Temporal pattern of skeletal muscle gene expression following endurance exercise in Alaskan sled dogs

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Brass EP, Peters MA, Hinchcliff KW, He YD, Ulrich RG. Temporal pattern of skeletal muscle gene expression following endurance exercise in Alaskan sled dogs. J Appl Physiol 107: 605-612, 2009. First published June 4, 2009; doi:10.1152/japplphysiol.91347.2008.—Muscle responses to exercise are complex and include acute responses to exercise-induced injury, as well as longer term adaptive responses. Using Alaskan sled dogs as an experimental model, changes in muscle gene expression were analyzed to test the hypotheses that important regulatory elements of the muscle’s adaptation to exercise could be identified based on the temporal pattern of gene expression. Dogs were randomly assigned to undertake a 160-km run (n = 9), or to remain at rest (n = 4). Biceps femoris muscle was obtained from the unexercised dogs and two dogs at each of 2, 6, and 12 h after the exercise, and from three dogs 24 h after exercise. RNA was extracted and microarray analysis used to define gene transcriptional changes. The changes in gene expression after exercise occurred in a temporal pattern. Overall, 569, 469, 316, and 223 transcripts were differentially expressed at 2, 6, 12, and 24 h postexercise, respectively, compared to controls, with a clear temporal pattern. RNA expression profiling has the potential to identify novel regulatory mechanisms and responses to exercise stimuli.

ribonucleic acid expression; metabolism

SKELETAL MUSCLE RESPONSES to an exercise stimulus are complex. The muscle responds in the short term by increasing ATP production to meet the energy demands of the exercise. However, exercise can also elicit a longer term response in which the muscle adapts to future work requirements through hypertrophy (1) and increasing oxidative metabolic capacity (19). These are major components of the classical training response. Additionally, exercise, either eccentric or concentric, can result in muscle injury characterized by sarcolemmal damage, with or without evidence of inflammation and pain (11, 26, 47). The skeletal muscle responses evoked are dependent on the intensity, duration, and type (eccentric, concentric) of exercise.

The skeletal muscle training response is manifest in dynamic changes in gene expression within skeletal muscle. Muscle gene expression has been studied using quantitation of one or more candidate mRNAs or using microarray methodologies to assess a broader collection of transcripts. For example, enhanced expression of mitochondrial genes and other genes involved in oxidative metabolism (6, 20) play a major role in muscle adaptation to training, and studies of specific transcripts have suggested important roles for the transcription regulators peroxisome proliferator-activated receptor-γ coactivator-1α, peroxisome proliferator-activated receptor (PPAR)-δ, nuclear respiratory factor (NRF)-1 and NRF-2 (2, 32, 37). Similarly, exercise-induced changes in VEGF mRNA have been linked to angiogenesis during training (4, 16, 33, 44). Novel exercise-associated changes in expression of extracellular matrix-related genes, potentially contributing to the regulation of angiogenesis, have also been defined by direct measurement of candidate transcripts (17, 44). However, the full richness of the muscle response to exercise has only become evident using microarray techniques (22, 27). This work has established that acute exercise or endurance training are associated with changes in multiple transcripts critical to several cellular processes, including intermediary metabolism, ion transport, signal transduction myocyte structure, and extracellular matrix composition (22, 27, 45, 50).

Despite this extensive body of work, the comprehensive, time-dependent changes in skeletal muscle gene expression have only been defined in a few model systems. Expression profiles are likely sensitive to the specific exercise protocol used, as has been shown for a single bout of exercise vs. a period of endurance training (22). Thus characterizations of time-dependent changes in well-defined models are critical for differentiating the responses to different training stimuli, as well as for understanding the specific triggers and mediators for skeletal muscle adaptation and how these processes might be deranged in disease.

Alaskan sled dogs used in competition are elite athletes that run distances up to 1,600 km and undergo regular exercise training. The rigorous training and competitive exercise bouts would be expected to induce classical training response, but also may be associated with muscle injury, as evidenced by plasma biomarkers and histology (9, 30). The present study utilized Alaskan sled dogs either before or after a prolonged endurance exercise training session to test the hypothesis that important regulatory elements of the muscle’s adaptation to exercise could be identified based on the temporal pattern of gene expression. A secondary hypothesis was that inflammatory responses resulting from the extreme exercise would be reflected in skeletal muscle gene expression. The results dem-
onstrate a coordinated change in expression of key transcriptional, signaling, and functional pathways after exercise.

**METHODS**

**Animals and related procedures.** All animal procedures were reviewed and approved by the Institutional Laboratory Animal Care and Use Committee at Ohio State University (approval no. 2004A0167). Subjects were 13 Alaskan sled dogs aged 4.5 ± 2.5 yr (mean ± SD) and weighing 23.3 ± 2.5 kg. Dogs were randomly assigned to two groups. One group of nine dogs subsequently ran 160 km in 24 h as two sessions of 80 km, separated by a 6-h rest period. The second group consisted of four dogs housed in unheated kennels, their usual housing, for the duration of the experiment. All dogs were from the same kennel. Dogs were fed a commercial kibble (Eukanuba, Iams, Dayton, OH) supplemented with frozen meat during the 8 wk preceding the study and throughout the study period. The dogs’ diet was consistent before, during, and after the exercise bout. All dogs had completed 1,590 ± 100 km of training runs in the 3 mo before the study. The dogs had not exercised for 72 h before the start of this study.

Dogs in the exercise group ran as a team pulling a lightly laden sled and driver over packed snow. Ambient temperatures were −20 to −10°C, with no wind. Dogs completed the two 80-km runs in 23 h, including the 6-h rest period.

Blood samples were collected by jugular venipuncture from all the dogs the day before exercise and within a 10-min window at 2, 6, 12, and 24 h after completing the second run. Samples were collected into evacuated glass tubes containing a clot enhancer.

Muscle samples were collected from each of two dogs within a 10-min window at 2, 6, and 12 h after completing exercise and from three dogs 24 h after completing exercise. Muscle samples were collected from each of the four unexercised dogs within 2 h of the other dogs initiating their exercise bout. Each dog had only one biopsy procedure performed. Dogs biopsied 2 h after exercise did not eat between completion of the run and the biopsy. Dogs biopsied at later time points were fed 1 h after the race, but not again before the biopsy. Muscle samples were collected from the biceps femoris muscle using a needle biopsy that yielded ~40–60 mg of muscle. The biopsy was performed after clipping and aseptic preparation of the skin overlaying the biopsy site. The dog was anesthetized with propofol (6 mg/kg iv, maintained as necessary with 2 mg/kg additional boluses), a cuffed orotracheal tube was placed, and the dog was ventilated with a hand-held ventilation bag. When adequate anesthesia had been obtained, a 5-mm incision was made in the skin. A sterile biopsy needle (12 g PGI EZ Core, Products Group International, Lyons, CO) was then inserted through the incision, and a sample of muscle collected. If necessary, repeated collections of muscle were made until a minimum of 40 mg of muscle had been collected from an individual dog. The skin incision was closed with tissue glue, and an antibiotic ointment applied. The dog was monitored closely until recovery from anesthesia was complete. Carprofen (4.4 mg/kg, orally) was administered when the dog had recovered sufficiently from anesthesia to have a gag reflex.

**RNA extraction and cRNA preparation.** Total RNA was isolated using the Promega SV total RNA isolation system (Promega, Madison, WI), according to the manufacturer’s instructions. RNA quantity and quality were determined through UV spectral analysis using a Agilent Bioanalyzer 2100 (Palo Alto, CA). Synthesis of Cy3- and Cy5-labeled cRNA was carried out by in vitro transcription using 5-µg total RNA and T7 RNA polymerase, as described by Hughes et al. (21).

**Microarray hybridization.** Labeled cRNAs were fragmented and hybridized to a custom ink-jet synthesized oligonucleotide microarray platform purchased from Agilent technologies, with 23,653 60-mer oligonucleotides representing 19,155 known dog transcripts and expressed sequence tags (plus control sequences). Hybridization conditions and scanning were done as previously described (21). All hybridizations were done in duplicate as dye-swap experiments against a mass balanced pool of RNA muscle samples from the animals that did not exercise to compensate for potential normalization dye bias. Processing of the raw scans was done using the Agilent feature extraction system, which performs error modeling of the data (49). All hybridizations passed quality control criteria, as previously described (7). In detail, fluorescent images obtained with the Agilent scanner were quantified using an in-house image processing code. Segmentation identified the most interesting region of each spot and pixels for local background estimation. Automated artifact recognitionflagged suspicious spots based on a neural net classifier that used measures such as color ratio, uniformity, and pixel intensity distribution shape. Typically 1% of spots was flagged as artifacts in this way. Although negative control spots were included on the array, processing used only local out-of-spot background pixels to estimate the background signal. Correction for sequence-specific cross-hybridizations was not attempted. The background-subtracted intensities from two channels were initially normalized by the mean intensity of each channel, excluding control spots. The second step in background subtraction balanced the background bias between the two color channels. This step was carried out by selecting low-intensity spots and performing a linear fit between intensities from the two colors. The offset is subtracted from the channel with extra background residual. The two background-balanced channels were further normalized and detrended by first binning the data, according to logarithmic intensity (geometric mean of both channels), and then fitting a linear relation between two channels using mean logarithmic intensity from each bin. The offset is due to the gain difference between two channels, and the coefficient to the logarithmic intensity reflects any nonlinearity of the scanner used. Both effects are corrected in the red channel. This correction is essential to avoid artifactual pattern similarities when looking for patterns composed of subtle expression changes. For each ratio measurement, a P value was assigned based on the Rosetta error model (49). To ensure feature quality and spot success rate on each microarray, 90% or greater of spots were required to pass a metric test. Ratios were reproducible within an intrachip standard deviation of the log (ratio) < 0.0607 and fluor-reversal pair standard deviation of the log (ratio) < 0.0792. The mean observed intrachip ratio assessed ratio accuracy and was not biased by >50% of the expected ratio. Spiked cRNA transcripts of known concentrations were used to ensure ratio sensitivity across the array chip and considered successful when at least one-half of these transcripts met their expected ratio within 50%.

Full microarray hybridization data are available through GEO accession GSE15117 (http://www.ncbi.nlm.nih.gov/geo/).

**Data processing and analysis.** Data consisting of gene annotation, log ratio, errors, and P values were loaded into the Rosetta Resolver system, version 7.0 (Rosetta BioSoftware, Seattle, WA), where replicates were combined into ratio experiments using an error-weighted average, as previously described (39, 49). Two types of replicates were combined: one where an experiment was defined as dye-swap pairs (individual animals), and another where an experiment was defined as all data belonging to a single time point (biological replicates). The Ingenuity Pathway Analysis (IPA) system, version 5.5 (Ingenuity Systems, Redwood City, CA) was used for pathway, network, and functional analysis of the biological replicate experiments at each individual time point. Before analysis in IPA, the dog transcripts were translated to the corresponding human homologs by requiring reciprocal BLAST hit and synteny support. The IPA Biological Function analysis was corrected for multiple testing using the Benjamin-Hochberg multiple-test correction method (24). K-means clustering (43) and Principal Component Analysis (PCA) (14) were being done using the Resolver system.
RESULTS

Traditional plasma biomarkers. All dogs completed the exercise regimen without evidence of muscle soreness. Serum activities of muscle-derived enzymes [creatine kinase (CK) and aspartate aminotransferase (AST)] were significantly increased by 2 h after completion of exercise (Table 1), consistent with skeletal muscle injury. There were smaller increases in the serum activity of alanine aminotransferase, and concentrations of phosphorus, cortisol, and creatinine after exercise.

Skeletal muscle gene expression changes associated with exercise. Heterogeneity in the group of dogs studied might introduce biological variability, independent of the exercise stimulus of interest. To ascertain the background biological variability, a PCA of all individual animals was done utilizing 3,394 transcripts, which represented all transcripts on the array, with an absolute fold change $\geq 1.5$ and ratio $P$ value $\leq 0.01$ in at least one animal. The analysis was done specifying that the retained data represent 95% of the variation in the dataset (Fig. 1). This analysis indicated that, although the study included a relatively small number of animals, gene expression profiles in animals sampled at the same time point were more similar than expression in animals sampled at different time points. The PCA also showed that, compared with the nonexercised control group, the greatest differences in gene expression occurred 2 and 6 h postexercise, with expression levels becoming more similar to the nonexercised control group 12 and 24 h postexercise.

To identify exercise-affected transcripts, significant change at each time point for each exercise group was defined as transcripts with a ratio $P$ value $\leq 0.01$ and an absolute change of $\geq 1.5$ compared with the unexercised control group. Using these criteria, 569, 469, 316, and 223 transcripts were differentially expressed at 2, 6, 12, and 24 h postexercise, respectively. A Venn diagram illustrates the overlap between all time points (Fig. 2).

A K-means cluster of 1,076 transcripts representing the union of the significant signatures at each time point shows a temporal pattern in gene expression after exercise (Fig. 3). Some transcripts demonstrated a significant increase in expression 2 h after exercise, with a return toward resting levels by 6 h (cluster group 5), while a second group of transcripts appeared to peak by 6 h after exercise and then returned to resting levels (cluster groups 1 and 2). Other transcripts showed sustained induction or repression over the 24 h after exercise (cluster groups 3 and 4). All 1,076 transcripts with corresponding fold change and $P$ values for each time point, including K-means cluster group and Venn diagram overlap annotation, are supplied in Supplemental Table 1. (The online version of this article contains supplemental data.)

Biological significance of gene expression changes. The IPA system was used to identify biological events of interest in

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**Table 1. Serum clinical chemistries**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>2 h After Exercise</th>
<th>6 h After Exercise</th>
<th>12 h After Exercise</th>
<th>24 h After Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT, U/l</td>
<td>60±19</td>
<td>80±21</td>
<td>89±27</td>
<td>88±24</td>
<td>94±28</td>
</tr>
<tr>
<td>AST, U/l</td>
<td>27±4</td>
<td>186±108</td>
<td>170±94</td>
<td>132±68</td>
<td>85±45</td>
</tr>
<tr>
<td>CPK, U/l</td>
<td>109±47</td>
<td>3,220±2,730</td>
<td>2,320±1,700</td>
<td>1,280±926</td>
<td>471±370</td>
</tr>
<tr>
<td>Phosphorous, mg/dl</td>
<td>3.8±0.6</td>
<td>4.9±0.6</td>
<td>5.3±0.5</td>
<td>5.0±0.6</td>
<td>3.7±0.6</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>0.9±0.1</td>
<td>1.0±0.1</td>
<td>1.0±0.2</td>
<td>1.1±0.1</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>Cortisol, mg/dl</td>
<td>2.1±0.7</td>
<td>3.6±2.0</td>
<td>1.7±0.6</td>
<td>1.9±0.5</td>
<td>1.5±0.5</td>
</tr>
</tbody>
</table>

Values are means ± SD. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CPK, creatine phosphokinase.

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**Fig. 1.** Principal component analysis (PCA) of expression profiles in individual animals. All of the transcripts on the array with an absolute fold change $\geq 1.5$ and ratio $P$ value $\leq 0.01$ in at least 1 animal were utilized in the PCA (3,394 transcripts). The analysis was done specifying that retained data represent 95% of the variation in the data set. Shown in the plot are principal components (PC) 1, 2, and 3, with PC1 representing the greatest variance in the data.

**Fig. 2.** Venn diagram illustrating the overlap of signatures at each time point. Signatures were defined as differentially expressed genes in the combined replicate experiments incorporating transcripts with a ratio $P$ value $\leq 0.01$ and an absolute fold change $\geq 1.5$ at each time point compared with the nonexercised control group. Indicated in blue are the 569 transcripts significant at 2 h, in green the 469 transcripts significant at 6 h, in yellow the 316 transcripts significant at 12 h, and in magenta the 223 transcripts significant at 24 h.
skeletal muscle after exercise. Simple compilation of the genes showing the greatest relative change in expression at each time point reinforced the temporal pattern of change in skeletal muscle gene expression after exercise (Table 2). Two hours after exercise, large increases in expression were observed for regulators of transcription [for example, cAMP responsive element modulator (CREM), CCAAT enhancer binding protein-δ (CEBPD), and nuclear receptor subfamily 4 group A member 2] and secreted regulatory factors [for example, brain-derived neurotrophic factor (BDNF), dermokine, and suprabasin]. There was also decreased expression of collagen type XXII α1 and the regulatory protein G0/G1 switch 2 h after exercise. Six hours after exercise, the list of 10 most affected transcripts contained only one gene product from the 2-h list (collagen type XXII α1) and included a number of transcripts with unclear roles in skeletal muscle. Twelve and 24 h after exercise, there was continued altered expression of genes involved in extracellular matrix formation and angiogenesis. Fos expression was markedly repressed in the dogs after exercise (Table 2).

IPA pathway and biological function enrichment analyses were used to identify potential coordinated functional links between transcripts with altered expression after exercise. Pathway analyses demonstrated changes in pathways involved in extracellular matrix formation, angiogenesis, and signal transduction, among other processes.

Table 2. Genes showing the largest magnitude of altered expression

<table>
<thead>
<tr>
<th>Transcript</th>
<th>2 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain-derived neurotrophic factor (BDNF)</td>
<td>+15.7</td>
<td>+2.7</td>
<td>NS</td>
<td>+1.5</td>
</tr>
<tr>
<td>Dermokine</td>
<td>+9.2</td>
<td>-1.8</td>
<td>-3.9</td>
<td>-3.9</td>
</tr>
<tr>
<td>Complement component 4 binding protein-β (C4BPB)</td>
<td>+7.9</td>
<td>+1.7</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>cAMP response element modulator (CREM)</td>
<td>+7.1</td>
<td>+3.7</td>
<td>-2.1</td>
<td>NS</td>
</tr>
<tr>
<td>Suprabasin (SBSN)</td>
<td>+7.1</td>
<td>NS</td>
<td>-1.8</td>
<td>-1.4</td>
</tr>
<tr>
<td>CCAAT enhancer binding protein-δ (CEBPD)</td>
<td>+6.8</td>
<td>NS</td>
<td>+1.9</td>
<td></td>
</tr>
<tr>
<td>Nuclear receptor subfamily 4, group A, member 2 (NR4A2)</td>
<td>+5.6</td>
<td>-2.1</td>
<td>-2.6</td>
<td>NS</td>
</tr>
<tr>
<td>Keratin 5 (KRT5)</td>
<td>+5.2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Collagen type XXIIα1 (C1Q20A1)</td>
<td>-5.1</td>
<td>-7.0</td>
<td>-2.6</td>
<td>-4.5</td>
</tr>
<tr>
<td>G0/G1 switch 2 (G0S2)</td>
<td>-6.5</td>
<td>NS</td>
<td>NS</td>
<td>-3.1</td>
</tr>
<tr>
<td>Cysteine-rich with EGF-like domains 2 (CRELD2)</td>
<td>+4.6</td>
<td>+6.5</td>
<td>+4.5</td>
<td>+2.6</td>
</tr>
<tr>
<td>Viral DNA polymerase transactivating protein 6</td>
<td>+3.7</td>
<td>+5.2</td>
<td>+2.5</td>
<td>NS</td>
</tr>
<tr>
<td>Solute carrier family 30 (zinc transporter) member 1 (SLC30A1)</td>
<td>+2.4</td>
<td>+4.4</td>
<td>NS</td>
<td>+1.5</td>
</tr>
<tr>
<td>Phosphomannomutase 1 (PMM1)</td>
<td>+2.5</td>
<td>+3.9</td>
<td>+3.6</td>
<td>+3.4</td>
</tr>
<tr>
<td>Ankrin repeat and SOCS box containing 5 (ASB5)</td>
<td>+3.7</td>
<td>+3.9</td>
<td>+2.0</td>
<td>NS</td>
</tr>
<tr>
<td>Jumonji domain containing 6 (JMJ6D)</td>
<td>+2.7</td>
<td>+3.8</td>
<td>+2.8</td>
<td>+1.7</td>
</tr>
<tr>
<td>SNW domain containing 1 (SNW1)</td>
<td>+3.6</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Cartilage oligomeric matrix protein (COMP)</td>
<td>-4.4</td>
<td>-5.3</td>
<td>NS</td>
<td>-4.9</td>
</tr>
<tr>
<td>Zona pellucida glycoprotein 2 (ZP2)</td>
<td>+3.4</td>
<td>+3.5</td>
<td>+5.4</td>
<td>+2.1</td>
</tr>
<tr>
<td>G protein coupled receptor 98 (GPR98)</td>
<td>NS</td>
<td>NS</td>
<td>+3.2</td>
<td>NS</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxykinase 1 (PCK1)</td>
<td>NS</td>
<td>NS</td>
<td>+3.1</td>
<td>NS</td>
</tr>
<tr>
<td>3-Hydroxy-3-methylglutaryl coenzyme A synthase 2 (HMGS2)</td>
<td>+4.2</td>
<td>+2.3</td>
<td>+3.0</td>
<td>+2.4</td>
</tr>
<tr>
<td>FK506 binding protein 1B (FKBP1B)</td>
<td>+2.9</td>
<td>+2.4</td>
<td>+2.9</td>
<td>+3.1</td>
</tr>
<tr>
<td>Fos-v-fos FBJ murine osteosarcoma viral oncogene homolog (Fos)</td>
<td>-3.1</td>
<td>-3.3</td>
<td>-13.5</td>
<td>-4.7</td>
</tr>
<tr>
<td>OTU domin containing 1 (OTUL1D)</td>
<td>-2.1</td>
<td>NS</td>
<td>-3.9</td>
<td>-1.3</td>
</tr>
<tr>
<td>Osteopontin (secreted phosphoprotein 1) (SPP1)</td>
<td>NS</td>
<td>+2.3</td>
<td>+3.4</td>
<td></td>
</tr>
<tr>
<td>Trinucleotide repeat containing 6B (TNRC6B)</td>
<td>-1.5</td>
<td>-3.2</td>
<td>NS</td>
<td>-6.5</td>
</tr>
<tr>
<td>Procollagen-lysine 1,2-oxoglutarate 5-dioxygenase (PLOD1)</td>
<td>-1.6</td>
<td>-3.2</td>
<td>-1.7</td>
<td>-5.6</td>
</tr>
<tr>
<td>Alcohol dehydrogenase 4 (class III) (ADH4)</td>
<td>-2.1</td>
<td>NS</td>
<td>-1.7</td>
<td>-4.3</td>
</tr>
</tbody>
</table>

The 10 genes showing the greatest relative change at each time point were identified. These genes with their relative changes in exercised animals compared with unexercised controls are shown at all after-exercise time points. NS indicates no significant change (P > 0.01, see Supplemental Table 1 for P values for each gene). Transcripts are presented in order of the earliest time point at which they were included in the 10 most changed transcript list.
in inflammation, oxidative stress, and intermediary metabolism (complete results in Supplemental Table 2, including specific transcripts contributing to pathway). Biological function analyses, based on the IPA categories of molecular and cellular functions, and physiological system development and function confirmed coordinated changes in expression of potential functional importance (Table 3). These analyses also suggested changes in transcription factors, signaling pathways, and tissue developmental mechanisms that may be important in the skeletal muscle response to exercise. Additionally, transcripts linked to immune function and cellular compromise were altered, potentially related to the exercise-associated muscle injury. Examples of induced transcripts potentially associated with injury or stress responses included HSP90B1, GCLC, TXNRD1, S100A8 and HERPUD1 (Supplemental Tables 1 and 2).

Signaling involving the PPAR-α appeared to be important to the postexercise responses, as this important transcription factor was included in many of the functional pathways identified by IPA (see Supplemental Tables 2 and 3). The expression of multiple transcripts encoding proteins known to interact with PPAR-α was affected after exercise (Fig. 4). Of particular note were the coordinate increases in the expression of the transcriptional coregulators cAMP responsive element modulator and CEBPD, and the cAMP-dependent protein kinase regulatory unit (PRKAR1A).

Given the marked and unexpected repression of expression observed for Fos, IPA was used to identify transcripts in the 2-h data set that encodes proteins with known Fos interactions (Fig. 5). This analysis showed coordinate repression of the early response gene early growth response 1 (EGR1), but induction of other related factors, including JunB.

Several candidate genes previously associated with exercise-induced changes in muscle were specifically examined. No significant changes over time were observed in mitochondrial transcription factor A, NRF-1, PPAR-δ, or heat shock protein-70 (data not shown).

### DISCUSSION

Skeletal muscle responds to an exercise stimulus with a complex array of short- and long-term adaptations that are incompletely understood. The current analysis of gene expression profiles over time permits description of the responses to exercise with increased clarity. Responses related to inflammation and stress, matrix remodeling, and changes in intermediary metabolism could be delineated, with a number of tran-
superscript factors and signaling pathways of potential importance also identified.

Changes in conventional biomarkers, including serum activities of AST and CK, and phosphorus concentration were consistent with leakage of intracellular constituents and possible sarcolemmal membrane injury as early as 2 h after exercise (Table 1). This is consistent with other data in sled dogs (30) and human marathon runners (25), but contrasts with eccentric exercise, where peak increases in AST and CK are delayed until 3–4 days after exercise (3, 26). This difference in time course might reflect different mechanisms of membrane injury or leakage between the two forms of exercise.

No histology was performed in the present study, so injury to the biopsied biceps femoris cannot be confirmed. Nonetheless, the biceps femoris would be involved in the running exercise, and the muscle injury response may have been evident at the gene expression level, as pathways related to inflammation and stress responses were affected by exercise (Supplemental Tables 2 and 3). Many of the specific transcripts induced in the present work were also induced in other models of skeletal muscle injury (41, 48). The functional significance of the changes in putative injury-response pathways should be interpreted with caution, as changes in transcripts associated with similar pathways have been seen with less extreme exercise stimuli (22, 27), and these genes may have other functional roles in muscle adaptation.

A large number of transcripts were included in the dog-specific microarray used. This, combined with the statistical approach used, was intended to identify genes whose expression was affected by exercise without prejudice to potential candidate genes. This rationale was further extended to the Ingenuity-based analysis to identify biological processes linked to changes in gene expression. Each candidate pathway or function incorporates a number of gene transcripts known to share regulatory mechanisms, cellular functions, or both. This approach demonstrates both the robustness of the analytic processes, as several such related responses were identified, and also the power of the overall approach in understanding complex biological responses.

Focusing on the transcripts with the largest relative change at each time point provides evidence of concordant changes and illustrates the potential of the approach used to identify novel regulatory elements in muscle adaptation (Table 2). For example, 2 h after exercise, an expected rise in BDNF (8) was observed. However, the upregulation of BDNF transcript also illustrates the caution that must be used in interpreting the expression data. The change in transcript levels may not correlate to changes in protein expression. The muscle biopsies used contain a number of different cell types, and muscle BDNF has been suggested to arise from nerves (15), vasculature (23), satellite cells (31), as well as myocytes (29). Peripheral nerve or neuromuscular junction plasticity may be part of the adaptation to exercise (10, 13, 34), and the neurotrophic properties of BDNF may be relevant to this process. Additionally, BDNF has been suggested to play roles in angiogenesis (23), an important component of training adaptation, as well as metabolic adaptation in skeletal muscle postexercise (29) and muscle progenitor biology (31).

Large increases in dermokine and suprabasin were also seen. These two secretory molecules have been suggested to have coordinated expression and to play a role in stratified epithelia (28), but their large changes here suggest an unexpected role in skeletal muscle. G0/G1 switch 2 has been suggested to be regulated by PPAR-α (52), but was downregulated 2 h after exercise, despite the increased expression of PPAR-α and associated proteins (Fig. 4). The list of transcripts with large relative changes postexercise included many others with unknown function in skeletal muscle. As these included transcripts with homologies to transcription factors, RNA splicing proteins, and secreted proteins (Table 2), they might suggest previously unrecognized pathways in muscle adaptation to exercise.

PPAR-α protein is expressed in human skeletal muscle (40), and this transcription factor plays an important role in skeletal muscle responses to exercise (35). Furthermore, the IPA analysis identified PPAR-α as a component of many of the pathways significantly coregulated postexercise (Supplemental Tables 2 and 3). Thus the increase in PPAR-α expression and associated genes was of particular interest (Fig. 4). Expression of the PPAR-γ coactivator-1α was also increased postexercise (Fig. 4, Supplemental Table 1). This is consistent with work suggesting PPAR-dependent signaling mediates many training-associated adaptations, including enhanced oxidative capacity and fatty acid oxidation. Like PPAR-α, expression of PPAR-δ has been suggested to play a major role in metabolic adaptation in skeletal muscle (12), but PPAR-δ expression was not significantly increased after exercise in the sled dogs. The induction of PRKAR1A as part of the PPAR-α network may enhance cAMP signaling, recently suggested to be important in muscle structural adaptations (36). Importantly, as the data supporting the Ingenuity analyses are not skeletal muscle specific, it is possible that not all proteins identified in the networks are actually expressed in skeletal muscle.

Fig. 5. Fos-related responses 2 h postexercise. Shown is a network of genes directly interacting with Fos based on significant changes 2 h postexercise and Ingenuity Pathway Analysis gene interaction annotation. Gene names shown in yellow are upregulated, and blue, downregulated. Ellipses represent transcriptional regulators; triangles, kinases; diamonds, peptidases; and rectangles, nuclear receptors. An arrowhead indicates the protein acted upon by the other connecting node. See Supplemental Table 1 for definition of protein abbreviations.
The repression of Fos expression (Table 2, Fig. 5) was surprising, given the previously reported induction of muscle Fos expression in various exercise models (5, 18). As for many other genes (22), the response in Fos expression thus appears to be dependent on the exercise protocol used. The observed decrease in Fos is also in contrast with induction of Fos observed in other skeletal muscle injury models (48). Network analysis suggests that Fos expression may be part of a more complex regulatory response in the muscle of dogs after exercise (Fig. 5). Thus, while expected increases in Jun and Myc (Supplemental Table 1) were observed, the transcriptional regulator EGR1 maintained coordinate expression with Fos. The significance of this altered pattern of expression in the muscle’s response to exercise is unknown.

A number of transcription factors were induced early after exercise. The induction of CEBPD has been associated with muscle wasting in dexamethasone-treatment models (51). The induction of CEBPD after exercise (Table 2, Fig. 4) suggests that this factor might not be specific for muscle wasting, but rather an important component for muscle remodeling or adaptation. This concept is supported by reports of induction of CEBPD after eccentric exercise (5). CEBPD expression is known to be regulated by corticosteroids (51), but the small increase in plasma cortisol concentration observed after exercise (Table 1) is unlikely to mediate the muscle changes observed. The potential interaction of CEBPD with known mediators of muscle training adaptation, such as PPAR-γ (42), supports an important role for this enhanced expression.

Several features of the present experimental design are important in the interpretation of the present data. The exercise model used in the present study was one of exceptional duration for mammals and likely enhanced the ability to detect both short- and long-term responses in gene transcription. However, while the data provide important insights into the temporal responses in skeletal muscle in response to exercise, any changes occurring during the course of exercise would not be identified. As noted by other groups (27, 46), interpretation of microarray expression data in exercise models must be done with caution due to technical factors, challenges in data analysis, and limitations in extrapolating results to protein expression and function. Practical considerations limited muscle sampling, and thus the events observed at 2 h after exercise and beyond were likely initiated at earlier time points. As a result, key early, transient changes in expression may not be reflected in the present analyses. Additionally, some of the changes in gene expression observed could result from diurnal (38), nutritional, or other physiological influences, independent of the exercise session. No effort was made to confirm the microarray data by direct, specific quantitation of mRNAs or proteins in the muscle. Also, the animals tested were elite athletes, and hence the baseline expression profile likely is not that typical of dogs, and the changes observed occurred against this atypical background. A different response profile might result if previously untrained dogs underwent an equivalent exercise session. The small number of dogs studied at each time point might have failed to detect changes in transcripts with low abundance, small absolute changes in expression, or highly variable changes in expression, despite physiologically important responses to exercise. As each sample was taken from a different dog, interindividual variation may have contributed to false-negative or false-positive identification of exercise-affected transcripts. Finally, the limitations of extending the dog transcripts to human gene annotations may have limited the canonical pathway and biological function analyses performed.

Exercise in elite sled dogs initiates a complex, but temporally orchestrated series of changes in skeletal muscle. RNA expression profiling provides a valuable tool for characterizing these changes and identifying potentially important regulatory pathways.

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