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# Time course-dependent changes in the transcriptome of human skeletal muscle during recovery from endurance exercise: from inflammation to adaptive remodeling

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**Neubauer O, Sabapathy S, Ashton KJ, Desbrow B, Peake JM, Lazarus R, Wessner B, Cameron-Smith D, Wagner K-H, Haseler LJ, Bulmer AC.** Time course-dependent changes in the transcriptome of human skeletal muscle during recovery from endurance exercise: from inflammation to adaptive remodeling. *J Appl Physiol* 116: 274–287, 2014. First published December 5, 2013; doi:10.1152/japplphysiol.00909.2013.—Reprogramming of gene expression is fundamental for skeletal muscle adaptations in response to endurance exercise. This study investigated the time course-dependent changes in the muscular transcriptome after an endurance exercise trial consisting of 1 h of intense cycling immediately followed by 1 h of intense running. Skeletal muscle samples were taken at baseline, 3 h, 48 h, and 96 h postexercise from eight healthy, endurance-trained men. RNA was extracted from muscle. Differential gene expression was evaluated using Illumina microarrays and validated with qPCR. Gene set enrichment analysis identified enriched molecular signatures chosen from the Molecular Signatures Database. Three hours postexercise, 102 gene sets were upregulated [family wise error rate (FWER), $P < 0.05$], including groups of genes related with leukocyte migration, immune and chaperone activation, and cyclic AMP responsive element binding protein (CREB) 1 signaling. Forty-eight hours postexercise, among 19 enriched gene sets (FWER, $P < 0.05$), two gene sets related to actin cytoskeleton remodeling were upregulated. Ninety-six hours postexercise, 83 gene sets were enriched (FWER, $P < 0.05$), 80 of which were upregulated, including gene groups related to chemokine signaling, cell stress management, and extracellular matrix remodeling. These data provide comprehensive insights into the molecular pathways involved in acute stress, recovery, and adaptive muscular responses to endurance exercise. The novel 96 h postexercise transcriptome indicates substantial transcriptional activity potentially associated with the prolonged presence of leukocytes in the muscles. This suggests that muscular recovery, from a transcriptional perspective, is incomplete 96 h after endurance exercise involving muscle damage.

skeletal muscular transcriptome; endurance exercise; muscle damage; time course of recovery; adaptive remodeling

SKELETAL MUSCLE IS A REMARKABLE tissue capable of rapid and significant regeneration from and adaptation to various kinds of stress (5–7). Regular endurance exercise is one of the most powerful stimuli for metabolic and morphological adaptive responses in skeletal muscle, leading to improved performance capacity and numerous health benefits (6, 7, 26, 58). Reprogramming of gene expression and specific signaling pathways are critical for the structural remodeling and functional adjustment of skeletal muscle in response to exercise-induced physiological and biochemical stimuli (7, 13, 30, 33, 34, 36, 46, 63). Acute bouts of exercise induce signaling cascades and a transient transcriptional stress response of early response genes in the immediate hours after exercise, followed by a second increase in transcriptional activity that can last for several days (21, 30, 34, 63). Long-term phenotype adaptations after frequent bouts of exercise (i.e., exercise training) result from cumulative transcriptional regulation and subsequent translation of specific gene subsets (6, 7, 13, 26).

Over the past years, human studies using whole genome microarrays have provided valuable information on the complex molecular processes and signaling pathways underlying the phenotypic muscular adaptations in response to endurance exercise (27, 34, 49, 51). However, no data are available on the human skeletal muscle transcriptome beyond 48 h after exercise in general. Furthermore, to our knowledge, no research has examined global gene expression in skeletal muscle tissue beyond 24 h endurance exercise involving a significant muscle-damaging component. Inflammatory responses in skeletal muscle after eccentric exercise involving muscle damage are known to occur for days (43, 44, 57). Therefore, the paucity of data on muscular transcriptomic responses to muscle-damaging endurance exercise beyond 24 h postexercise prevents a thorough understanding of the cellular and molecular events occurring during later recovery phases.

The major aim of the current study was to investigate the time course of changes in the transcriptome of skeletal muscle during recovery from an experimental exercise trial consisting of 1 h of cycling immediately followed by 1 h of running. These transcriptomic changes were expected to be indicative of the molecular pathways that are activated and contribute to acute stress, recovery, and adaptive responses to endurance

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exercise involving a muscle-damaging component. Crucially, we aimed to extend upon previous investigations (27, 34, 49, 51) by exploring the time course of transcriptomic responses beyond 24 h, i.e., 3, 48, and 96 h postexercise in healthy, endurance-trained individuals. The rationale behind the selected endurance exercise protocol was that the underlying physiological and biochemical demands would elicit both muscular and systemic stress and subsequently induce muscle damage and a systemic inflammatory response, similar to short-distance triathlon or duathlon events (38, 56). Furthermore, we expected that the exercise-induced muscle damage would be followed by a transmigration of blood-borne neutrophils and macrophages into the exercised skeletal muscle (43, 44). On the basis of the concept that full muscular recovery even after moderate muscle damage in healthy and trained individuals can take days (44), we hypothesized that the time course-dependent transcriptomic responses to the endurance exercise trial would indicate a prolonged crosstalk between accumulated leukocytes and the muscle. To verify this hypothesis, we specifically aimed to identify coordinated changes in the expression of groups of genes (55) that are functionally related to inflammation, leukocyte transmigration to the muscle, and chemokine signaling. Furthermore, we specifically focused on the transcriptomic profile at 96 h postexercise, which would, hypothetically, reveal the signaling mechanisms involved in resolving muscular inflammation and adaptive remodeling of skeletal muscle tissue.

## MATERIALS AND METHODS

*Participants.* Eight healthy, endurance-trained men volunteered to participate in this study. The participants' characteristics were reported previously (40). Briefly, the study participants (age: 25.0 ± 4.1 yr; mean ± SD) had a relative peak oxygen consumption ($\dot{V}O_{2\,peak}$) of 56.3 ± 6.7 ml·kg$^{-1}$·min$^{-1}$, with a gas exchange threshold of 60.1 ± 4.5% of $\dot{V}O_{2\,peak}$. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Griffith University Human Research Ethics Committee (ethics no. MSC/16/10/HREC).

*Prescribed training period and preliminary testing.* All study participants had been engaged in regular endurance training (including cycling and running) for at least 1 year before their study participation. After study enrolment, the participants undertook a prescribed 6-wk training program (weekly net endurance exercise time of 6.6 ± 1.1 h/wk) to ensure a similar training status among them. This training program included cycle-to-run transition training for specifically preparing them to commence the 2-h endurance exercise trial in a competitive manner. After this training program, they performed a tapering period of 5 days, after which they abstained from any intense exercise for 48 h. Subsequently, the participants performed an incremental exercise test to volitional fatigue to determine their physiological characteristics on a cycle ergometer, as described previously (40).

*Study design.* Muscle and blood samples were taken 1 wk before the endurance exercise trial (pre-exercise; baseline) and then at 3, 48, and 96 h thereafter (postexercise). These sampling time points were chosen because they represent distinct time points of muscular recovery from stress responses to intense endurance exercise (34, 49), exercise-induced muscle damage (33), and leukocyte accumulation in skeletal muscle after eccentric exercise (43). In the week subsequent to the preliminary testing and before the pre-exercise sampling, the participants performed recovery training (that was of very moderate intensity and duration) including a 48-h resting period before the pre-exercise sampling to avoid any effects of the biopsies on their performance or physiological variables during the trial. After the exercise trial, they abstained from any exercise until the final sampling. To avoid circadian effects, all samples were taken between 1.00 and 1:30 PM.

*Standardization of diet.* For the 24 h preceding each sampling, including the day of the exercise trial, the participants followed a standardized diet, as reported previously (40). Briefly, the standardized diet consisted of ~9 g of carbohydrate (CHO)/kg body mass, ~1.4 g protein/kg body mass, and ~1.4 g fat/kg body mass and contained moderate amounts of antioxidants (~18 mg of vitamin C, ~6 mg of vitamin E), because very high doses of antioxidants may interfere with the physiological response of skeletal muscle to exercise (12). Approximately 2 h before the exercise trial, study participants consumed a breakfast (containing ~2 g of CHO/kg body mass). During the exercise trial, the participants ingested 3 ml/kg body mass of a beverage (Gatorade) containing 6% (or 60 g/l) CHO and ~2.3 mmol/l of sodium every 15 min (equivalent to 50 g of CHO/h). After the exercise trial, they consumed the same beverage in a volume equivalent to 150% of their pre- to postexercise loss of BM for rehydration. Within 1 h after completion of the trial, the participants consumed a standardized sandwich snack providing 621 kcal of energy, 55 g of CHO, 22 g of protein, and 33 g of fat.

*Experimental endurance exercise protocol.* As reported previously (40), the participants completed a laboratory based, highly controlled experimental exercise trial consisting of 1 h of continuous ergometer cycling immediately followed by 1 h of continuous treadmill running, both performed at a predetermined intensity. Briefly, this endurance exercise protocol was selected because of its underlying physiological and biomechanical demands, which induced moderate muscle damage and a systemic inflammatory response similar to that of short-distance triathlon (38) or duathlon events (56). The cycling bout was performed on a cycling ergometer at 105% of the power output attained at the gas exchange threshold as assessed by the initial incremental exercise test. For the running bout, performed on a motor-driven treadmill, the study participants were encouraged to select a running speed that approximated a 10-km time trial pace, and a lower limit of treadmill speed was imposed to ensure that the oxygen consumption values were not below those achieved during the preceding cycling bout. During the exercise trial, gas exchange was measured and global ratings of perceived exertion (RPE) using the Borg 6–20 scale were collected at 15-min intervals.

*Skeletal muscle biopsies and RNA extraction.* A minimally invasive, fine-needle microbiopsy technique was applied that allowed multiple samples over time with minimal discomfort for the participants (18). Muscle biopsies were taken percutaneously from the vastus lateralis muscle of the right thigh. The microbiopsies were performed using 14 gauge (20 mm throw length), spring-loaded biopsy needles (Quick-Core; Cook Medical, Australia) after subcutaneous injection of a local anesthetic (2% lignocaine) to anesthetize the overlying tissue. Serial muscle biopsies (i.e., pre-, and 3, 48, and 96 h postexercise) were collected from the same muscle but with a distance of at least 2 cm from the initial sampling site to prevent interference from the previous biopsy. To further reduce the risk of biopsy-induced local trauma, three muscle samples were acquired through a single skin puncture from the same biopsy site at each sampling time point. Thereby, a total of ~30–45 mg of tissue was obtained for each biopsy time point. The muscle tissue samples were immediately immersed in RNAlater RNA stabilization solution (Ambion, Applied Biosystems, Australia), and stored overnight (4°C). On the next day, fat and connective tissue were carefully removed, and the muscle tissue was minced using a sterile scalpel. Subsequently, the muscle tissue samples were transferred into a tube containing RNeasy Lysis Buffer RLT and beta-mercaptoethanol, homogenized by passing the tissue sequentially through sterile 18-, 21-, 23-, and 25-gauge needles using a sterile 2.5-ml syringe, and then treated with proteinase K (Qiagen, Doncaster, Victoria, Australia) for further lysis (10 min, 55°C). Subsequently, total RNA from skeletal muscle samples was

extracted by the use of the RNeasy Mini columns (Qiagen). RNA concentrations and estimation of purity were determined by measuring the absorbance of the eluate at 260 and 280 nm (Nanodrop, Thermo Scientific). One microgram of RNA was then reverse transcribed into cDNA using a first-strand cDNA synthesis kit (Thermo Scientific).

*Microarray gene expression measurement and statistical analysis.* RNA was reverse transcribed using a T7-promoter-oligo (dT) primer followed by the Ambion labeling procedure. Relative transcript abundance in the biotin-labeled cRNA samples was measured using Illumina HT12 v3 microarrays and processed using the Illumina (San Diego, CA) iScan platform. Data were exported after background correction in GenomeStudio software. Analysis was performed in the R (v 2.15.1) statistical environment. Background corrected raw data were quantile normalized using the Bioconductor Lumi package (version 2.10.0), and array quality was assessed using ArrayQualityMetrics. The 47,323 transcript probes on the array were nondifferentially filtered to remove 18,732 transcript probes that were below the threshold of reliable detection according to the default Illumina scanner software. Differential expression between the baseline values and each postexercise time point was tested using the Bayesian moderated *t*-statistic available in Limma in a paired design to account for within-subject correlation. All reported probe *P* values were adjusted to control the false discovery rate (FDR) to 0.05 within each comparison using the Benjamini-Hochberg method in Limma. These *P* values are presented for a number of comparisons without additional adjustment.

*Identification of coordinated changes in the expression of groups of functionally related genes by gene set enrichment analysis.* Gene set enrichment analysis (GSEA) (55) was used for detecting coordinated changes in the gene expression of groups of genes (i.e., gene sets) that are characteristic of a biological process, a cellular component, or a molecular function according to Gene Ontology (GO) terms (55). Using the GSEA algorithm, all analyzed genes were ranked in order of evidence (the signed moderated *t*-statistic from Limma) of most upregulated to most downregulated. It was tested whether prespecified sets of genes were enriched at the top (i.e., upregulated) or at the bottom (i.e., downregulated) of this ranked list. These gene sets were obtained from the Molecular Signatures Database (MSigDB) (http:/www.broadinstitute.org/gsea). To determine whether any of these gene sets showed significant differences between pre-exercise and one of the postexercise time points (i.e., whether a group of functionally related genes was significantly up- or downregulated), the GSEA permutation FWER (*P* value) was used to determine statistical significance. Gene sets were considered as significantly enriched at or below an FWER *P* value of 0.05. Other results identified by GSEA included the core enrichment of gene sets, consisting of a subset of genes that contributed most to the enrichment results.

*Heatmap generation.* The Institute of Genomic Research (TIGR) MultiExperiment Viewer (TMEV) software (50) was used to generate heatmaps illustrating potential relationships between the transcriptional activity in the muscle and exercise-induced muscle damage as indicated by plasma muscle damage markers myoglobin and creatine kinase (CK) activity [reported previously (40)]. Figure 4 shows a heatmap indicating the relative change in transcript abundance from baseline to 3 h postexercise for each subject. Differential expression data from the Illumina arrays were quantile normalized and colors assigned according to change in ($\log_2$) fold expression values +4 (red) to −4 fold (green). Individual subjects array data were then arranged from left to right, from those experiencing the least to the greatest degree of muscle damage as indicated by pre- to 3 h postexercise changes in plasma myoglobin concentrations.

*Quantitative real-time polymerase chain reaction validation of gene expression.* Eight candidate genes were selected for confirmation of differential gene expression with quantitative real-time, reverse transcriptase polymerase chain reaction (qPCR) using TaqMan gene expression assays and an Applied Biosystems StepOnePlus instrument (both, Applied Biosystems, Life Technologies). These genes were selected based on the following: *1*) substantial differential regulation as indicated by the microarray data and *2*) their potential relevance for exercise-associated phenotypic changes in skeletal muscle with a focus on mitochondrial biogenesis (47), muscular inflammation (14, 32, 57), stress response (32, 34, 36, 49), and tissue remodeling (33, 41, 49). Integrin beta 2 (CD18) (ITGB2), which is an adhesion molecule exclusively expressed by cells of the hematopoietic lineage, primarily neutrophils and monocytes/macrophages (17, 35), and CD68 molecule (CD68), which is a marker for M1 macrophages (14, 43, 57), were used as marker genes for leukocyte accumulation in skeletal muscle tissue. Predesigned primers for the TaqMan assays were used to analyze mRNA levels of the following target genes (official gene symbol and assay ID in parentheses): peroxisome proliferator-activated receptor γ, coactivator 1 α (PPARGC1A, Hs01016719_m1), jun B proto-oncogene (JUNB, Hs00357891_ms1), chemokine (C-C motif) ligand 2 (CCL2, Hs00234140_m1), heme oxygenase 1 (HMOX1, Hs01110250_m1), tissue inhibitor of metallopeptidases 1 (TIMP1, Hs00171558_m1), follistatin-like 1 (FSTL1, Hs00907496_m1), ITGB2 (Hs00164957), and CD68 (Hs02836816_g1). The reaction was prepared according to the Taqman Fast Advanced Master Mix protocol in a total volume of 10 μl with target gene primers and probe and endogenous control gene primers and probe in the same reaction (Applied Biosystems, Life Technologies). All samples were analyzed in triplicate. Quantitative PCR data was normalized as described by Vandesompele et al. (59). Using all pre- and postexercise samples of each study participant, cyclophilin (PPIA) and β-2-microglobulin (B2M) were evaluated among several potential control genes and validated as the most stably expressed endogenous control genes over time (stability value $M = 0.684$) by the use of the geNorm algorithm (59). Quantitative PCR data were then normalized to the geometric mean of these two endogenous control genes, and fold-changes in transcript abundance were expressed relative to pre-exercise (i.e., baseline) values.

*Statistical analysis.* All statistical analyses except for the microarray data (described above) were performed using PASW Statistics 17 (IBM SPSS). All nonmicroarray data were normally distributed as determined by the Kolmogorov-Smirnov test. In accordance with the paired-design statistical analysis of the microarray data, paired *t*-tests determined the significance of the change in the differential expression of the eight target genes measured by qPCR. Pearson correlations were used to examine significant relationships between the fold-changes of the expression of genes and plasma muscle damage

Table 1. *Physiological variables assessed during the experimental endurance exercise trial*

| | Phase |
|---|---|
| *Cycling phase* | |
| Mean oxygen consumption during the exercise trial, $ml \cdot kg^{-1} \cdot min^{-1}$ | 40.9 ± 7.5 |
| Percentage of peak oxygen consumption, % | 73.7 ± 11.0 |
| Mean heart rate during the exercise trial, beats/min | 162.8 ± 19.1 |
| Percentage of peak heart rate, % | 87.3 ± 10.3 |
| Mean power output during the exercise trial, W | 207 ± 37 |
| Percentage of peak power output, W* | 51 ± 5 |
| *Running phase* | |
| Mean oxygen consumption during the exercise trial, $ml \cdot kg^{-1} \cdot min^{-1}$ | 48.1 ± 5.2 |
| Percentage of peak oxygen consumption, % | 87.3 ± 8.1 |
| Mean heart rate during the exercise trial, beats/min | 177.6 ± 9.5 |
| Percentage of peak heart rate, % | 95.0 ± 4.4 |
| Mean treadmill (running) speed during the exercise trial, km/h | 11.8 ± 0.8 |

Values are presented as mean ± SD. Percentage of peak heart rate and peak oxygen consumption were eternined during the incremental spiroergometry testing.

markers, which have been reported previously (40). Statistical significance was set at $P < 0.05$.

## RESULTS

*Experimental exercise trial.* All study participants successfully completed the 2-h experimental exercise trial. The RPE (6–20 Borg scale) increased significantly ($P < 0.01$) from initial stages of exercise (RPE at 5 min; cycling: 12 ± 1; running: 14 ± 1) to the end of each exercise phase (cycling: 15 ± 2; running: 17 ± 1). Physiological variables assessed during the exercise trial, indicative of the exercise intensity and the demands of the endurance exercise protocol are summarized in Table 1.

*Microarray analysis.* After adjusting to maintain a false discovery rate of 0.05, microarray analysis revealed that a total of 109 individual transcripts were differentially altered at 3 h

Table 2. *Selected upregulated gene sets 3 h postexercise functionally related with leukocyte transmigration, immune and chaperone activation, or related to their identification of the occurrence of binding sites for transcription factors. Also presented is the time course of representative genes contributing to the core enrichment of these gene sets*

| Selected Upregulated Gene Sets 3 h Postexercise | | | Representative Genes Contributing to the Core Enrichment | | 3 h Postexercise | | 48 h Postexercise | | 96 h Postexercise | |
|---|---|---|---|---|---|---|---|---|---|---|
| Size | ES | FWER *P* value | Gene symbol | Gene name | Adjusted *P* value | FC | Adjusted *P* value | FC | Adjusted *P* value | FC |
| *KEGG Pathogenic escherichia coli infection [Overlapping gene sets: e.g., KEGG regulation of actin cytoskeleton (P = 5.11 e-15), and KEGG leukocyte transendothelial migration (P = 2.46 e-10)]* | | | | | | | | | | |
| 53 | 0.65 | 0.000 | TUBB6 | Tubulin, beta 6 | 0.083 | 1.8 | 0.923 | 1.1 | 0.515 | 1.5 |
| | | | TUBA1B | Tubulin, alpha 1b | 0.099 | 1.5 | 0.827 | 1.8 | 0.135 | 2.0 |
| | | | ACTB | Actin, beta | 0.106 | 5.4 | 0.828 | 0.5 | 0.385 | 3.6 |
| | | | ITGB1 | Integrin, beta 1 | 0.135 | 1.7 | 0.827 | 1.4 | 0.442 | 1.5 |
| | | | CTNNB1 | Catenin (cadherin-associated protein), beta 1, 88 kDa | 0.137 | 1.8 | 0.827 | 1.3 | 0.783 | 1.2 |
| | | | ACTG1 | Actin, gamma 1 | 0.140 | 2.9 | 1.000 | 1.0 | 0.556 | 1.8 |
| | | | RHOA | Ras homolog gene family, member A | 0.170 | 1.6 | 0.868 | 0.8 | 0.764 | 1.3 |
| | | | ROCK2 | Rho-associated, coiled-coil containing protein kinase 2 | 0.192 | 2.2 | 0.839 | 1.2 | 0.722 | 1.5 |
| *V$CREB Q2 [Genes with promoter regions matching annotation for cAMP responsive element binding protein 1 (CREB1)]* | | | | | | | | | | |
| 165 | 0.58 | 0.000 | PPARGC1A | Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha | 0.000 | 5.9 | 0.896 | 0.9 | 0.732 | 0.8 |
| | | | MAFF | V-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian) | 0.001 | 5.5 | 0.827 | 0.8 | 0.809 | 1.2 |
| | | | NR4A3 | Nuclear receptor subfamily 4, group A, member 3 | 0.004 | 4.4 | 0.827 | 0.8 | 0.837 | 0.9 |
| | | | FOS | FBJ murine osteosarcoma viral oncogene homolog | 0.004 | 6.7 | 0.909 | 1.1 | 0.757 | 1.3 |
| | | | ATF3 | Activating transcription factor 3 | 0.052 | 2.9 | 0.827 | 0.7 | 0.968 | 1.0 |
| | | | EGR1 | Early growth response 1 | 0.033 | 3.4 | 0.952 | 0.9 | 0.610 | 1.6 |
| *Response to biotic stimuli [Overlapping gene sets: e.g., KEGG Cytokine-cytokine receptor interaction (P = 9.25 e-10)]* | | | | | | | | | | |
| 89 | 0.58 | 0.000 | HSPH1 | Heat shock 105 kDa/110 kDa protein 1 | 0.020 | 3.6 | 0.893 | 0.9 | 0.838 | 1.1 |
| | | | HSPA1B | Heat shock 70 kDa protein 1B | 0.024 | 5.4 | 0.827 | 0.5 | 0.735 | 1.5 |
| | | | DNAJB4 | DnaJ (Hsp40) homolog, subfamily B, member 4 | 0.027 | 2.5 | 0.908 | 1.1 | 0.795 | 1.1 |
| | | | CXCL12 | Chemokine (C-X-C motif) ligand 12 | 0.046 | 2.3 | 0.833 | 0.8 | 0.483 | 1.6 |
| | | | DNAJB5 | DnaJ (Hsp40) homolog, subfamily B, member 5 | 0.109 | 2.6 | 0.828 | 0.7 | 0.878 | 1.2 |
| *TTCNRGNNNNTTC V$ Heat shock transcription factor (HSF) Q6 [Overlapping gene sets: e.g., V$HSF1 01, genes with promoter regions around transcription start site for HSF1 (P = 0 e0)]* | | | | | | | | | | |
| 53 | 0.65 | 0.000 | HSPH1 | Heat shock 105 kDa/110 kDa protein 1 | 0.020 | 3.6 | 0.893 | 0.9 | 0.838 | 1.1 |
| | | | HSPA1B | Heat shock 70 kDa protein 1B | 0.024 | 5.4 | 0.827 | 0.5 | 0.735 | 1.5 |
| | | | SERPINH1 | Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1 | 0.049 | 2.7 | 0.962 | 0.9 | 0.744 | 1.6 |
| | | | DNAJB5 | DnaJ (Hsp40) homolog, subfamily B, member 5 | 0.077 | 3.3 | 0.828 | 0.7 | 0.878 | 1.2 |
| *KEGG Antigen processing and presentation* | | | | | | | | | | |
| 72 | 0.58 | 0.000 | HSPA5 | Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa) | 0.005 | 2.7 | 0.827 | 1.8 | 0.616 | 1.4 |
| | | | HSPA1B | Heat shock 70 kDa protein 1B | 0.024 | 5.4 | 0.827 | 0.5 | 0.735 | 1.5 |
| | | | HSP90AA1 | Heat shock protein 90 kDa alpha (cytosolic), class A member 1 | 0.082 | 6.1 | 0.827 | 1.4 | 0.802 | 0.8 |
| | | | HSPA1L | Heat shock 70 kDa protein 1-like | 0.092 | 1.8 | 0.922 | 1.1 | 0.865 | 0.9 |
| | | | CTSL1 | Cathepsin L1 | 0.140 | 1.4 | 0.827 | 1.9 | 0.009 | 2.4 |
| *V$ Serum response factor (SRF) Q4 [Genes with promoter regions around transcription start site for SRF]* | | | | | | | | | | |
| 163 | 0.50 | 0.002 | FOS | FBJ murine osteosarcoma viral oncogene homolog | 0.004 | 6.7 | 0.909 | 1.1 | 0.757 | 1.3 |
| | | | THBS1 | thrombospondin 1 | 0.006 | 4.2 | 0.851 | 1.3 | 0.296 | 2.2 |
| | | | EGR1 | early growth response 1 | 0.033 | 3.4 | 0.952 | 0.9 | 0.610 | 1.6 |
| | | | SERPINH1 | serpin peptidase inhibitor, clade H (heat shock protein 47), member 1 | 0.049 | 2.7 | 0.962 | 0.9 | 0.744 | 1.6 |
| | | | JUNB | jun B proto-oncogene | 0.056 | 2.6 | 0.827 | 1.3 | 0.729 | 1.3 |
| | | | CTGF | connective tissue growth factor | 0.073 | 2.2 | 0.827 | 1.4 | 0.735 | 1.2 |

ES, enrichment score for the gene set (i.e., the degree to which this gene set is overrepresented at the top or bottom of the ranked list of genes in the expression dataset); FWER *P* value, familywise-error rate (i.e., a conservative estimated probability that the normalized enrichment score represents a false positive finding); FC, fold-change relative to baseline.

Table 3. *Selected upregulated gene sets 48 h postexercise functionally related with cytoskeleton dynamics including the time course of representative genes contributing to the core enrichment of this gene set*

| Selected Upregulated Gene Sets 48 h Postexercise | | | Representative Genes Contributing to the Core Enrichment | | 3 h Postexercise | | 48 h Postexercise | | 96 h Postexercise | |
|---|---|---|---|---|---|---|---|---|---|---|
| Size | ES | FWER *P* value | Gene symbol | Gene name | Adjusted *P* value | FC | Adjusted *P* value | FC | Adjusted *P* value | FC |
| *Leading edge [Overlapping gene sets: e.g., Cytoskeleton organization and biogenesis (P = 0 e0), KEGG Regulation of actin cytoskeleton (P = 1.4 e-3), and Regulation of Rho protein signal transduction (P = 6.55 e-15)]* | | | | | | | | | | |
| 39 | 0.59 | 0.006 | S100A11 | S100 calcium binding protein A11 | 0.648 | 1.1 | 0.827 | 2.4 | 0.121 | 2.0 |
| | | | FGD2 | FYVE, RhoGEF and PH domain containing 2 | 0.247 | 0.7 | 0.827 | 2.0 | 0.393 | 1.6 |
| | | | ITGA5 | Integrin, alpha 5 (fibronectin receptor, alpha polypeptide) | 0.208 | 1.3 | 0.827 | 1.6 | 0.556 | 1.3 |
| | | | MYO5A | Myosin VA (heavy chain 12, myoxin) | 0.741 | 0.9 | 0.827 | 1.7 | 0.281 | 1.7 |
| | | | S100A6 | S100 calcium binding protein A6 | 0.244 | 0.7 | 0.827 | 1.5 | 0.235 | 1.6 |
| | | | ITGB1 | Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12) | 0.135 | 1.7 | 0.827 | 1.4 | 0.442 | 1.5 |
| | | | MYH9 | Myosin, heavy chain 9, nonmuscle | 0.010 | 2.3 | 0.827 | 1.4 | 0.170 | 1.6 |

postexercise compared with pre-exercise (103 genes upregulated, 6 genes downregulated). No transcripts were significantly regulated from pre- to 48 h postexercise. From pre- to 96 h postexercise, 62 transcripts were differentially regulated (61 genes upregulated including the M2 macrophage gene CD163, one gene downregulated). The microarray data of the current study have been deposited in the NCBI Gene Expression Omnibus website (GEO, http://www.ncbi.nlm.nih.gov/geo) and are accessible through GEO Series accession number GSE43856.

*Enrichment of groups of functionally related genes determined by gene set enrichment analysis.* All significant differentially regulated gene sets between baseline and all three time points postexercise are summarized in Tables 5–7. At 3 h postexercise, GSEA identified 102 enriched gene sets with a FWER $P$ value $< 0.05$ (all gene sets were enriched for highly upregulated genes). Genes that contributed to the core enrichment of the top-ranked gene set (referred to as KEGG pathogenic *Escherichia coli infection*) at 3 and 96 h postexercise, overlapped with gene sets functionally related to the regulation of the actin cytoskeleton, leukocyte transendothelial migration, and/or gap junction communication. Between pre- and 48 h postexercise, there were only modest and nonsignificant changes in the differential expression of individual genes. However, GSEA revealed coordinated changes in the differential expression of groups of functionally related genes at 48 h postexercise, resulting in the significant (FWER, $P < 0.05$) enrichment of 19 gene sets (2 gene sets were enriched for upregulated genes and 17 gene sets for downregulated genes). At 96 h postexercise, 81 gene sets were significantly (FWER, $P < 0.05$) enriched (80 gene sets were enriched for upregulated genes, and 1 gene set was enriched for downregulated genes). Selected differently regulated gene sets at 3, 48, and 96 h postexercise including representative genes contributing to the core enrichment of these gene sets are shown in Tables 2–4.

*Quantitative PCR validation of the expression of selected genes.* To validate changes in transcript abundance within the microarray analysis, qPCR was used to assess the differential expression of eight representative genes, including PPARGC1A, JUNB, CCL2, HMOX1, TIMP1, FSTL1, ITGB2, and CD68. At 3 h postexercise the following genes were all upregulated: PPARGC1A (10.3-fold), JUNB (3.8-fold), CCL2 (5.0-fold), HMOX1 (5.1-fold), TIMP1 (2.3-fold), FSTL1 (1.6-fold), and CD68 (1.7-fold) (for all $P < 0.05$). Compared with pre-exercise, PPARGC1A significantly decreased 48 h (0.6-fold, $P < 0.05$) and 96 h postexercise (0.5-fold; $P < 0.05$). For TIMP1 and CD68, progressive increases were observed at 48 h postexercise (for both 3.5-fold) and at 96 h postexercise (4.4-fold, and 8.6-fold, respectively; for both $P < 0.05$). Furthermore, 96 h postexercise the transcription of the following genes was increased: CCL2 (2.8-fold; $P = 0.053$), HMOX1 (10.5-fold; $P < 0.05$), FSTL1 (3.6-fold; $P = 0.069$), and ITGB2 (5.5-fold; $P < 0.05$). The time course of the expression changes for these genes compared with the microarray data is shown in Figs. 1 and 2. These results indicated a firm consistency between qPCR and microarray data, which is also confirmed by the correlation between these two data sets. The Spearman coefficient for the correlation between qPCR and microarray data after log2 transformation on the mean differential expression of eight genes (including PPARGC1A, JUNB, CCL2, HMOX1, TIMP1, FSTL1, ITGB2, and CD68) at 3, 48, and 96 h after the exercise trial compared with baseline was 0.86.

*Associations between gene expression of CD68 and HMOX1 in skeletal muscle and plasma muscle damage markers.* The time course of inflammatory and muscle damage markers have been reported previously (40). A strong correlation was observed between the pre- to 3 h postexercise changes in plasma myoglobin concentrations, and the pre- to 48 h postexercise fold-change of CD68 gene expression in skeletal muscle ($r = 0.85$, $P < 0.01$) (Fig. 3). The pre- to 3 h postexercise change in plasma myoglobin concentrations also correlated with the fold-changes of muscular HMOX1 gene expression from pre- to 3 h ($r = 0.79$, $P < 0.05$) and from pre- to 48 h postexercise ($r = 0.79$, $P < 0.05$). Similarly, pre- to 48 h postexercise changes in the plasma CK activity correlated with the pre- to 48 fold-change of HMOX1 gene expression in muscle ($r = 0.86$, $P < 0.05$). Furthermore, the pre- to 3 h and the pre- to 48 h postexercise fold-changes of HMOX1 gene expression both correlated with the pre- to 48 h postexercise CD68 gene expression in skeletal muscle ($r = 0.96$, $P = 0.001$, and $r = 0.78$, $P < 0.05$, respectively).

## DISCUSSION

The present data provide comprehensive and novel insights into the time course-dependent changes of the skeletal muscular transcriptome after endurance exercise involving moderate muscle damage. These transcriptomic phases are indicative of the molecular pathways that are transcription-

Table 4. *Selected upregulated gene sets 96 h postexercise functionally related with proteolysis, inflammatory and immune responses, and remodeling of the extracellular matrix. Also presented is the time-course of representative genes contributing to the core enrichment of these gene sets*

| Selected Upregulated Gene Sets 96 h Postexercise | | | Representative Genes Contributing to the Core Enrichment | | 3 h Postexercise | | 48 h Postexercise | | 96 h Postexercise | |
|---|---|---|---|---|---|---|---|---|---|---|
| Size | ES | FWER *P* value | Gene symbol | Gene name | Adjusted *P* value | FC | Adjusted *P* value | FC | Adjusted *P* value | FC |
| | | | | *KEGG Lysosome* | | | | | | |
| 114 | 0.68 | 0.000 | CD68 | CD68 molecule | 0.355 | 0.7 | 0.827 | 4.1 | 0.002 | 5.9 |
| | | | CTSL1 | Cathepsin L1 | 0.140 | 1.4 | 0.827 | 1.9 | 0.009 | 2.4 |
| | | | NPC2 | Niemann-Pick disease, type C2 | 0.555 | 1.1 | 0.827 | 1.7 | 0.014 | 2.3 |
| | | | LIPA | Lipase A, lysosomal acid, cholesterol esterase | 0.225 | 1.4 | 0.936 | 0.9 | 0.035 | 2.9 |
| | | | CTSS | Cathepsin S | 0.973 | 1.0 | 0.827 | 1.3 | 0.068 | 1.9 |
| | | | CTSK | Cathepsin K | 0.335 | 0.8 | 0.827 | 0.6 | 0.128 | 1.7 |
| | | | | *Reactome signaling by platelet-derived growth factor (PDGF)* | | | | | | |
| 59 | 0.66 | 0.000 | COL1A2 | Collagen, type I, alpha 2 | 0.159 | 2.9 | 0.973 | 1.1 | 0.001 | 3.0 |
| | | | COL1A1 | Collagen, type I, alpha 1 | 0.221 | 1.9 | 0.827 | 2.4 | 0.003 | 11.9 |
| | | | COL5A2 | Collagen, type V, alpha 2 | 0.797 | 1.1 | 0.827 | 2.2 | 0.009 | 3.0 |
| | | | THBS2 | Thrombospondin 2 | 0.040 | 1.7 | 0.995 | 1.0 | 0.052 | 2.0 |
| | | | SPP1 | Secreted phosphoprotein 1 | 0.584 | 1.1 | 0.827 | 3.0 | 0.053 | 3.6 |
| | | | | *KEGG Extracellular matrix (ECM) receptor interaction* | | | | | | |
| 68 | 0.65 | 0.000 | COL1A2 | Collagen, type I, alpha 2 | 0.159 | 2.9 | 0.973 | 1.1 | 0.001 | 3.0 |
| | | | COL1A1 | Collagen, type I, alpha 1 | 0.221 | 1.9 | 0.827 | 2.4 | 0.003 | 11.9 |
| | | | COL5A2 | Collagen, type V, alpha 2 | 0.797 | 1.1 | 0.827 | 2.2 | 0.009 | 3.0 |
| | | | THBS2 | Thrombospondin 2 | 0.040 | 1.7 | 0.995 | 1.0 | 0.052 | 2.0 |
| | | | SPP1 | Secreted phosphoprotein 1 | 0.584 | 1.1 | 0.827 | 3.0 | 0.053 | 3.6 |
| | | | TNC | Tenascin C | 0.166 | 1.4 | 0.827 | 1.4 | 0.063 | 2.0 |
| | | | | *Immune system process* | | | | | | |
| 261 | 0.530 | 0.000 | THY1 | Thy-1 cell surface antigen | 0.713 | 1.1 | 0.827 | 2.6 | 0.001 | 4.6 |
| | | | ITGB2 | Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit) | 0.656 | 1.1 | 0.827 | 1.9 | 0.016 | 2.7 |
| | | | CCL2 | Chemokine (C-C motif) ligand 2 | 0.019 | 2.4 | 0.829 | 1.5 | 0.032 | 3.4 |
| | | | CTSS | Cathepsin S | 0.973 | 1.0 | 0.827 | 1.3 | 0.068 | 1.9 |
| | | | CCR1 | Chemokine (C-C motif) receptor 1 | 0.372 | 1.2 | 0.372 | 1.2 | 0.090 | 1.7 |
| | | | | *Inflammatory response* | | | | | | |
| 98 | 0.58 | 0.001 | CCL13 | Chemokine (C-C motif) ligand 13 | 0.188 | 1.5 | 0.827 | 1.4 | 0.003 | 2.4 |
| | | | AIF1 | Allograft inflammatory factor 1 | 0.296 | 0.8 | 0.827 | 1.6 | 0.058 | 2.2 |
| | | | CCR1 | Chemokine (C-C motif) receptor 1 | 0.372 | 1.2 | 0.372 | 1.2 | 0.090 | 1.7 |
| | | | S100A9 | S100 calcium binding protein A9 | 0.030 | 3.2 | 0.967 | 1.1 | 0.096 | 3.9 |
| | | | | *Proteolysis* | | | | | | |
| 156 | 0.54 | 0.001 | TIMP1 | TIMP metallopeptidase inhibitor 1 | 0.141 | 1.6 | 0.827 | 2.4 | 0.002 | 4.2 |
| | | | CTSS | cathepsin S | 0.973 | 1.0 | 0.827 | 1.3 | 0.068 | 1.9 |
| | | | UBE2C | ubiquitin-conjugating enzyme E2C | 0.231 | 1.3 | 0.827 | 1.5 | 0.114 | 2.0 |
| | | | | *Extracellular region* | | | | | | |
| 292 | 0.5 | 0.001 | EMILIN2 | elastin microfibril interfacer 2 | 0.206 | 1.3 | 0.827 | 1.9 | 0.001 | 3.2 |
| | | | COL1A2 | collagen, type I, alpha 2 | 0.159 | 2.9 | 0.973 | 1.1 | 0.001 | 3.0 |
| | | | PLA2G2A | phospholipase A2, group IIA (platelets, synovial fluid) | 0.056 | 1.6 | 0.827 | 2.3 | 0.002 | 4.7 |
| | | | TGFBI | transforming growth factor, beta-induced, 68 kDa | 0.124 | 1.6 | 0.827 | 1.9 | 0.005 | 3.4 |
| | | | COL5A2 | collagen, type V, alpha 2 | 0.797 | 1.1 | 0.827 | 2.2 | 0.009 | 3.0 |
| | | | VCAN | versican | 0.077 | 2.4 | 0.945 | 1.1 | 0.032 | 3.6 |
| | | | ECM1 | extracellular matrix protein 1 | 0.494 | 0.8 | 0.827 | 2.0 | 0.058 | 2.2 |
| | | | FSTL1 | follistatin-like 1 | 0.175 | 1.9 | 0.871 | 0.7 | 0.142 | 3.1 |
| | | | | *KEGG chemokine signaling* | | | | | | |
| 152 | 0.54 | 0.001 | CCL13 | chemokine (C-C motif) ligand 13 | 0.188 | 1.5 | 0.827 | 1.4 | 0.003 | 2.4 |
| | | | CCL2 | chemokine (C-C motif) ligand 2 | 0.019 | 2.4 | 0.829 | 1.5 | 0.032 | 3.4 |
| | | | CCR1 | chemokine (C-C motif) receptor 1 | 0.372 | 1.2 | 0.372 | 1.2 | 0.090 | 1.7 |
| | | | CCL18 | chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated) | 0.203 | 1.3 | 0.843 | 1.2 | 0.121 | 2.0 |

ally activated and potentially contribute to the early (3 h postexercise), mid-term (48 h), and late (96 h) responses to an acute bout of intense, prolonged exercise. The 3 h postexercise transcriptome indicates leukocyte transmigration into the muscle, immune and chaperone activation, and the activation of downstream targets of the cyclic AMP responsive element binding protein (CREB) 1. Furthermore, these data show that endurance exercise induces the in-

Table 5. *Top 50 of 102 differentially regulated gene sets in skeletal muscle 3 h after the experimental endurance exercise trial as identified by gene set enrichment analysis*

| Name of the Gene Sset | Size | ES | NES | FWER *P* Val. |
|---|---|---|---|---|
| *Positively regulated gene sets 3 h postexercise* | | | | |
| KEGG Pathogenic escherichia coli infection | 53 | 0.654 | 2.420 | 0.000 |
| V$ Cyclic AMP responsive element binding protein (CREB) Q2 | 165 | 0.529 | 2.376 | 0.000 |
| TTCNRGNNNNTTC V$ Heat shock transcription factor (HSF) Q6 | 89 | 0.580 | 2.363 | 0.000 |
| Response to biotic stimulus | 97 | 0.579 | 2.362 | 0.000 |
| Biocarta G1 pathway | 22 | 0.743 | 2.306 | 0.000 |
| V$ Activating transcription factor (ATF) 01 | 166 | 0.515 | 2.296 | 0.000 |
| KEGG antigen processing and presentation | 72 | 0.575 | 2.276 | 0.001 |
| ATPase activity coupled to transmembrane movement of ions | 20 | 0.762 | 2.274 | 0.001 |
| V$ Cyclic AMP responsive element binding protein (CREB) Q2 01 | 135 | 0.522 | 2.272 | 0.001 |
| V$ Serum response factor (SRF) Q4 | 163 | 0.505 | 2.252 | 0.002 |
| Reactome platelet degranulation | 71 | 0.564 | 2.249 | 0.002 |
| V$ Serum response factor (SRF) Q5 01 | 164 | 0.505 | 2.248 | 0.002 |
| V$ Serum response factor (SRF) Q6 | 177 | 0.501 | 2.246 | 0.002 |
| Endomembrane system | 197 | 0.494 | 2.245 | 0.002 |
| GCCATNTTG V$ Yin Yang (YY)-1 Q6 | 278 | 0.479 | 2.234 | 0.004 |
| KEGG Spliceosome | 120 | 0.514 | 2.218 | 0.004 |
| Reactome formation of tubulin folding intermediates by chaperonin containing t-complex (TriC) polypeptide (CCT) | 21 | 0.731 | 2.212 | 0.004 |
| Biocarta p53 hypoxia pathway | 22 | 0.714 | 2.21 | 0.004 |
| Reactome metabolism of proteins | 204 | 0.488 | 2.207 | 0.004 |
| YYCATTCAWW unknown | 113 | 0.518 | 2.207 | 0.004 |
| CAGCTTT, microRNA (MIR-)320 | 186 | 0.486 | 2.207 | 0.004 |
| Biocarta cellcycle pathway | 19 | 0.753 | 2.204 | 0.004 |
| V$ Activator protein (AP) 1 Q6 01 | 160 | 0.500 | 2.204 | 0.004 |
| V$ Yin Yang (YY)-1 01 | 164 | 0.500 | 2.201 | 0.005 |
| Antiapotosis | 105 | 0.524 | 2.200 | 0.005 |
| V$ Serum response factor (SRF) C | 154 | 0.495 | 2.200 | 0.005 |
| Reactome influenza life cycle | 134 | 0.508 | 2.197 | 0.005 |
| Vesicle membrane | 25 | 0.695 | 2.192 | 0.005 |
| Cytoplasmic vesicle part | 23 | 0.700 | 2.191 | 0.005 |
| ATACTGT, microRNA (MIR)-144 | 158 | 0.493 | 2.184 | 0.006 |
| V$ Transcription factor (TCF)-11 01 | 134 | 0.502 | 2.184 | 0.006 |
| Reactome intrinsic pathway for apoptosis | 28 | 0.66 | 2.183 | 0.006 |
| Cytoplasmic vesicle membrane | 23 | 0.7 | 2.177 | 0.007 |
| V$ NGFIC [(early growth response (EGR4)] 01 | 154 | 0.493 | 2.176 | 0.007 |
| Cellular macromolecule catabolic process | 90 | 0.531 | 2.175 | 0.007 |
| ATPase activity coupled to transmembrane movement of ions phosphorylative mechanism | 16 | 0.758 | 2.173 | 0.007 |
| GTPase activity | 88 | 0.533 | 2.168 | 0.007 |
| Reactome processing of capped intron-containing premRNA | 131 | 0.504 | 2.165 | 0.008 |
| Membrane coat | 16 | 0.759 | 2.165 | 0.008 |
| Reactome gene expression | 403 | 0.45 | 2.164 | 0.008 |
| Fatty acid metabolic process | 54 | 0.578 | 2.162 | 0.009 |
| V$ Cyclic AMP responsive element binding protein (CREBP)-1 Q2 | 151 | 0.491 | 2.161 | 0.009 |
| V$NFMUE1 Q6 | 167 | 0.487 | 2.152 | 0.012 |
| Hydrolase activity acting on acid anhydrides | 197 | 0.470 | 2.151 | 0.012 |
| Reactome hemostasis | 224 | 0.466 | 2.144 | 0.013 |
| CAATGCA, microRNA (MIR)-33 | 69 | 0.549 | 2.141 | 0.013 |
| V$ serum response factror (SRF) 01 | 42 | 0.602 | 2.141 | 0.013 |
| Reactome formation and maturation of mRNA transcript | 145 | 0.482 | 2.139 | 0.014 |
| Reactome regulation of gene expression in beta cells | 89 | 0.522 | 2.137 | 0.014 |
| V$ Signal transducer and activator of transcription (STAT) 5A 01 | 158 | 0.484 | 2.132 | 0.016 |
| Hydrolase activity acting on acid anhydrides | 197 | 0.470 | 2.151 | 0.012 |

Size, number of genes in the gene set after filtering out those genes not in the expression dataset; NES, normalized ES (i.e., the ES for the gene set after it has been normalized across analyzed gene sets).

creased transcription of follistatin-like 1 (FSTL1; 3 h postexercise), which has recently been identified as a myokine, potentially involved in vascularization and regeneration after muscle injury (42). The 48 h postexercise transcriptome suggests actin cytoskeleton remodeling. The late and so far unknown 96 h postexercise transcriptome, including increased transcript abundance of tissue inhibitor of metalloproteinase 1 (TIMP1), chemokine (C-C motif) ligand 2 (CCL2), and heme oxygenase 1 (HMOX1), indicates extensive remodeling of the muscular extracellular matrix (ECM), cellular stress management, and chemokine signaling. These sustained inflammatory processes in skeletal muscle 96 h postexercise might be related to the prolonged presence of leukocytes in the exercised muscles. Finally, the currently reported dataset suggests that the muscle damaging component of the endurance exercise trial may, in part, have been responsible for providing the stimulus for many of the observed transcriptional responses.

*Indications for leukocyte infiltration into skeletal muscle and early regulatory interactions between muscle and leukocytes.* In a complementary part of this study (40), our data suggested

Table 6. *All differentially regulated gene sets in skeletal muscle 48 h after the experimental endurance exercise trial as identified by gene set enrichment analysis*

| Name of the gene set | Size | ES | NES | FWER *P* val. |
|---|---|---|---|---|
| *Positively regulated gene sets 48 h postexercise* | | | | |
| Leading edge | 39 | 0.594 | 2.212 | 0.006 |
| Ruffle | 26 | 0.645 | 2.139 | 0.023 |
| *Negatively regulated gene sets 48 h postexercise* | | | | |
| Reactome electron transport chain | 61 | −0.698 | −3.145 | 0.000 |
| Reactome glucose regulation of insulin secretion | 133 | −0.569 | −3.014 | 0.000 |
| KEGG Parkinsons disease | 104 | −0.565 | −2.822 | 0.000 |
| KEGG Oxidative phosphorylation | 104 | −0.534 | −2.681 | 0.000 |
| Mitochondrial respiratory chain | 23 | −0.722 | −2.571 | 0.000 |
| Reactome regulation of insulin secretion | 168 | −0.474 | −2.563 | 0.000 |
| Reactome integration of energy metabolism | 189 | −0.470 | −2.519 | 0.000 |
| KEGG Huntingtons disease | 151 | −0.445 | −2.410 | 0.000 |
| Structural constituent of ribosome | 79 | −0.505 | −2.393 | 0.000 |
| KEGG Alzheimers disease | 139 | −0.454 | −2.381 | 0.000 |
| Mitochondrial part | 132 | −0.441 | −2.286 | 0.010 |
| Mitochondrial membrane part | 48 | −0.523 | −2.249 | 0.016 |
| Mitochondrial envelope | 87 | −0.466 | −2.248 | 0.016 |
| KEGG Cardiac muscle contraction | 56 | −0.505 | −2.215 | 0.023 |
| Mitochondrion | 311 | −0.380 | −2.209 | 0.024 |
| Mitochondrial inner membrane | 60 | −0.491 | −2.200 | 0.028 |
| Mitochondrial membrane | 78 | −0.456 | −2.194 | 0.033 |

that the transcriptional activation and priming of circulating neutrophils was, to a certain extent, induced by the muscle damaging component of the endurance exercise protocol. The current data of the skeletal muscle transcriptomic response provide several indications that blood-borne leukocytes, potentially including neutrophils, transmigrated and infiltrated into the muscle tissue during recovery from exercise. First, the top-ranked set of upregulated genes functionally related with actin cytoskeleton dynamics and with communication via gap junctions 3 h postexercise (Table 2) might reflect gap-junction coupling of leukocytes with endothelial cells, modulating their transendothelial migration into the muscle (65). Second, in accordance with recent studies (14, 17), leukocyte accumulation in exercised muscles was indicated by the gradually increased abundance of leukocyte-specific transcripts in muscle tissue (Fig. 2). These qPCR-validated surrogate genes were ITGB2 (also known as CD18), which is characteristic for neutrophils and macrophages (35), and CD68, which is a specific marker for M1 macrophages (14, 43, 57). Notably, the pre- to 3 h postexercise changes in plasma myoglobin correlated with the pre- to 48 h postexercise fold-change of CD68 gene expression in muscle tissue (Fig. 3), which may suggest that macrophage accumulation in the muscle was related to the extent of muscle damage. Plasma/serum markers of muscle damage do not always accurately reflect the degree of muscle damage (44), and muscle damage can vary because of factors including age, training status, hydration status, body mass, and genetic variability (22, 43, 44). In the present study, however, factors potentially influencing muscle damage markers were minimized (due to dietary standardization, a similar training status, and age among the participants, etc.) and largely limited to individual (i.e., genetic) variations. Furthermore, the relationship between increases in plasma myoglobin and the transcription of CD68 in muscle is in agreement with the proposed concept that leukocyte accumulation is a gradual process dependent on the extent of muscle damage (44). The information acquired from the heatmap (Fig. 4) showing the study participants ranked for pre- to 3 h postexercise plasma myoglobin changes vs. all significantly different regulated genes at 3 h postexercise also suggests that the transcriptional regulation of many of these genes was related to muscle damage.

The increased transcription of CCL2 (Fig. 2) and chemokine (C-X-C motif) ligand 12 (CXCL12) 3 h postexercise suggests two potential chemokine candidates that could have modulated early interactions between muscle and leukocytes (15, 57). CCL2 is expressed by both monocytes and injured muscle cells to assist in recruiting monocytes/macrophages and to support muscle repair (31). CXCL12 (also known as stromal derived factor-1, SDF1) and its receptor chemokine (C-X-C motif) receptor 4 (CXCR4) could play an important role in the migration of muscle cells during muscle regeneration and development (15). Moreover, CXCL12/CXCR4 signaling is proposed to regulate neutrophil homeostasis under stress conditions (10). Together with the paired neutrophil transcriptome data showing an increased transcription of CXCR4 in circulatory neutrophils at the same time point (3 h postexercise) (40), we speculate that this could be a potential mechanism by which neutrophils may engage in crosstalk with muscle tissue. Additional research is warranted to verify this hypothesis.

*Transcriptional immune and chaperone activation 3 h postexercise.* The observed coordinated upregulation of genes functionally related with antigen processing and presentation 3 h postexercise (Table 2) indicates early muscular inflammatory and immune responses involved in the breakdown and removal of damaged tissue through macrophages and/or muscle-endogenous proteolytic systems (2), similar to previous findings (30, 49). The concomitant enrichment of several heat shock protein (HSP)−70 members (e.g., HSPA5, HSPA1B) might reflect a mechanism for facilitating antigen presentation (28), but avoiding inappropriate proteolytic activity (52, 64). Additional stressors that could have induced HSP70 gene expression include hyperthermia, hypoxia, and oxidative stress (28). Moreover, the enrichment of HSPs with promoter regions for the heat shock transcription factor 1 (HSF1), including heat shock 105 kDa/110 kDa protein 1 (HSPH1) and HSPA1B (Table 2), suggests that HSF1 signaling was involved in their transcriptional activation. These data support the concept that chaperones contribute to the regulation of muscle repair and regeneration after mechanical stress (52, 64).

*Transcriptional activation of transcription factors and downstream targets of CREB-1 3 h postexercise.* Our endurance exercise protocol rapidly induced the transient transcriptional upregulation of several transcription factors 3 h postexercise. In accordance with previous studies, we observed the increased transcription of peroxisome proliferator-activated receptor γ coactivator α (PPARGC1A) (7, 34, 47), nuclear receptor subfamily 4 group A member 3 (NR4A3) (34), and the immediate early response genes FBJ murine osteosarcoma viral oncogene homolog (FOS), and jun B proto-oncogene (JUNB) (33, 48). The 5.9-fold increase in the transcription of PPARGC1A (Fig. 1) (the translated protein is known as PGC-1 α), a proposed "master regulator" of mitochondrial biogenesis (47) also contributed to the core enrichment of a set of genes

Table 7. *Top 50 of 80 positively regulated gene sets and the only negatively regulated gene set in skeletal muscle 96 h after the experimental endurance exercise trial as identified by gene set enrichment analysis*

| Name of the Gene Set | Size | ES | NES | FWER *P* val |
|---|---|---|---|---|
| *Positively regulated gene sets 96 h postexercise* | | | | |
| KEGG Pathogenic Escherichia coli infection | 53 | 0.753 | 2.690 | 0.000 |
| KEGG Lysosome | 114 | 0.676 | 2.665 | 0.000 |
| Reactome platelet degranulation | 71 | 0.671 | 2.521 | 0.000 |
| Reactome formation of platelet plug | 151 | 0.601 | 2.508 | 0.000 |
| Reactome platelet activation | 134 | 0.612 | 2.490 | 0.000 |
| Reactome hemostasis | 224 | 0.563 | 2.427 | 0.000 |
| Reactome signaling by platelet-derived growth factor (PDGF) | 59 | 0.658 | 2.378 | 0.000 |
| KEGG extracellular matrix (ECM) receptor interaction | 68 | 0.655 | 2.365 | 0.000 |
| Endoplasmic reticulum | 250 | 0.535 | 2.361 | 0.000 |
| Cellular cation homeostasis | 71 | 0.623 | 2.361 | 0.000 |
| Immune system process | 261 | 0.528 | 2.354 | 0.000 |
| Reactome further platelet releasate | 21 | 0.806 | 2.349 | 0.000 |
| Cation homeostasis | 73 | 0.617 | 2.347 | 0.000 |
| Chemical homeostasis | 106 | 0.583 | 2.346 | 0.000 |
| Peptidyl tyrosine modification | 21 | 0.773 | 2.338 | 0.001 |
| Leading edge | 39 | 0.694 | 2.336 | 0.001 |
| Defense response | 212 | 0.539 | 2.332 | 0.001 |
| Reactome platelet activation triggers | 47 | 0.666 | 2.329 | 0.001 |
| KEGG Leishmania infection | 62 | 0.632 | 2.316 | 0.001 |
| Ion homeostasis | 87 | 0.596 | 2.298 | 0.001 |
| Inflammatory response | 98 | 0.584 | 2.281 | 0.001 |
| KEGG Antigen processing and presentation | 72 | 0.611 | 2.277 | 0.001 |
| Immune response | 184 | 0.536 | 2.269 | 0.001 |
| Homeostatic process | 146 | 0.541 | 2.256 | 0.001 |
| Response to wounding | 142 | 0.543 | 2.255 | 0.001 |
| Proteolysis | 156 | 0.541 | 2.253 | 0.001 |
| Reactome neural cell adhesion molecule (NCAM) 1 interactions | 32 | 0.707 | 2.245 | 0.001 |
| KEGG Chemokine signaling pathway | 152 | 0.543 | 2.239 | 0.001 |
| Extracellular region | 292 | 0.503 | 2.237 | 0.001 |
| Reactome lysosome vesicle biogenesis | 21 | 0.760 | 2.235 | 0.001 |
| Ruffle | 26 | 0.748 | 2.234 | 0.001 |
| KEGG Fc gamma receptor-mediated phagocytosis | 86 | 0.574 | 2.234 | 0.001 |
| Actin cytoskeleton organization and biogenesis | 89 | 0.573 | 2.227 | 0.001 |
| Reactome signaling in immune system | 288 | 0.503 | 2.213 | 0.001 |
| Response to biotic stimulus | 97 | 0.558 | 2.213 | 0.001 |
| Response to external stimulus | 226 | 0.507 | 2.206 | 0.001 |
| Locomotory behavior | 66 | 0.600 | 2.203 | 0.004 |
| G protein coupled receptor binding | 35 | 0.672 | 2.198 | 0.005 |
| Peptidyl tyrosine phosphorylation | 19 | 0.773 | 2.198 | 0.005 |
| Cell migration | 67 | 0.585 | 2.180 | 0.008 |
| Chemokine receptor binding | 28 | 0.703 | 2.179 | 0.008 |
| Reactome the role of NEF in HIV replication and disease pathogenesis | 27 | 0.699 | 2.175 | 0.009 |
| Peptidyl amino acid modification | 46 | 0.628 | 2.168 | 0.009 |
| Cellular homeostasis | 99 | 0.555 | 2.167 | 0.009 |
| Cytoskeleton organization and biogenesis | 172 | 0.516 | 2.165 | 0.009 |
| Biocarta glycogen synthase kinase (GSK) 3 pathway | 26 | 0.703 | 2.164 | 0.009 |
| Endoplasmic reticulum part | 87 | 0.556 | 2.162 | 0.009 |
| Chemokine activity | 27 | 0.698 | 2.147 | 0.011 |
| KEGG focal adhesion | 175 | 0.509 | 2.146 | 0.012 |
| Actin filament based process | 97 | 0.551 | 2.144 | 0.012 |
| *Negatively regulated gene sets 96 h postexercise* | | | | |
| Reactome electron transport chain | 61 | −0.621 | −2.618 | 0.000 |

involved in lipid metabolism (Table 5). This finding agrees with the concept that a single bout of intense endurance exercise, performed by endurance-trained individuals with an already enhanced capacity for fat utilization, further stimulates transcriptional responses (7), which can promote phenotypic adaptations upon repeated exercise bouts (i.e., exercise training) (6, 13, 26, 46). Other upregulated transcription factors included early growth response 1 (EGR1) and v-maf musculoaponeurotic fibrosarcoma (MAF) oncogene homolog F (MAFF). MAFF is a member of the small MAF transcription factors, which are proposed as crucial regulators of stress signaling (25) but have not previously been reported in the context of exercise. PPARGC1A, NR4A3, FOS, EGR1, and MAFF all contributed to the core enrichment of a set of genes with promoter regions for CREB1 (Table 2). This suggests that CREB1 signaling, which has been implicated in skeletal muscle metabolism, regeneration, and adaptation (3) might have been involved in the activation of these transcription factors.

Furthermore, these data provide indications in support of the recently discovered endurance exercise-induced transcriptional activation of the actin-binding rho activating protein (ABRA), also known as striated muscle activator of Rho signaling

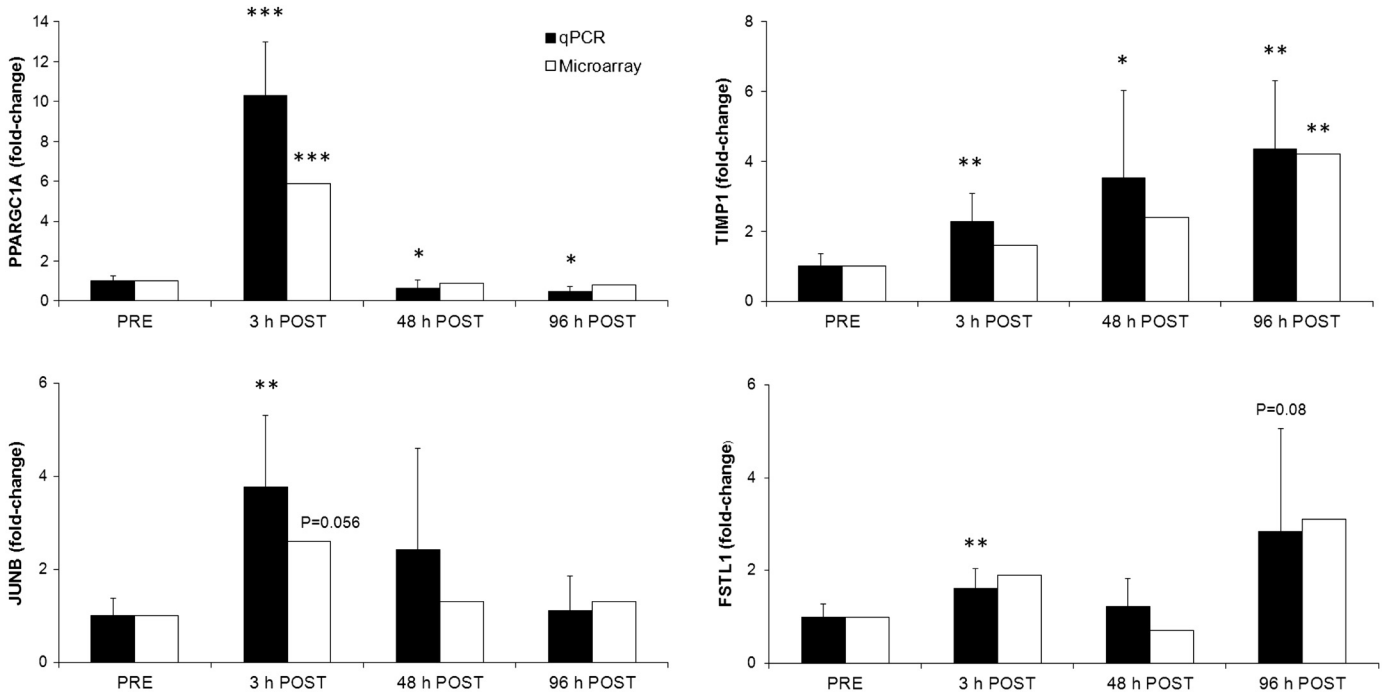


Fig. 1. Fold-changes in the gene expression of peroxisome proliferator-activated receptor γ, coactivator 1 α (PPARGC1A), jun B proto-oncogene (JUNB), tissue inhibitor of metallopeptidases 1 (TIMP1), and follistatin-like 1 (FSTL1) in exercised skeletal muscle tissue from baseline (PRE) to 3, 48, and 96 h after (POST) the experimental exercise trial. Presented are quantitative (q) PCR (mean fold-changes ± SD) and microarray data (mean fold-changes) for 8 study participants. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, change significantly different than baseline. For observed trends the $P$ value is indicated.

(STARS) (62), which increased 7.6-fold 3 h postexercise. STARS has been proposed as a muscle-specific signaling mechanism linking biomechanical stress with downstream transcriptional regulators such as serum response factor (SRF) (29). SRF, in turn, plays an essential role in the transcriptional regulation of actin cytoskeleton dynamics and the sarcomere structure (29, 37). In support of recent findings (33), showing that the STARS/SRF pathway is involved in the early remodeling, repair, and hypertrophic muscle responses to acute eccentric (resistance) exercise, the present data indicate an increased transcription of genes with SRF-binding sites 3 h postexercise, including EGR1, JUNB (Fig. 1), and connective tissue growth factor (CTGF) (Table 2). The upregulation of SRF target genes could have led to the upregulation of a top-ranked group of genes related to the formation of matrix-cell-cytoskeleton connections, so called leading edges, at 48 h postexercise (Table 3). Leading edge formation is crucial for the reorganization of actin filaments and cytoskeleton dynamics (37).

*Indications for sustained inflammatory processes and accumulated leukocytes 96 h postexercise.* To our knowledge, we report for the first time data on the human skeletal muscle transcriptome beyond 48 h after endurance exercise. The current observation that 83 gene sets were differently regulated 96 h postexercise (the majority of which was upregulated) demonstrates the importance of molecular events at such a late phase of recovery from intense, prolonged exercise. Crucially, the positive enrichment of several sets of genes functionally related to inflammatory and immune responses 96 h postexercise might be explained by the prolonged leukocyte presence in exercised muscles. Consistent with this concept, the more than fivefold increase in the leukocyte-specific genes ITGB2 (CD18) and CD68 at 96 h postexercise suggest that leukocytes had accumulated in skeletal muscle at this time point (Fig. 2). Furthermore, the more than fourfold increased transcription of the M2 macrophage gene CD163 in muscle 96 h postexercise could reflect a gradual phenotypic switch from M1 to M2 macrophages, critical for the shift from pro- to anti-inflammatory responses, and for promoting growth and regeneration of muscle (1, 57).

With respect to the sustained muscular inflammatory responses, the coordinated upregulation of genes involved in chemokine signaling (Table 4), including CCL2, at 96 h postexercise supports the concept that chemokines regulate interactions between immune and muscle cells during different phases of muscle regeneration (15, 19). The transcription of CCL2 increased in a biphasic manner (Fig. 2). The first peak in CCL2 transcripts (3 h postexercise, as discussed above) may reflect an inflammatory response, whereas the second peak 96 h postexercise might indicate mechanisms by which CCL2 directly promotes muscle repair (31).

Whereas most other HSP transcripts increased only 3 h postexercise, the transcription of HMOX1 increased at 3 and then again at 96 h postexercise (Fig. 2). HMOX1 has anti-inflammatory and cell-/tissue-protective functions (64) and it might be induced in skeletal muscle to minimize oxidative damage (36, 46). HMOX1-mediated protection from oxidative stress may be stimulated either by increased metabolism and removal of heme (e.g., from myoglobin) or by the formation of the potent antioxidant bilirubin (4). Moreover, HMOX1 has been associated with the regulation of unrestrained calcineurin activity in response to exercise-induced muscle damage (33). Therefore, the more than fourfold increased HMOX1 transcription 3 h postexercise might have been related to oxidative (36),

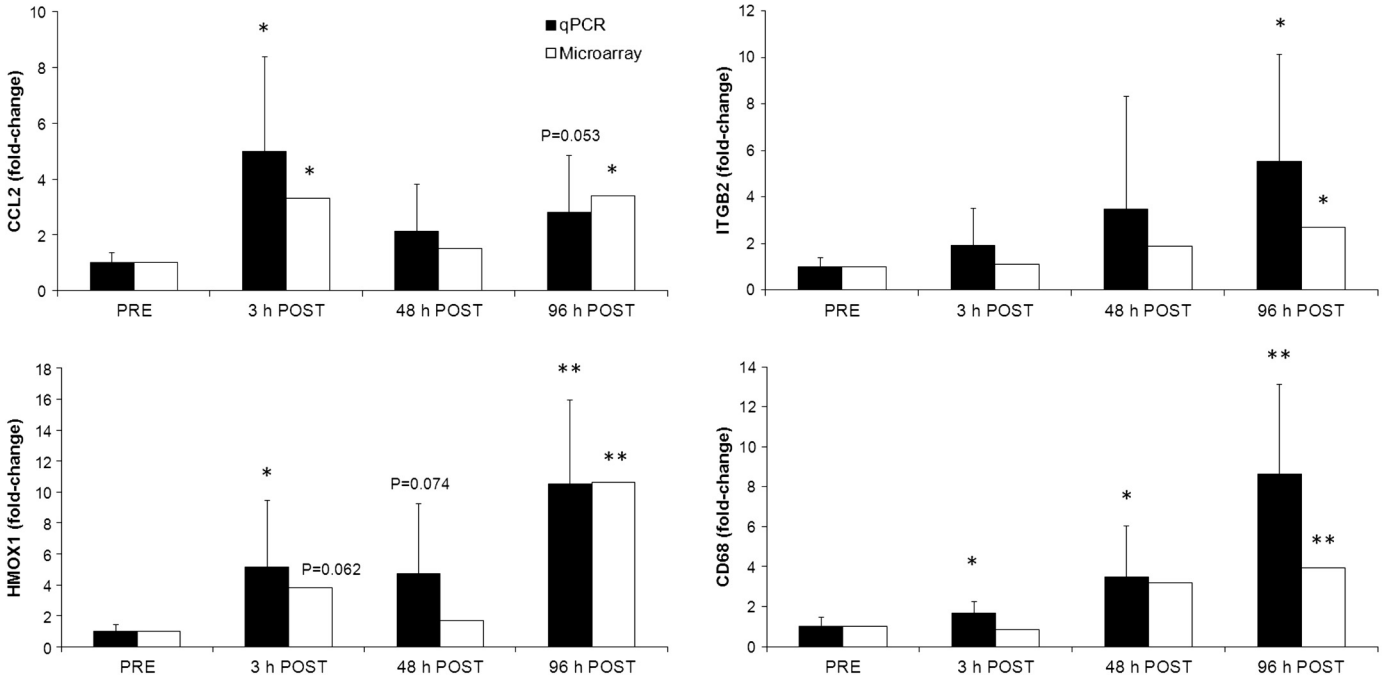


Fig. 2. Fold-changes in the gene expression of chemokine (C-C motif) ligand 2 (CCL2), heme oxygenase 1 (HMOX1), and the leukocyte-specific genes integrin beta 2 (ITGB2) and CD68 in exercised skeletal muscle tissue from baseline (PRE) to 3, 48, and 96 h after (POST) the experimental exercise trial. Presented are qPCR (mean fold-changes ± SD) and microarray data (mean fold-changes) for 8 study participants. *P < 0.05, **P < 0.01, change significantly different than baseline. For observed trends the *P* value is indicated.

mechanical stress (33), and/or the intracellular increase of calcium (24) associated with strenuous exercise. The proposed association of HMOX1 with muscle damage is supported by correlations between the HMOX1 gene expression in muscle and plasma muscle damage markers. Another correlation between the gene expression changes of HMOX1 and CD68 in muscle suggests that the accumulation of macrophages in skeletal muscle was associated with the upregulated HMOX1 transcription. Furthermore, based on the observed increased CD163 transcripts in muscle 96 h postexercise and previous research showing that macrophages upregulate HMOX1 upon CD163 ligation (45), it may be speculated that M2 macrophages, at least partly, were responsible for the 9-fold increased HMOX1 transcription at the same time point.

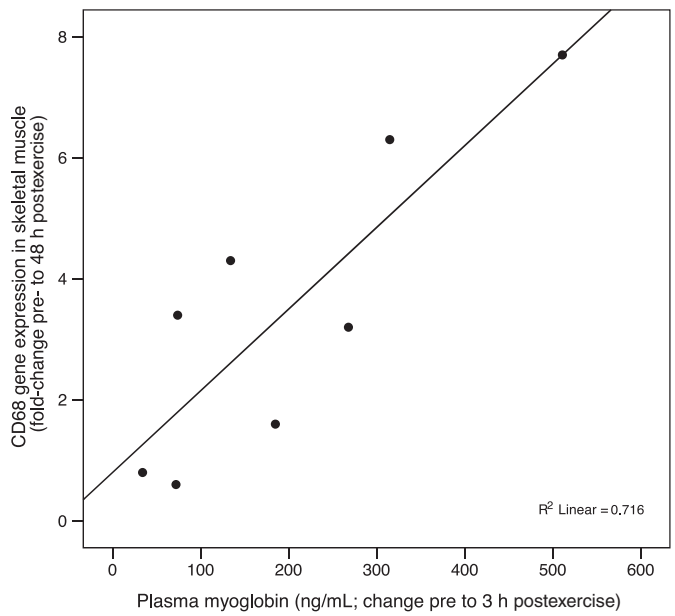


Fig. 3. Correlation between the pre- to 3 h postexercise increase in the plasma concentration of myoglobin and the pre- to 48 h postexercise fold-change of CD68 gene expression in skeletal muscle (used as a marker for macrophage accumulation) ($r = 0.85$; $P < 0.01$).

*Interactions with muscle extracellular matrix and adaptive remodeling 96 h postexercise.* Remodeling of the intramuscular connective tissue or extracellular matrix (ECM) has been proposed as a requirement for subsequent muscular adaptation after endurance exercise (16, 58). Concomitant with this concept, the molecular signature of the skeletal muscle transcriptome at 96 h postexercise reflected substantial transcriptional activity related to crosstalk with, and structural rearrangement of the ECM (Table 4). The enrichment of genes involved in antigen presentation and processing, as well as proteolytic and, especially, lysosomal processes [e.g., proteases including cathepsin 1 (CTSL1)] (2), suggests that the breakdown of tissue was not complete 96 h postexercise. The activation of protein degradation pathways is associated with stress on cell and tissue integrity, which could explain the sustained appearance of plasma myoglobin, as reported previously (40). However, targeted protein and tissue degradation, in particular cleavage of muscle ECM, may be critical for muscle tissue repair and adaptive remodeling (9, 32). Similar to previous studies (51), structural ECM remodeling was indicated by an increased transcription of a number of genes encoding for collagen (e.g., COL1A2, COL1A1, COL5A2). Other upregulated extracellular genes included elastin microfibril interfacer 2 (EMILIN2), transforming growth factor beta induced (TGFBI), and versican (VCAN). All of these genes have specific functions in the

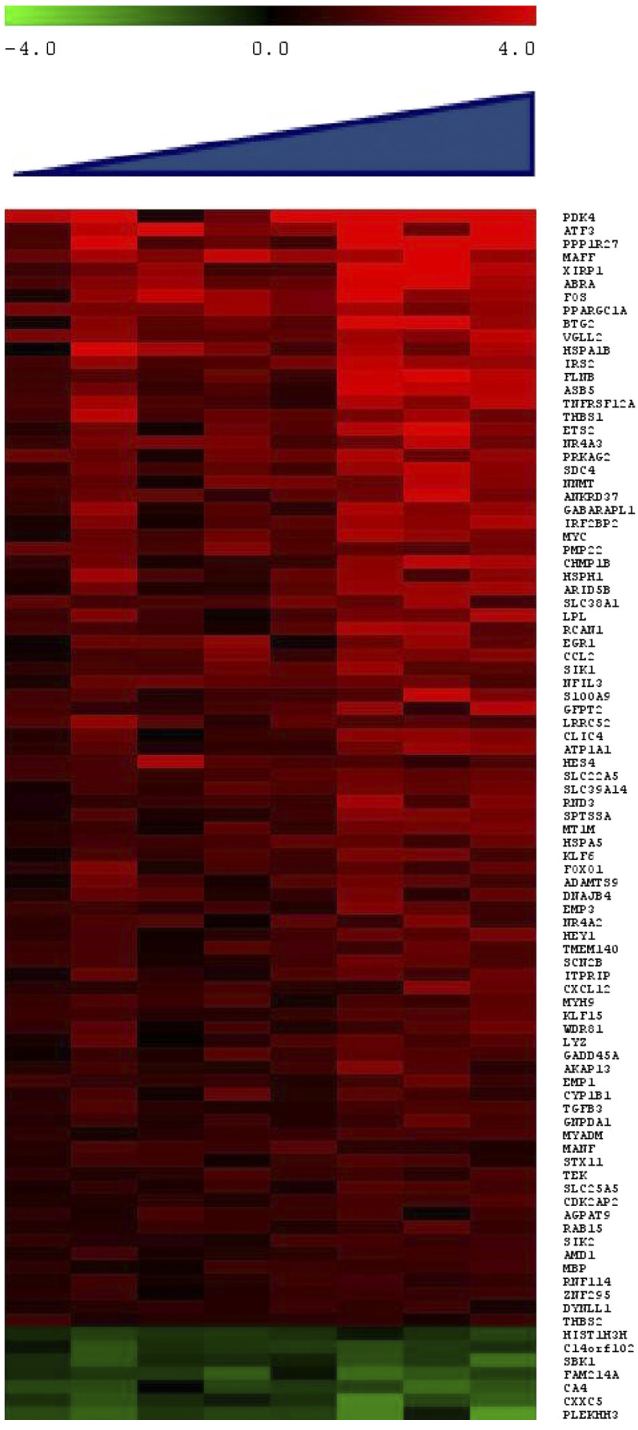


Fig. 4. Heat map showing significantly differentially regulated genes in skeletal muscle 3 h after the experimental endurance exercise trial. Eight study participants are ranked for the least to the greatest degree of muscle damage as indicated by the pre- to 3 h postexercise changes in plasma myoglobin concentrations (from the least to the greatest change from *left* to *right*).

regulation of tissue homeostasis (EMILIN2) (39), cell-matrix adhesion (TGFBI) (23), or facilitation of muscle fiber fusion during muscle regeneration (VCAN) (54); however, they have previously not been reported in exercised human muscle.

Additionally, these data indicate the transcriptional activation of pro- and antiangiogenic factors 96 h postexercise (Table 4), which is in accordance with a model suggesting that appropriate exercise-induced vascular remodeling requires a balanced response of promoters and inhibitors of capillary growth (11, 16, 20). Transcriptionally activated antiangiogenic and remodeling-associated factors included TIMP1. TIMP1 has been shown to increase after acute resistance exercise together with matrix metalloproteinase (MMP) genes (8) and after endurance exercise training (20). In the absence of a transcriptional induction of MMP activity at the currently investigated time points, the increased transcription of TIMP1 may rather indicate an MMP-independent response to counterregulate inappropriate endothelial cell proliferation and angiogenesis (11, 53). Finally, FSTL1, a recently identified myokine involved in revascularization after muscle injury (42), contributed to the enrichment of a set of genes associated with the ECM 96 h postexercise (Table 4). FSTL1 has been shown to respond to strength training in humans (41). To our knowledge, we report for the first time that endurance exercise increased the transcription of FSTL1 (3 h postexercise) and, with a trend for increased transcript levels, again at 96 h postexercise.

*Study limitations.* We acknowledge that the present findings do not allow us to conclude whether genes were explicitly expressed by muscle cells, infiltrating leukocytes, or other cell types present in muscle tissue. Furthermore, although it was not possible to include a representative (resting) control group within the scope of this study, because of practical and ethical reasons associated with four muscle biopsy samplings, we recognize the possibility that multiple muscle biopsies may, at least partly (61), or may not (60) affect gene expression. Therefore, we aimed to reduce related potential confounding effects to a minimum by applying a validated, minimally invasive microbiopsy technique (18) and by taking additional measures (e.g., multiple sampling through a single puncture at each time point) to further reduce the risk for biopsy-induced trauma. In addition, because the muscle samplings were at least 48 h apart from each other, we are confident that the endurance exercise protocol was the major factor behind the observed transcriptional responses.

*Perspectives and significance.* The current study demonstrates that a single bout of endurance exercise induces distinct time course-dependent transcriptomic responses in skeletal muscle tissue of trained individuals, which are sustained until, at least, 96 h after exercise. The presented data suggest that many of the coordinate changes in the expression of individual genes and functionally related gene groups might have occurred in response to exercise-induced muscle damage and/or were associated with the subsequent accumulation of leukocytes in the muscle tissue. According to our hypothesis, the time course of changes in the muscular transcriptome indicates significant regulatory interactions between the muscle, accumulated leukocytes, and the ECM throughout recovery until at least 96 h after the acute endurance exercise bout. The novel transcriptome 96 h postexercise in particular reveals substantial transcriptional activity at such a late stage of recovery, most of which is functionally related with inflammatory and immune responses, cell stress management, as well as crosstalk with and extensive remodeling of the muscular ECM. The exact posttranscriptional regulation and functional consequences of the transcriptomic changes in terms of phenotypic adaptations require further research. Considering that in particular ECM-related gene expression changes in skeletal muscle may reflect

physiological adaptation to exercise (58) and that leukocyte accumulation in exercised muscle is associated with halted recovery of muscle function (43), the current results have important physiological implications. Collectively, the presented data indicate that muscular recovery from endurance exercise involving moderate muscle damage, from a transcriptional perspective, is still incomplete 96 h after the initiating bout of exercise ended.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

## AUTHOR CONTRIBUTIONS

Author contributions: O.N., S.S., J.M.P., D.C.-S., K.-H.W., L.J.H., and A.C.B. conception and design of research; O.N., S.S., B.D., A.C.B. and performed experiments; O.N., K.J.A., R.L., B.W., and A.C.B. analyzed data and interpreted results of experiments; O.N. prepared figures and drafted manuscript; O.N., B.W., J.M.P., D.C.-S., and A.C.B. edited and revised manuscript; O.N., S.S., K.J.A., B.D., R.L., B.W., J.M.P., D.C.-S., K.-H.W., L.J.H., and A.C.B. approved final version of manuscript.

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