout in both TG and CG. After the second acute bout, CG showed an upregulated response \((P < 0.03)\) similar to the one registered after the first bout. Furthermore, TNF-\(\alpha\) protein concentration was lower in TG after the second eccentric bout compared with the first exercise bout and with CG \((P < 0.02)\).

**Discussion**

The present study investigated the effects of eccentric training on responses of the TLR4 signaling pathway to acute eccentric exercise in young men. As hypothesized, the results indicated that an acute bout of eccentric exercise provoked a proinflammatory response, which was associated with TLR4 signaling pathway activation. Furthermore, a 6-wk eccentric training program reduced the proinflammatory response to acute eccentric exercise through a downregulation of TLR4-NF-\(\kappa\)B pathway-associated factors.

CD4 and TLR4 mRNA expression and protein concentration suggested a proinflammatory response induced by the first acute eccentric exercise. This finding contrasts other investigations that reported a downregulated response of CD4-TLR4 after an aerobic exercise bout (21, 29, 35). However, while eccentric exercise has been described as a proinflammatory stimulus (31), aerobic exercise is often accompanied by a downregulation of immune and inflammatory responses (32). The idea that TLR4 pathway activation is dependent on factors such exercise modality, environmental conditions, and studied population has been already suggested elsewhere (27). However, a recent investigation carried out by the same group (4) found higher TLR4 activity in blood monocytes after acute strenuous bicycle exercise, which may indicate that intensity, rather than exercise modality, determines TLR4 activity.

The eccentric training did not influence CD4 and TLR4 mRNA levels. Since some studies have shown that CD4-TLR4 expression is downregulated after a training period in rodents (18, 40) and humans (12), it is important to highlight the different purposes of the previous studies (chronic responses) compared with the present investigation (acute response). On the other hand, eccentric training induced a reduc-
tion in protein concentration of CD14 and TLR4 in TG after the second acute eccentric bout compared with the first bout and with CG. Thus it appears that the eccentric training was the principal factor reducing CD14-TLR4 protein concentration. In support, another study (13) employing a similar protocol reported attenuated PBMC NF-κB activation in response to eccentric training. Thus eccentric training seems to induce anti-inflammatory adaptations after a repeated bout of eccentric contractions. The discrepancy between CD14 and TLR4 mRNA levels and protein concentration may indicate posttranscriptional adaptations (e.g., binding of micro-RNA) of CD14 and TLR4 in response to eccentric training (7).

Results from the present investigation showed that CD14-TLR4 activation through MyD88-dependent pathway plays an important role in exercise-induced inflammatory responses, since an acute eccentric exercise bout increased both MyD88 and TRAF6 protein expression in PBMC. This increase concords with results from adipose tissue in rats (33). In contrast, the overexpression of these proteins was lower in PBMC following the eccentric training program. Recent research has proved that MyD88 protein expression in PBMC from obese athletes remained unchanged after a 10-wk endurance training program (28). It is tempting to speculate that eccentric training represents a stronger stimulus for decreasing MyD88 protein concentration than aerobic training. However, since training population may also influence the training adaptations, research comparing the effect of different training programs on MyD88 activity in similar populations is warranted.

TRAF6 is a crucial molecule in the TLR4 signaling pathway, playing a critical role in the TLR-induced NF-κB pathway (22). It is known that cells isolated from TRAF6-deficient mice fail to activate NF-κB in response to IL-1 or LPS (19), and depletion of TRAF6 blocks the activation of both NF-κB and p38 mitogen-activated protein kinase (MAPK) in denervated skeletal muscle (30). The most frequently activated form of NF-κB is a heterodimer composed of p65 and p50, which is kept in the cytoplasm as a latent and inactive form by interaction with IκB proteins. Cell stimulation triggers the rapid phosphorylation of IκB proteins by a multiprotein complex termed the IKK complex, resulting in NF-κB dissociation and nuclear translocation (17). Biochemical evidence indicates that TRF6 possesses ubiquitin ligase activity that controls NF-κB (36), it is tempting to propose that the decrease in NF-κB activation through IKK activation and subsequent degradation of IκB (10). Our data revealed that increased expression of TRAF6 coincided with IKK and IκB phosphorylation and nuclear translocation of p65 subunits after an acute bout of eccentric exercise, which supports the role of TLR4 signaling in NF-κB activation. These effects were prevented by the 6-wk period of eccentric training.

CD14-TLR4 activation through MyD88-independent pathway was also involved in the inflammatory response after an acute eccentric bout and in the adaptations that followed eccentric training. Thus TRIF protein levels increased after the first acute eccentric bout, which may indicate that TRIF also recruits TRAF6, and that these molecules cooperate to facilitate NF-κB activation. Furthermore, the difference between TG and CG after the second bout of exercise showed that these effects were ameliorated by the eccentric training.

TNF-α is one of the principal proinflammatory cytokines and one of the NF-κB end-products (38). The upregulation of TNF-α mRNA expression found in our study supports data from other investigations in rats (23) and humans (5), emphasizing the systemic proinflammatory response after eccentric exercise (31). However, TG presented a reduction in TNF-α mRNA abundance after the second bout, which would indicate that the eccentric training protocol mediated the downregulation of TNF-α mRNA levels. Keeping in mind that the TLR4 signaling cascade is one of the principal pathways activating NF-κB (36), it is tempting to propose that the decrease in CD14, TLR4, and TRAF6 protein levels (via both MyD88-dependent or -independent pathway) leads to a reduction in NF-κB activation, resulting in a downregulation of TNF-α at both transcriptional and posttranscriptional levels. The observed TNF-α downregulation would ultimately suggest a reduction in the proinflammatory response associated with an acute eccentric exercise. However, it should be acknowledged that TNF-α is also regulated by other transcription factors, such as activator protein-1 (25), which is phosphorylated and activated through the MAPK signaling pathway. In addition, TRAF6 also plays an essential role in the activation of MAPKs, such as ERK, c-Jun N-terminal kinase (JNK), and p38 MAPK (p38) (17). Albeit caution is warranted when comparing studies analyzing different tissues, ERK-1/2 protein data reported here are supported by others (15), where multiple cardiac MAPKs (ERK-1/2, JNK-1/2, and p38) were activated by a single bout of exercise in untrained rats. Interestingly, MAPK activation in response to a single acute bout of exercise was abolished after 12 wk of training, which resembles the response observed in TG after the second eccentric bout. Thus these data imply that the effects of training on TNF-α expression in PBMC were also due, at least in part, to changes in the MAPK pathway.

One variable that may influence muscle damage and the subsequent inflammatory response to eccentric exercise is contraction velocity (6). Therefore, to reduce any confounding effects, contraction velocity was measured during the two acute eccentric bouts. No differences in contraction velocity were found between the first and the second acute eccentric bout, which suggest that both bouts were similar. Muscle soreness results supported the molecular data, indicating that eccentric training increased muscle damage protection if compared with the mere repetition of two acute eccentric bouts. Furthermore, the training protocol employed in the present investigation promoted increased maximal dynamic (1RM) and isometric strength and power, which is in accordance with several other studies (11, 13, 37).

In summary, this study examined the molecular inflammatory signaling associated with acute exercise and training-induced adaptations. Acute eccentric exercise increased TLR4-mediated NF-κB and MAPK activation, and TNF-α levels in PBMC, which suggests a proinflammatory response. In addition, a 6-wk eccentric training program reduced TLR4-mediated activation of the proinflammatory response through MyD88-dependent and -independent pathways.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).
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