Resistance exercise increases nuclear factor-kappa B activity in human skeletal muscle

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Running title – Activation of NF-κB by exercise
ABSTRACT

Intense resistance exercise causes a significant inflammatory response. Nuclear factor-kappa B (NF-κB) has been identified as a prospective key transcription factor mediating the post-exercise inflammatory response. The purpose of this study was to determine whether a single bout of intense resistance exercise regulates NF-κB signaling in human skeletal muscle. Muscle biopsy samples were obtained from the vastus lateralis of five recreationally active, but not strength trained males [21.9 ± 1.3 yr] prior to, and at 2 and 4 hours following, a single bout of intense resistance exercise. A further five subjects (4 males, 1 female) [23 ± 0.89 yr] were recruited as a non-exercise control group to examine the effect of the muscle biopsy protocol on key markers of skeletal muscle inflammation. Protein levels of IκBα and phosphorylated-NF-κB (p65), as well as the mRNA expression of inflammatory myokines MCP-1, IL-6 and IL-8 were measured. Additionally, NF-κB (p65) DNA binding to the promoter regions of MCP-1, IL-6 and IL-8, was investigated. IκBα protein levels decreased, while p-NF-κB (p65) protein levels increased 2 hours post-exercise and returned to near baseline levels by 4 hours post-exercise. Immunohistochemical data verified these findings, illustrating an increase in p-NF-κB (p65) protein levels, and nuclear localization at 2 hours post-exercise. Furthermore, NF-κB DNA binding to MCP-1, IL-6 and IL-8 promoter regions increased significantly 2 hours post exercise as did mRNA levels of these myokines. No significant change was observed in the non-exercise control group. This novel data provides evidence that intense resistance exercise transiently activates NF-κB signaling in human skeletal muscle during the first few hours post exercise. These findings implicate NF-κB in the transcriptional control of myokines known to be central to the post-exercise inflammatory response.

Key words – myokines, interleukin-6, interleukin-8, monocyte chemoattractant protein 1, tumor necrosis factor alpha, inflammation, biopsy control group.
INTRODUCTION

It is well established that intense muscular contractions common to resistance exercise subject myofibers to large mechanical forces with the capacity to cause muscle trauma (10, 37, 40, 43). Not surprisingly, this form of muscular injury can be acutely detrimental to force output, functionality and promotes sensations of pain (37, 40, 43). This process is characterized by a transient inflammatory response involving the activation and recruitment of immune cells, including neutrophils and macrophages, into the damaged tissue (10, 40). These mononuclear cells remove cellular debris from the injured area and release a series of cytokines and chemokines, which play a key role in facilitating processes involved in cellular inflammation and tissue repair (1, 7, 9). A considerable number of myokines have been identified in high concentrations in both in-vivo skeletal muscle (5, 31) and plasma (38) models following intense exercise, such as interleukin-1 (IL-1), IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) (for a comprehensive review see references (25, 35, 39). Despite the known link between resistance exercise and skeletal muscle inflammation, the transcription factors governing this response are not well defined.

Nuclear factor-κB (NF-κB), a pleiotropic transcription factor, acts as a central regulator of stress signaling pathways and has been identified as a potential regulator of exercise-induced inflammation (2, 3, 6, 8, 13, 18, 34). The responsiveness of NF-κB to cellular stress suggests an involvement in the post-exercise inflammatory cascade. In mammalian tissue, the Rel/NF-κB family of eukaryotic transcription factors is composed of five structurally related subunits. RelA (p65), cREL, RelB contain transactivation domains and are thus capable of initiating gene transcription, while p50/p105 and p52/p100 are typically considered repressors of gene activation due to the absence of a transactivation domain (3, 36). The classical and most common member of the NF-κB family is the p65/p50 heterodimer, with both subunits capable of inducing DNA binding, but only the p65 subunit is capable of initiating transcription (2, 36). Under basal conditions NF-κB remains sequestered within the cytoplasm in an inactive complex bound to the inhibitor protein IκB (13, 29, 46). Upon stimulation the IκB kinase (IKK) complex regulates the degradation of IκB proteins via phosphorylation and subsequent polyubiquitination, permitting the nuclear localisation of NF-κB subunits.
Transcriptional activity of NF-κB is a highly complex and tightly regulated phenomenon which can be induced by over 150 currently identified stimuli (36). Acute exercise has consistently been shown to trigger a transient rise in various components of the NF-κB signaling pathway in rodent skeletal muscle in vivo (19, 20, 22, 26, 41). Studies using human peripheral blood lymphocytes support the findings of rodent studies in identifying exercise as a potent stimulus for NF-κB activation in response to both continuous and high intensity intermittent exercise protocols (12, 17, 23, 24, 45). Remarkably our understanding of the exercise-induced regulation of NF-κB in human muscle tissue is limited (14, 42). Further, previous studies have identified NF-κB binding domains on the promoter regions of IL-6, IL-8, and MCP-1, which are key pro-inflammatory myokines known to be up regulated following intense exercise (27, 28, 30). It has not been investigated whether the exercise-induced increase in these pro-inflammatory myokines is transcriptionally regulated by NF-κB.

To enhance our understanding of the resistance exercise-induced regulation of NF-κB activity in human skeletal muscle, the current study aimed to firstly, determine the abundance of key components of the NF-κB signaling pathway, including IκB and NF-κB (p65) phosphorylation, secondly, determine the DNA binding of NF-κB to the promoter regions of several proinflammatory myokines, IL-6, IL-8 and MCP-1 and finally, measure the gene expression of these myokines following exercise. We hypothesized that NF-κB signaling would increase following resistance exercise, and that this would be represented by reduced levels of IκB and increased NF-κB (p65) phosphorylation, NF-κB binding to the promoter region on genes coding IL-6, IL8 and MCP-1, and correspondingly the mRNA expression these target genes.
METHODS

Participant Characteristics

Ten recreationally active participants (exercise = 5 male; control = 4 male, 1 female) were recruited for this study (exercise: 21.9 ± 1.3 years, 179.4 ± 6.3 cm, 80.1 ± 9.8 kg and BMI 24.9 ± 2.5 kg.m$^{-2}$; control: 23 ± 0.89 years, 175.8 ± 5.14 cm, 74 ± 4.76 kg and BMI 23.8 ± 2.1 kg.m$^{-2}$). All participants completed a medical history questionnaire that was used to identify and exclude participants with a diagnosed condition or illness that would endanger participants during strenuous exercise.

Ethical Approval

Before the experimental procedures each subject received oral and written information about the experimental protocols before giving written consent to participate. All experimental procedures involved in the study were previously approved by the Deakin University Ethics Committee (DUEC). All participants were informed on the nature and possible risk of the experimental procedure before written informed consent was obtained. All muscle sampling procedures were performed in accordance with the Helsinki declaration and were formally approved by the DUEC.

Experimental Procedures

In the exercise group, five participants were required to complete a familiarization session at least seven days prior to completing the exercise trial. Participants undertook a 5-repetition maximum (5RM) test for three leg based exercises; the Smith-machine assisted squat, the leg press and the leg extension. This data was substituted into the validated Brzycki equations to determine the participants’ 1RM. On the day of testing participants reported to the laboratory in a 24 hour fasted state having abstained from caffeine, alcohol, tobacco and exercise. Each participant completed a single bout of intense resistance exercise. Following a brief warm-up, each participant completed three sets of 8-12 repetitions of three aforementioned leg based exercises at 80% of their 1RM. The exercises were completed as a circuit with 1 minute rest periods permitted between exercises and a 3 minute rest interval between sets. Muscle samples were obtained under local anesthesia (Xylocaine 1%) from the vastus lateralis of either leg using the percutaneous needle biopsy technique (4) modified to include suction (15). To minimize the potential for interference, serial biopsy samples were collected at least 2 cm from previous biopsy sites. Excised tissue was immediately immersed in liquid nitrogen.
and stored at -80°C until further analysis. Muscle biopsy samples were obtained prior to exercise and at 2 and 4 hours post-exercise.

To control for possible effects of the biopsy procedure five participants reported to the laboratory in a 24 hour fasted state having abstained from caffeine, alcohol, tobacco and exercise. Muscle samples were obtained at rest (0 hrs) and at 2 and 4 hours post the initial biopsy.

**Western Blot Analysis**

Samples were homogenized in 15 µl of lysis buffer (20 mM Tris-HCl, 5 mM EDTA, 10 mM Na-pyrophosphate, 100 mM NaF, 2 mM Na3VO4, 1% Igepal, 10 µg/ml Aprotinin, 10 µg/ml Leupeptin, 3 mM Benzamindine, 1 mM PMSF) per 1 mg of tissue sample. The lysate was rotated at 4°C for 60 minutes before being centrifuged at 13,000 rpm at 4°C for 10 minutes. Total protein concentration was identified using a BCA Protein Assay Kit (Pierce, Rockford, Illinois, USA) according to the manufacturer’s instructions. Protein samples (50-75 µg) were denatured in loading buffer and separated by 10% SDS-PAGE, and transferred to a nitrocellulose (IκBα) or PVDF (phosphorylated p65) membrane via wet transfer. Membranes were blocked for 90 minutes at room temperature in 5% cold fish gelatin in 10% (v/v) tris buffered saline with 0.1% Tween 20 (TBST) (IκBα) or 5% skim milk/TBST (NF-κB). Antibodies, IκBα and phosphorylated NF-κB p65 Ser 536 (Santa Cruz Biotechnology, Santa Cruz, California, USA) diluted in 5% skim milk/TBST were applied and incubated overnight at 4°C. Membranes were subsequently washed with TBST and incubated for 1 hour at room temperature with HRP-conjugated anti-rabbit diluted in 5% skim milk/TBST (Chemicon, Melbourne, Australia). Proteins were visualized by enhanced chemiluminescence (Perkin Elmer Lifesciences, Boston, MA, USA) using a Kodak Digital Science Image Station 2000M (Model: 440CF, Eastman Kodak Company, USA) and quantified by densitometry software (Version 4.0.5, © 1994-2005 Eastman Kodak Company USA).

**Immunofluorescence**

Immunofluorescence was performed as described previously (44). Antibodies, phosphorylated NF-κB p65 Ser 536 (Cell Signaling Technology, Danvers, MA, USA) and desmin (Sigma, St. Louis, MO) were diluted in 3% (w/v) bovine serum albumin (BSA) in
phosphate buffered saline (PBS) overnight at 4°C. Sections were incubated with Alexa Fluor
goat anti-mouse 488 and Alexa Fluor donkey anti-rabbit 594 (Invitrogen, Australia) diluted in
3% BSA/PBS. Nuclei were stained by incubating with the DNA binding dye, Bisbenzimide
Hoechst 33285 (Sigma, St Louis, MO) for 10 min, followed by three washes for 5 min each.
Fluorescence was viewed on an Olympus IX70 fluorescent microscope (Olympus, Australia)
and digital images were then collected (Spot RT slide camera; Magna-Fire Software).

Electrophoretic Mobility Shift Assays (EMSA)

Nuclear protein was extracted from 30 mg of muscle tissue using NE-PER® Nuclear and
Cytoplasmic Extraction Reagents (Pierce, Rockford, Illinois, USA) according to the
manufacturer’s instructions. A Western blot analysis probing for α-tubulin was performed to
ensure there was no contamination of cytosolic protein within the nuclear extract. To
establish downstream targets of NF-κB p65, biotinylated DNA probes were designed to
specifically target the NF-κB binding sites on the promoter regions of IL-6, IL-8 and MCP-1
(Table 1). The NF-κB binding sites for these targets were identified using Genomatix
software (Genomatix Software Inc., Minnesota, USA) (http://www.genomatix.de/). EMSA
was performed as per manufacturer’s instructions (Panomics Fremont, California, USA).
Nine micrograms (2 µl) of nuclear protein was incubated with 2 µl of 5x binding buffer, 1 µl
of poly d(I-C) (1 µg/µL), and 1 µl of biotinylated transcription factor probe for 30 min at
15°C. To confirm specificity of the DNA binding, competition assays were performed with a
100x concentrated non-biotinylated DNA probe and a mutated DNA probe.

A 10% non-denaturing polyacrylamide gel was pre-run in 0.5x TBE (Tris/Borate/EDTA) for
10 min at 120 V. Samples containing loading buffer were loaded and separated at 4°C for 55
min at 120 V. Proteins were transferred onto a Biodyne Nylon Membrane (Pall Life Sciences
Corporation, Pensacola, Florida, USA) via wet transfer in 0.5x TBE for 30 min at 300 mA.
The oligonucleotides were fixed on the membrane using a UV crosslinker (Vilber Lourmat,
France) for 10 minutes. Membranes were blocked in 20 ml 1x blocking buffer (Panomics,
Fremont, California, USA) for 15 min with gentle agitation. Subsequently 1 ml of the
blocking buffer was removed and vortexed with 20 µl of streptavidin-HRP (Panomics,
Fremont, California, USA) for 10 s and returned to the membrane at room temperature for 15
min. Membranes were washed three times with 20 ml of a 1x wash buffer (Panomics,
Fremont, California, USA) for 8 minutes and then incubated in 20 ml of 1x detection buffer
(Panomics, Fremont, California, USA) for 5 minutes at room temperature. The blot was
visualized by exposing the membrane to 2 ml of chemiluminescence working substrate solution (Panomics, Fremont, California, USA) for 5 min. Membranes were exposed for 60 minutes and images obtained using a Kodak Digital Science Image Station (Model 440CF, Eastman Kodak Company, USA). Densitometry readings were obtained as described above.

**RNA Extraction and RT-PCR**

Total cellular RNA was extracted as previously described (44), using the ToTALLY RNA Kit (Ambion, Austin, TX). RNA quality and concentration was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). First-strand cDNA was generated from 0.5 µg total RNA using the AMV RT kit (Promega, Madison, WI). RT-PCR was performed using the Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). PCR was performed in duplicate with reaction volumes of 20 µl, containing SYBR Green 1 (Applied Biosystems, Foster City, CA), forward and reverse primers and cDNA template (1.25 ng/µl). Data were analyzed using a comparative critical threshold (Ct) method where the amount of target normalized to the amount of endogenous control relative to control value is given by $2^{-\Delta\Delta Ct}$. The efficacy of GAPDH as an endogenous control was examined using the equation $2^{-\Delta Ct}$. It was considered an appropriate control for this study when no changes in the expression of the gene were observed (data not shown). Primers were designed using Primer Express software package version 3.0 (Applied Biosystems, Foster City, CA) from gene sequences obtained from GenBank (Table 2). Primers were designed spanning intron-exon boundaries to prevent amplification of the target region for any contaminating DNA. Primer sequence specificity was also confirmed using BLAST. A melting point dissociation curve was generated by the PCR instrument for all PCR products to confirm the presence of a single amplified product.

**Statistical Analysis**

Data obtained from the resistance exercise trial and in the biopsy control group was expressed as mean ± SD. For the exercise group and the biopsy control group statistical significance was determined via a one-way ANOVA followed by a Tukey’s multiple comparison post-hoc. $P <0.05$ was considered statistically significant.
RESULTS

NF-κB subunit p65 is phosphorylated via IκBα degradation pathway following resistance exercise

To examine NF-κB activation following resistance exercise, p65 phosphorylation and IκBα protein levels were measured in vastus lateralis samples from human subjects following a single bout of resistance exercise. Protein expression of the NF-κB inhibitory protein IκBα was significantly reduced 0.6 fold 2 hours post exercise (P<0.05; Fig. 1A) and returned to basal levels by 4 hours post exercise. Phosphorylated NF-κB subunit p65 (Ser 536) protein expression was increased significantly 7.3 fold at 2 hours post exercise (P<0.05; Fig. 1B) and also returned to resting levels by 4 hours.

To further explore NF-κB activation, the cellular localization of p65 protein was examined in muscle tissue sections collected at rest and 2 hours post exercise. Prior to exercise, minimal fluorescence was evident for phosphorylated p65 (Ser 536) in the tissue samples (Figure 2A). Following acute exercise, phosphorylated p65 was evident and co-localized with many of the nuclei (Figure 2B).

NF-κB DNA binding to promoter regions of inflammatory myokines following resistance exercise

To determine DNA binding activity of NF-κB following exercise, EMSA was performed. NF-κB binding to the promoter regions of genes coding for inflammatory myokines, IL-6 (Figure 3A), MCP-1 (Figure 3B) and IL-8 (Figure 3C) was significantly increased (P <0.05) at 2 hours post exercise and returned to basal levels by 4 hours. Competition assays were run using a non-biotinylated NF-κB (p65) probe and a biotinylated mutated probe to control for specificity. Further, as shown in Figure 3A, mutation of the DNA binding sites in the IL-6 probe inhibited the binding to the nuclear protein extracts.

Genes associated with NF-κB signaling are up regulated following acute exercise

We next sought to determine if the increased NF-κB activation was associated with a concomitant increase in the expression of potential downstream inflammatory genes, IL-6, IL-8 and MCP-1. In response to an exercise stimulus IL-6, MCP-1 and IL-8 mRNA expression was significantly increased 2 hours post exercise (P<0.01) (Figure 4). By 4 hours post exercise these mRNA increases were significantly lower than at 2 hours (P<0.05), but were still greater than the pre-exercise levels (P<0.05).
To confirm that the elevations in gene expression observed following exercise were not induced by the multiple needle biopsy procedure, we examined the mRNA expression of IL-6, MCP-1 and IL-8 in a resting muscle sample as well as in samples taken 2 and 4 hours after the first resting sample. No exercise was performed between biopsies (Figure 5). Analysis of the non-exercised control group was restricted to the mRNA expression of inflammatory myokines. In contrast to the large changes observed with exercise (IL-6 >400 fold, MCP-1 >35 fold, IL-8 > 30,000 fold at 2 hours) the biopsy protocol did not yield any significant increases in the mRNA expression of IL-6 (1.45 fold at 2 hours, 6.53 fold at 4 hours), MCP-1 (1.35 fold at 2 hours, 5.94 fold at 4 hours) or IL-8 (1.7 fold at 2 hours, 2.68 fold at 4 hours). Further, the female subject that participated in this trial did not differ from the male counterparts with respect to gene expression following the repeated biopsy protocol.
DISCUSSION

Inflammation is a well described pathological event, which plays an essential role in skeletal muscle repair following exercise-induced tissue injury (37, 40, 43). Despite the well documented importance of post-exercise inflammation, the molecular mechanisms that mediate this response remain poorly understood. NF-κB lies at the nexus of multiple signaling pathways involved in inflammation and protein turnover, and has been the target of much recent research as a potential transcription factor mediating physiological responses to exercise. Whilst a considerable body of literature is accumulating supporting the up-regulation of NF-κB following exercise in rodent models, surprisingly, this is yet to be consistently observed in human trials. Thus, the aim of the current study was to investigate the influence of an acute bout of resistance exercise on key components of the NF-κB signaling pathway in human skeletal muscle tissue. This research further provides insight into the consequential downstream targets of NF-κB in response to an exercise stimulus.

The present study demonstrates several new and important findings regarding the regulation of NF-κB following acute resistance exercise. Firstly, we show for the first time that an acute bout of high-intensity resistance exercise is sufficient to induce a transient increase in phosphorylated p65 protein expression and a concomitant decrease in IκBα protein expression at 2 hours post exercise. Immunohistochemical analysis demonstrated that p65 was predominately localized to the nucleus at 2 hours post exercise implicating a role for NF-κB in post exercise gene expression. Subsequently, we explored the activity of potential p65 target genes within exercise-induced muscle damage, specifically identifying p65 as a transcriptional regulator of proinflammatory myokines MCP-1, IL-6 and IL-8 via a series of EMSAs. Using Genomatix promoter identification software we identified specific κB binding sites on the promoter region of genes coding MCP-1, IL-6 and IL-8. This data revealed for the first time that NF-κB binding to the promoter region on all three respective genes increased significantly at 2 hours post exercise and returned to basal levels by 4 hours. The mRNA expression of MCP-1, IL-6 and IL-8 exhibited a similar trend, showing a large increase at 2 hours post exercise, and modest increases at 4 hours. Collectively, these findings present the first comprehensive investigation of the regulation of NF-κB following acute exercise and establish this transcription factor as a key regulator of post-exercise inflammatory myokine gene expression.
Previous literature describing the regulation of NF-κB following acute exercise is remarkably sparse in human skeletal muscle tissue. In contrast to the present findings, Durham et al. (2004), also in healthy young participants, observed significantly reduced NF-κB binding to nuclear protein at 10 minutes post exercise and no change at 60 minutes post exercise (14). The discrepancies that exist between this model of research and the current findings may provide further insight into the regulatory patterns of NF-κB following exercise. A pertinent difference between the two exercise studies is the time course of muscle biopsy sampling. The 2 hour time point we employed was specifically used to coincide with peak concentrations of inflammatory myokines and resultant neutrophilia (16, 39, 43). This may suggest that muscle biopsies at 10 minutes and 1 hour post exercise may have been too early to observe an increase in key markers of the NF-κB signaling pathway.

More recent research supports this premise, identifying an increase in p50/p65 DNA binding activity at 210 minutes after a moderate intensity cycling bout, whilst no change was observed in total IκBα, IκBβ, p50 or p65 protein expression (42). Preceding an increase in NF-κB DNA binding activity Tantiwong et al., (2010) demonstrated an increase in the mRNA expression of inflammatory cytokines MCP-1 and IL-6 at 40 minutes post exercise (42), suggesting that the regulation of these cytokines following exercise occurs in an NF-κB independent manner. Contrastingly the present study suggests IL-6, IL-8 and MCP-1 act downstream of NF-κB, identifying these myokines as key transcriptional target genes of NF-κB following acute resistance exercise. These findings establish an interesting possibility; previous research has identified inflammatory myokines as both key activators and downstream target genes of the NF-κB signaling pathway (36). Therefore, it is possible that NF-κB and inflammatory myokines may coexist as part of a positive-enhancer loop activated under conditions of acute cellular stress and inflammation. Future research needs to determine whether these molecules can also act to activate key components within the NF-κB signaling pathway in response to an exercise stress.

A further novel finding of this research is that the muscle biopsy protocol that we employed was not a sufficient stimulus to significantly increase inflammatory gene expression. Inflammation represents a common protective response to a cellular disturbance or stress. It is therefore widely speculated that the invasive nature of a muscle biopsy procedure may activate pathways involved in inflammation, establishing a confounding variable when analyzing the effect of an exercise stress on inflammatory gene expression and muscle...
Contrastingly a recent trial examined the effect of a single bout of concentric and eccentric exercise on inflammatory gene expression in human muscle tissue, and used the contralateral limb as a control, completing concentric contractions only (21). This research identified no significant change in the mRNA expression of key inflammatory genes including MCP-1 in the control limb. Findings from the current research support this premise and show for the first time no significant change in the mRNA expression of MCP-1, IL-6 and IL-8 in a non-exercise control group subject to three muscle biopsies within the same limb. As the change in protein expression of both IκBα and phosphorylated p-65 was transient in nature and returned to baseline levels at 4 hours following exercise, it was not necessary to include the measurement of these proteins in the non-exercise control group.

CONCLUSION

NF-κB has emerged as a transcription factor of pre-eminent importance in human physiology via its regulation of various biological pathways (36). Significant research efforts have yielded a considerable body of literature implicating a role for NF-κB in regulating the inflammatory response to various stressors. Continuing investigations are needed to discover a role for NF-κB in mediating the inflammatory and regenerative responses to exercise. The present research provides novel evidence demonstrating NF-κB as a key transcriptional regulator of the inflammatory response to acute resistance exercise. This research is the first to illustrate an increase in phosphorylated p65 protein expression and a concomitant decrease in IκBα protein expression in human skeletal muscle within the first 4 hours following acute resistance exercise. Further, this research shows for the first time that NF-κB regulates the expression of inflammatory myokines IL-6, IL-8 and MCP-1 following acute resistance exercise, identifying NF-κB is an important regulator of post exercise inflammatory pathways. The results from this work will inform future research aimed at understanding and manipulating the post-exercise inflammatory response to enhance processes in muscle regeneration. Future research needs to consider how modulating components of the NF-κB signaling pathway and cytokine gene expression influences post-exercise inflammation and skeletal muscle hypertrophy.
Figure Legends

**Figure 1. Activation of NF-κB subunit p65 and IκBα following resistance exercise**
Representative Western blots for (A) IκBα and (B) phosphorylated NF-κB subunit p65 (Ser 536) measured in muscle biopsy samples taken at rest, 2 and 4 hours post acute resistance exercise. Results are mean values ± SD. Significantly different from resting values, *P <0.05; n = 5 subjects per time point.

**Figure 2. Nuclear localization of phosphorylated NF-κB subunit p65 following acute exercise**
Immunohistochemical analysis of phosphorylated NF-κB subunit p65 (Ser 536) in human skeletal muscle at rest (A) and at 2 hours post exercise (B). Sections were probed with antibodies raised against phosphorylated p65 (Ser 536) (red) and desmin (green), while Bisbenzimide was used to stain the nuclei (blue). Note in Figure 2B that the co-localization of phosphorylated NF-κB subunit p65 (Ser 536) (pink) with several nuclei (blue) appears pink.

**Figure 3. NF-κB DNA binding is increased following resistance exercise**
NF-κB subunit p65 binding to the promoter of three downstream target genes IL-6 (A), MCP-1 (B) and IL-8 (C) at 2 and 4 hours post exercise. The graphs show NF-κB subunit p65 binding relative to binding at rest (n = 5). The representative blots in this diagram have been cut and reordered to assist with the visualization of the controls and the overall interpretation of the data. Results are mean values ± SD. Significantly different from resting values, *P <0.05.

**Figure 4. Increased expression of NF-κB target genes**
RT-PCR analysis of IL-6 (A), MCP-1 (B) and IL-8 (C) in skeletal muscle of young males at rest and 2 and 4 hours post exercise. The graphs show mRNA expression normalized to GAPDH, relative to resting values (n = 5). Results are mean values ± SD. Significantly different from resting values, *P <0.05. Significantly different from 2 hours, φP <0.05.
Figure 5. No change in expression of NF-κB target genes in repeat biopsy control subjects

RT-PCR analysis of IL-6 (A), MCP-1 (B) and IL-8 (C) in skeletal muscle of young males at rest and 2 and 4 hours following the initial biopsy. The graphs show mRNA expression normalized to GAPDH, relative to resting values (n = 5). Results are mean values ± SD.
ACKNOWLEDGEMENTS

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REFERENCES


Figure 5

A

IL-6 mRNA fold change from 0 hours values

B

MCP-1 mRNA fold change from 0 hours values

C

IL-8 mRNA fold change from 0 hours values
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<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Primer Sequence</th>
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<td></td>
<td></td>
<td>Forward</td>
</tr>
<tr>
<td>MCP-1</td>
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<td>TAA ACT TGG GGA ATT TAC AGA T</td>
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<td>NM_000600</td>
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<td>NM_000584</td>
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</table>

Promoter sequences on each gene were identified using Genomatix software (Genomatix Software Inc., Minnesota, USA) (http://www.genomatix.de/). The location of the NF-κB promoter sequence was established using Ensemble Genome software (http://www.ensembl.org.html).
Table 2. Summary of primer sequences for RT-PCR

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<td>IL-8</td>
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Primer sequences were designed using Primer Express version 3.0 software (Applied Biosystems) using sequences accessed through GenBank and checked for specificity using Nucleotide-Nucleotide Blast search.