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Alterations in oxidative gene expression in equine skeletal muscle following exercise and training

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Eivers SS, McGivney BA, Fonseca RG, MacHugh DE, Menson K, Park SD, Rivero JL, Taylor CT, Katz LM, Hill EW. Alterations in oxidative gene expression in equine skeletal muscle following exercise and training. *Physiol Genomics* 40: 83–93, 2010. First published October 27, 2009; doi:10.1152/physiolgenomics.00041.2009.—Intense selection for elite racing performance in the Thoroughbred horse (*Equus caballus*) has resulted in a number of adaptive physiological phenotypes relevant to exercise; however, the underlying molecular mechanisms responsible for these characteristics are not well understood. Adaptive changes in mRNA expression in equine skeletal muscle were investigated by real-time qRT-PCR for a panel of candidate exercise-response genes following a standardized incremental-step treadmill exercise test in eight untrained Thoroughbred horses. Biopsy samples were obtained from the gluteus medius before, immediately after, and 4 h after exercise. Significant ($P < 0.05$) differences in gene expression were detected for six genes (*CKM*, *COX4I1, COX4I2, PDK4, PPARGC1A*, and *SLC2A4*) 4 h after exercise. Investigation of relationships between mRNA and velocity at maximum heart rate (VHR_{max}) and peak postexercise plasma lactate concentration ($[La]T_1$) revealed significant ($P < 0.05$) associations with postexercise *COX4I1* and *PPARCG1A* expression and between $[La]T_1$ and basal *COX4I1* expression. Gene expression changes were investigated in a second cohort of horses after a 10 mo period of training. In resting samples, *COX4I1* gene expression had significantly increased following training, and, after exercise, significant differences were identified for *COX4I2, PDK4,* and *PPARGC1A*. Significant relationships with VHR_{max} and $[La]T_1$ were detected for *PPARGC1A* and *COX4I1*. These data highlight the roles of genes responsible for the regulation of oxygen-dependent metabolism, glucose metabolism, and fatty acid utilization in equine skeletal muscle adaptation to exercise.

mRNA; physiology; performance

SKELETAL MUSCLE RESPONDS TO alterations in bioenergetic demands through changes in gene expression (17, 33, 39, 54). Exercise-induced hypoxia is a key signal for such responses (22) that result in mitochondrial biogenesis, improved ATP provision (23), and muscle hypertrophy via coordinated molecular events (21). A single bout of endurance exercise generates multiple stresses in skeletal muscle that leads to increased oxygen consumption (4). Following exercise, skeletal muscle recovery is promoted by decreasing glucose consumption (31), elevated fatty acid oxidation (66), and the resynthesis of muscle glycogen (42).

Thoroughbred horses have a large offset between oxygen supply and demand during exercise (12) and therefore provide a valuable biological model to improve our understanding of the molecular response of skeletal muscle to exercise. The elite athletic phenotypes observed in Thoroughbred horses are a result of 400 years of selective breeding for structural and functional adaptations that enable superior performance. Thoroughbred horses have a range of extreme physiological characteristics enabling both high anaerobic and aerobic metabolic capabilities (14). The aerobic capacity or maximum oxygen uptake ($\dot{V}O_{2max}$) of Thoroughbreds (>200 ml $O_2 \cdot kg^{-1} \cdot min^{-1}$) is superior to other athletic species of similar size (24) and is obtained by an extraordinary oxygen carrying capacity and delivery assisted by adaptations within the respiratory and cardiovascular systems (11). As well as a large lung volume, high maximum hemoglobin concentration and cardiac output, adaptations include a large muscle mass (~55%)-to-body weight ratio, high skeletal muscle mitochondrial density and oxidative enzyme activity, and considerable intramuscular stores of energy substrates (primarily glycogen) (20). In Thoroughbred horses (as in humans), the $\dot{V}O_{2max}$ is generally restricted by oxygen supply to the mitochondria rather than by mitochondrial oxidative capacity (26), and as a result during maximal exercise increased oxygen requirements that cannot be fulfilled can lead to an exercise-induced arterial hypoxemia (12). It has been proposed that a greater efficiency of oxygen supply could therefore increase maximal performance in racehorses (16).

In most living organisms the requirement for cellular oxygen homeostasis has resulted in an efficient and rapid molecular response system that senses hypoxia, leading to the induction of an array of adaptive genes that facilitate increased oxygen supply and support anaerobic ATP generation (61). This response is governed by the transcription factor HIF (hypoxia-inducible factor). Because of the large requirement of the musculature for oxygen during exercise in the Thoroughbred, we investigated the gene expression responses of a panel of HIF-responsive genes, including glycolytic enzymes (56) and genes encoding key proteins for oxidative metabolism that are expressed in hypoxic conditions (18).

The transcriptional coactivator PGC-1α has emerged as a critical control factor in skeletal muscle adaptation to exercise acting via transcriptional coactivation of genes responsible for fatty acid oxidation, oxidative phosphorylation, mitochondrial

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Table 1. *Subject characteristics, physiological and biochemical details for group A, group B, and group C*

	Group A		*Group B*		*Group C*	
	Mean	SE(±)	Mean	SE(±)	Mean	SE(±)
Horses, *n*	8		12		9	
Age, mo	50	0.65	21	1.42	32	1.48
Pre-exercise body weight, kg	565.75	13.71	446.92	8.12		
Postexercise body weight, kg	560.75	13.27			447.11	9.65
Height, cm	165.25	1.44	154.58	0.87	162.44	1.43
Resting heart rate, beats/min	32.63	1.42			49.33	4.65
Maximum heart rate, beats/min	217.50	3.32			226.11	2.58
Velocity at maximum heart rate, m/s	12.43	0.24			13.69	0.17
Distance, m	4362.87	102.71			5430.89	126.93
Work days, *n*					10	1.68
Races, *n*					3	3.21
Pre-exercise lactate T_0, mmol/l	2.42	0.07			1.40	0.18
Postexercise lactate T_1, mmol/l	12.95	1.57			13.34	0.93
Postexercise lactate T_1 (5 mins), mmol/l	13.25	1.21			14.77	0.96
Postexercise lactate T_2 (4 h), mmol/l	2.96	0.32			1.03	0.31
Preexercise muscle biopsy T_0, h-min	1.19	0.03			1.34	0.23
Postexercise muscle biopsy T_1, min-s	6.46	0.07			3.20	0.24
Postexercise muscle biopsy T_2, h-min	4.14	0.02			3.56	0.11

biogenesis, muscle fiber type composition, and angiogenesis (5). The central role of PGC-1α in exercise led us to also investigate exercise-induced changes in expression of the gene encoding PGC-1α (*PPARGC1A*).

A single bout of acute exercise induces multiple stresses in skeletal muscle (34, 53), and the responses to these stressors can be divided into two broad categories: the return to homeostasis and the adaptive response. The adaptive response is the process whereby skeletal muscle responds to repeated exercise bouts (training) in ways that cumulatively lead to an enhanced ability to maintain muscle homeostasis during exercise. This training response involves both morphological changes such as hypertrophy and metabolic responses such as an increased capacity for oxidative substrate metabolism in mitochondria and a shift toward oxidizing a higher proportion of fats and less glucose during exercise (1, 10). The role of genes involved in oxidative metabolism may therefore alter with training. Here, we report changes in Thoroughbred skeletal muscle gene expression following a period of exercise training.

The products of many genes are likely to influence system-wide physiological responses. As Thoroughbreds have an unusually large muscle mass we investigated whether the expression of genes responsible for skeletal muscle adaptation affect overall physiological performance by exploring relationships between skeletal muscle mRNA expression profiles and measures of *1*) overall athletic ability [velocity at maximal heart rate (VHR_{max})] and *2*) anaerobic capacity (postexercise lactate concentration).

While studies investigating gene expression during human exercise exist (50, 54), the role of molecular signaling pathways in the phenotypic adaptation of skeletal muscle to exercise training in the horse has not been reported. This study reveals underlying molecular responses to exercise and training adaptations in equine skeletal muscle and the effect of such responses on system-wide physiological performance.

MATERIALS AND METHODS

All animal procedures were approved by the University College Dublin, Animal Research Ethics Committee; a license was granted from the Department of Health and Children (Ireland); and owners' consent was obtained for all horses.

Subjects

Details of subject cohorts are outlined in Table 1.

Group A. Four-year-old untrained Thoroughbred horses (castrated males, $n = 8$) were raised at the same farm for the previous 12 mo and destined for National Hunt racing with the same trainer.

Group B. Two-year-old ($n = 12$) untrained Thoroughbred horses ($n = 9$ females, $n = 3$ entire males) were raised on the same farm for the previous 2 mo and destined for Flat racing with the same trainer. *Group B* undertook a regular exercise regime with the same trainer for 10 mo (for details see Supplemental Information).[1]

Group C. A subset of $n = 9$ horses ($n = 6$ females, $n = 3$ entire males) from *group B* participated in an exercise test following the 10 mo period of training.

Exercise Protocol

Group A (untrained) and *group C* (trained) participated in a standardized incremental-step exercise test (52) on a high-speed equine treadmill (Sato, Knivsta, Sweden) (for details see Supplemental Information). HR was measured continuously by telemetry (Polar Equine S810i heart rate monitor system; Polar Electro, Warwick, UK).

Blood samples for plasma lactate:. Venous blood samples were collected before, immediately after exercise, 5 min after exercise, and 4 h postexercise and placed in fluoride oxalate tubes for the determination of plasma lactate concentrations using the YSI 2300 STAT Plus lactate analyzer (YSI UK, Hampshire, UK) (*group A*) or the L-Lactate randox kit and RX imola instrument (Randox, Antrim, UK) (*group C*).

Muscle Biopsy Sampling

Percutaneous needle muscle biopsies (29) were obtained from the dorsal compartment of the gluteus medius muscle (13) using a 6 mm diameter, modified Bergstrom biopsy needle (Jørgen KRUUSE, Veterinary Supplies). The gluteus medius is one of the largest locomotory muscles and is important for the generation of propulsive forces during exercise (49) and shows considerable adaption to training (57). Biopsy samples intended for mRNA gene expression analyses were preserved in RNA*later* (Ambion, UK).

[1] The online version of this article contains supplemental material.

Biopsy samples intended for muscle fiber type analyses were systematically frozen by immersion in isopentane (47).

Muscle biopsy samples for *group A* and *group C* were collected at rest pre-exercise (T_0), immediately postexercise (T_1), and 4 h postexercise (T_2). For *group B*, muscle biopsy samples were collected at rest (T_0) before training and at rest (T_0) following training. The time points were chosen to examine both the immediate and delayed response to endurance exercise (33) and the response to training.

RNA Isolation and Purification

Total RNA was extracted from ~100 mg tissue, using a protocol combining TRIzol reagent (Invitrogen, Paisley, UK), DNase treatment (RNase-free DNase; Qiagen, Crawley, UK) and RNeasy Mini-Kit (Qiagen). RNA was quantified using a Nano Drop ND1000 spectrophotometer V 3.5.2 (NanoDrop Technologies, Wilmington, DE), and RNA quality and purity were assessed using the 18S/28S ratio and RNA integrity number (RIN) on an Agilent Bioanalyser with the RNA 6000 Nano LabChip kit (Agilent Technologies Ireland, Dublin, Ireland). The RNA isolated from *group A* had an average RIN of 8.43 ± 0.08 (range 8.0–9.0), and *group B* and *C* had an average RIN of 7.63 ± 0.07 (range 6.3–8.3).

cDNA Synthesis and Real-time Quantitative RT-PCR

We reverse transcribed 2 μg of total RNA from each sample to cDNA with 50 μM oligo(dT) primers using a SuperScript III first-strand synthesis SuperMix kit (Invitrogen). Real-time quantitative RT-PCR (qRT-PCR) reactions were carried out as described by O'Gorman and colleagues (37) using the Applied Biosystems 7500 Fast qRT-PCR system [Apperla (ABI), Dublin, Ireland]. Reactions were performed in duplicate and control samples (no template controls) were run for each primer set.

Prior to the availability of the annotated horse genome sequence (EquCab2.0) PCR primers were designed by a comparative genomics approach. *Homo sapiens* or *Bos taurus* transcript sequences were extracted from the Ensembl genome browser (58) for each gene. The equine nucleotide sequence with greatest homology to the human/bovine transcript was identified along with its chromosomal location by basic local alignment search tool (BLAST) searching EquCab1.0 (3). The EquCab2.0 database was interrogated for titin (*TTN*) sequence. Primers for real-time qRT-PCR were designed to span an exon-exon boundary using the Vector NTI Advance software package (Invitrogen) or Primer3-web 0.3.0 version (www.primer3.sourceforge.net) and were commercially synthesized (Invitrogen and MWG Biotech). Primers were checked for sequence homology to other genomic regions using the National Center for Biotechnology Information BLAST (2). On availability of EquCab2.0, in silico PCR on the UCSC Genome Browser confirmed amplicon locations except the *ACTB* gene. The *ACTB* primers were a perfect match at two independent chromosome locations, the correct location for *ACTB* (Table 2), and the actin-α1, skeletal muscle gene (*ACTA1*) located on chromosome 12:6,869,114-6,869,212. For each primer pair, a preliminary qRT-PCR assay was performed to evaluate optimal primer concentrations, by titrating 100, 300, and 900 nM final concentrations, and disassociation curves were examined to evaluate the presence of nonspecific products or primer dimer artifacts. Details of primer sets, including those for the panel of reference genes, are given in Table 2.

Housekeeping Gene Normalization and Calculations

A panel of putative reference or "housekeeping" genes [*GAPDH,* glyceraldehyde-3-phosphate dehydrogenase (69); *TTN,* titin (17)*; B2M,* β-2-microglobulin (32); and *ACTB,* actin-β (36)] was evaluated for appropriateness using geNorm version 3.4 for Microsoft Excel (64). The average pairwise variation of each gene against all other genes was calculated for constant expression stability (*M*). Reference genes were ranked by *M* (the accepted limit is <1.5). The genes that displayed the greatest stability (lowest *M*) over the three time course samplings (T_0, T_1, T_2) were *GAPDH* and *TTN* ($M = 0.602$). Glycolytic enzymes have been identified as target genes of HIF-1 (56); therefore, *GAPDH* was excluded. Consequently, *TTN* was used as a single standard reference gene for these experiments.

Amplification efficiencies (*E*) were calculated for each primer pair using 1:6 serial dilutions of a template cDNA (pooled from the individuals studied). The amplification efficiency of each gene was determined to be ≥1.9 (90–102%) (62). Significant changes in gene expression were determined from the Ct (cycle threshold) values using the $2^{-\Delta\Delta Ct}$ method (30) where $2^{-\Delta\Delta Ct} = (Ct_{\text{gene of interest}} - Ct_{\text{reference gene}})_{Tx} - (Ct_{\text{mean gene of interest}} - Ct_{\text{mean reference gene}})_{T0}$. T_x corresponds to the time of interest. Relative fold changes in gene expression were determined across the time course for *group A* (T_0 T_1, T_2), *group B* (T_0, untrained; T_0, trained), and *group C* (T_0, T_1, T_2). Data were normalized to the reference gene (*TTN*) relative to resting gene expression (T_0), which represents a fold change of 1.

Data Analysis

Normal distribution of the gene expression data was confirmed using the one-sample Kolmogorov-Smirnov test (SPSS, Chicago, IL). *PDK4, PRKAA2,* and *PPARGC1A* did not have normal distributions, and therefore the $\log_{10}$ transformation was applied [$\log_{10}(2^{-\Delta\Delta Ct})$]. Student's *t*-test (parametric, paired, two-tailed test) identified significant differences ($\alpha = 0.05$) in gene expression between samples at each time point.

Linear regression and correlation analyses determined relationships between gene expression ($2^{-\Delta\Delta Ct}$) and physiological variables {VHR_{max} and peak postexercise lactate concentration ($[La]T_1$)} (GraphPad Software, San Diego, CA). The correlation coefficient (*r*), 95% confidence interval, significance value (*P*), and best-fit linear regression (including 95% confidence interval bands) were calculated.

Muscle Fiber Type and Myosin Heavy Chain Analyses

Muscle biopsy samples were cross-sectioned serially in a cryostat for immunocytochemistry (45, 47) followed by immunoperoxidase staining protocol with avidin-biotin complex (46).

Image Analysis and Morphometry

Serial sections were visualized and digitized within 2–3 h after staining (45). To establish muscle fiber type composition, at least 250 muscle fibers were identified in each muscle biopsy specimen. To correlate quantitative data between immunohistochemistry and myosin heavy chain (MyHC) electrophoresis, the hybrid type IIax fibers were split so that one-half were combined with type IIa and one half with type IIx fibers (46). These revised data were used to correlate with MyHC isoform electrophoretical composition.

MyHC Electrophoresis

MyHC electrophoresis was performed following the sodium dodecyl sulfate polyacrylamide gel electrophoresis protocol validated for horse muscle (48). Gels were scanned (GS-700; Bio-Rad, Hercules, CA), and a quantification of each MyHC isoform was obtained in relative terms for each muscle biopsy specimen (Multi-Analyst version 1.0, Bio-Rad).

RESULTS

This study describes mRNA transcript profiles for a panel of candidate genes in equine skeletal muscle following a single bout of exercise and after a 10 mo period of training.

Table 2. *Equine oligonucleotide primers for real-time qRT-PCR*

Gene Symbol	Gene Name	Forward Primer Sequence (5′-3′)	Reverse Primer Sequence (5′-3′)	Amplicon Size, bp	Chr	Location
		Reference Genes				
ACTB	actin-β	GTCACCCACACTGTGCCCATCTACG	CTTCATGAGGTAGTCCGTCAGGTCC	99	13	2,464,594-2,464,692
B2M	β2-microglobulin	GCGTGTTCCGAAGGTTCAGG	CGGTCGACTTTCATCTTCTCTCC	144	1	144,494,328-144,494,471
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	GAGATCAAGAAGGTGGTGAAGC	CATCGAAGGTGGAAGAGTGG	121	18	70,952,995-70,953,115
TTN	titin	GCATGACACAACTGGAAAGC	AACTTTGCCCTCATCAATGC	113	18	56,685,621-56,685,862
		Genes of Interest				
AK1	adenylate kinase 1	CAACGAGGAGACCATCAAGAAGC	ATGCCCCGTTTCTCGTAGAAGG	84	25	31,255,356-31,255,439
ALDOA	aldolase A, fructose-bisphosphate	TGAGAACACAGAGGAGAACCGACG	ACGCCCATCATCTGCTTTCTGG	130	13	19,618,764-19,618,893
CKM	creatine kinase, muscle	AACTACAAGCCCGAAGAG	CATCATCCAGAGTGAAGC	130	10	15,887,783-15,887,912
COX4I1	cytochrome c oxidase subunit IV isoform 1	ACGAGAGCTTTGCTGAAATG	TGCTTCTCCCAGATCAGAAG	109	3	32,772,517-32,772,625
COX4I2	cytochrome c oxidase subunit IV isoform 2 (lung)	TACACCAACTACCACGCCCAGC	AGCTGCCCTTCTCCTTCTCCTTC	106	22	22,683,852-22,683,957
ENO3	enolase 3 (β, muscle)	GTGGCAGCATCAGAGTTCTATCG	AGCTTCTCCCCAGTGATGTGC	92	11	49,570,216-49,570,307
HIF1A	hypoxia-inducible factor 1, α-subunit (basic helix-loop-helix transcription factor)	GACTTCCAGTTACGTTCC	GTTAGTGACGGTAGGAAGA	132	24	8,988,772-8,988,903
LDHA	lactate dehydrogenase A	CCGTGTTATCGGAAGTGGTTGC	AGAATCTCCATGCTCCCCAAGG	121	7	86,630,874-86,630,994
LONP1	lon peptidase 1, mitochondrial	GGAGAAGGAGGACAAGGACGC	TGAGGAGTGGTTGTCCAGCAG	133	7	3,471,557-3,471,689
PDK1	pyruvate dehydrogenase kinase, isozyme 1	GGATCAGGAACCGACACAATGATG	TGAACATTCTGGCTGGTGACAGG	103	18	51,624,272-51,624,374
PDK2	pyruvate dehydrogenase kinase, isozyme 2	GGTGCTAGAGTACAAGGACA	TAGAAGCGGTCCAGGAAGTA	80	11	25,838,750-25,838,829
PDK4	pyruvate dehydrogenase kinase, isozyme 4	GCTGGTTTTGGTTATGGCTTGC	TCCACAGACTCAGAAGACAAAGCC	137	4	38,965,192-38,966,723
PGK1	phosphoglycerate kinase	GAATGGGATGCCTTTGCTAAGG	AGCACAGCAAGTAGCAGTGTCTCC	114	20	47,802,289-47,802,402
PPARGC1A	peroxisome proliferator-activated receptor γ, coactivator 1α	TCTACCTAGGATGCATGG	GTGCAAGTAGAAACACTGC	93	3	100,878,084-100,878,176
PRKAA2	protein kinase, AMP-activated, α2 catalytic subunit	TGCCACCTCTTATAGCAGACAGCC	CTGACTTCGGATTCCAAGATGCC	119	2	3,150,720-3,150,838
SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3	GGAGACCACCACCAATGTCTAAGC	TCCTGGGTTACAACCTGATGAGG	110	6	35,459,954-35,460,063
SLC2A4	solute carrier family 2 (facilitated glucose transporter), member 4	TGTGGCATTCTTTGAGATTGGC	CCATGCCAATGATGAAGTTGC	137	11	50,291,318-50,291,454

Exercise Parameters

To provide context for the gene expression data physiological measurements during the exercise experiments were recorded. In both exercise groups (*group A* and *group C*), lactate concentrations reached levels >13 mmol/l, consistent with the work intensity reflecting a contribution of anaerobic metabolism to energy production (51).

Group A. Following warm-up, the exercise test comprised an average of six (range 5–7) incremental steps achieving a mean maximum velocity of 12.4 ± 0.2 m/s and a mean distance of 4,362.9 ± 102.7 m for an average duration of 8.77 ± 0.5 min. Mean $[La]T_1$ was 13.3 ± 1.2 mmol/l (Fig. 1), which was significantly ($P < 0.0001$) higher than pre-exercise values.

Group C. Following warm-up, the exercise test comprised an average of nine (range 8–10) incremental steps achieving a mean maximum velocity of 13.7 ± 0.2 m/s and a mean distance of 5,430.9 ± 126.9 m for an average duration of 11.33 ± 0.29 min. Mean $[La]T_1$ was 14.3 ± 1.0 mmol/l, which was significantly ($P < 0.0001$) higher than pre-exercise values.

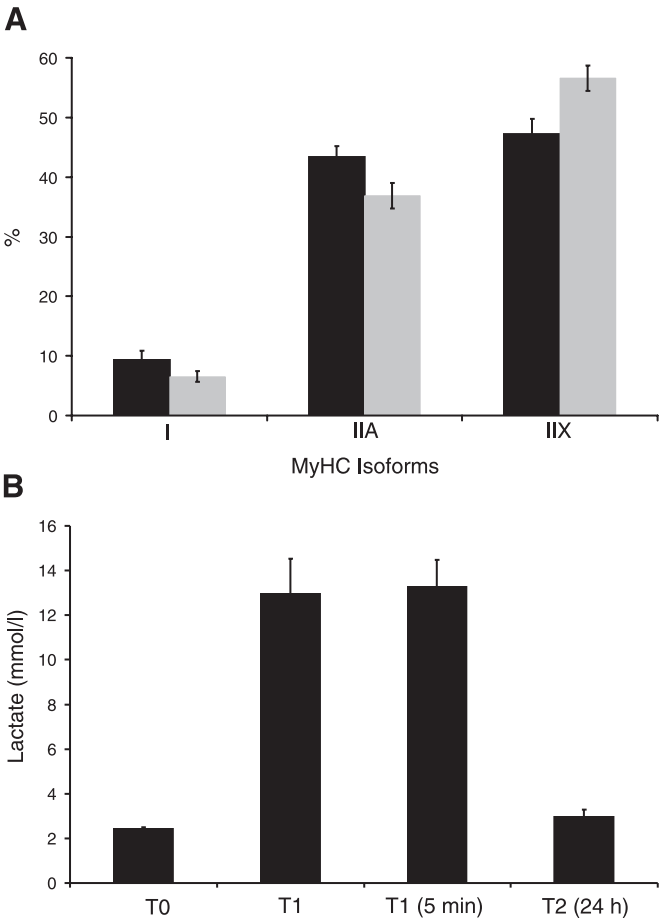

Fig. 1. *A*: skeletal myosin heavy chain (MyHC) composition, muscle fiber type distribution (%) in gluteus medius biopsy samples in *group A* ($n = 8$) derived by gel electrophoresis (light gray), immunohistochemistry (black bars); data are expressed as means ± SE. *B*: blood plasma lactate concentrations (mmol/l) for *group A* at rest (T_0), immediately postexercise (T_1), and 4 h postexercise (T_2).

Muscle Fiber-Type and MyHC Composition in Untrained Horses

In *group A* type II fibers were most abundant (>90%). The relative proportions of each myosin heavy chain (MyHC, %) determined by electrophoresis and immunohistochemistry were: MyHC-I (9.37, 6.54); MyHC-IIa (43.39, 36.88); and MyHC-IIx (47.24, 56.58) (Fig. 1). The mean proportions of MyHC-I, MyHC-IIa, and MyHC-IIx derived electrophoretically were similar to the relative proportions of type I, IIa and IIx fiber types determined by immunohistochemistry (MyHC I, $R^2 = 0.847$, $P < 0.001$; MyHC IIa, $R^2 = 0.665$, $P < 0.05$; MyHC IIx, $R^2 = 0.795$, $P < 0.01$).

Skeletal Muscle mRNA Expression Following Exercise

To investigate the effect of exercise on skeletal muscle mRNA expression we analyzed by qRT-PCR the gene expression responses of 18 genes with functions in the following KEGG pathways and gene ontology biological processes: glycolysis/gluconeogenesis (*ALDOA, ENO3, GAPDH, LDHA, PGK1*); oxidative phosphorylation (*COX4I1, COX4I2*); ATP-dependent proteolysis (*LONP1*); insulin signaling pathway (*PPARGC1A, PRKAA2*); phosphocreatine biosynthetic process (*CKM*); phosphotransferase activity (*AK1*); response to hypoxia (*HIF1A*); carbohydrate metabolic process and glucose metabolic process (*PDK1, PDK2, PDK4*); and glucose transport (*SLC2A3, SLC2A4*). For *group A* qRT-PCR was performed for all genes before exercise (T_0), immediately postexercise (T_1), and 4 h postexercise (T_2). Table 3 shows the relative mean fold differences between pre- and postexercise time points (T_0 vs. T_1; T_0 vs. T_2).

Analyses of mRNA profiles revealed no significant transcriptomic differences immediately postexercise (T_0 vs. T_1). However, 4 h postexercise (T_2) six of the 18 genes had significant ($P < 0.050$) differences relative to pre-exercise levels: *CKM* (1.3-fold, $P = 0.013$); *COX4I1* (1.4-fold, $P = 0.003$); *COX4I2* (−2.0-fold, $P < 0.001$); *PDK4* (7.4-fold, $P = 0.007$); *PPARGC1A* (6.0-fold, $P = 0.005$); and *SLC2A4* (1.4-fold, $P = 0.007$). Graphical representations of mean fold changes following exercise are shown relative to pre-exercise values in Fig. 2.

To validate these findings we performed a follow-on study in a cohort of trained horses (*group C*). A set of four genes (*COX4I1, COX4I2, PDK4,* and *PPARGC1A)* that had the greatest differences between time points and also had a significant relationship with a physiological variable in *group A* was selected. Table 4 shows the mean fold changes relative to pre-exercise mRNA expression (T_0 vs. T_1, T_0 vs. T_2). Analyses of mRNA profiles revealed no significant fold differences immediately postexercise (T_0 vs. T_1). Three of the four genes had significant ($P < 0.05$) fold differences 4 h postexercise (T_0 vs. T_2): *COX4I2* (−1.5-fold, $P = 0.005$); *PDK4* (2.3-fold, $P = 0.046$); and *PPARGC1A* (3.4-fold, $P < 0.001$). Graphical representations of mean fold changes following exercise are shown relative to pre-exercise values in Fig. 3.

To investigate whether the basal expression of these genes changed with training, we created mRNA profiles for the same set of four genes in *group B*. *COX4I1* gene expression in-

Table 3. *Relative gene expression fold changes between time points following treadmill exercise in group A*

		T_0 vs. T_1		T_0 vs. T_2	
	Gene Symbol	Fold Change	*P* Value	Fold Change	*P* Value
ATP-dependent proteolysis	*LONP1*	1.03	0.695	−1.07	0.449
Carbohydrate/glucose metabolic process	*PDK1*	1.01	0.742	−1.03	0.452
	PDK2	−1.08	0.242	−1.08	0.183
	PDK4	3.01	0.222	**7.51**	**0.007**
Glucose transport	*SLC2A3*	1.61	0.236	1.16	0.673
	SLC2A4	1.14	0.067	**1.42**	**0.007**
Glycolysis/gluconeogenesis	*ALDOA*	1.19	0.328	1.01	0.898
	ENO3	1.15	0.360	1.02	0.816
	GAPDH	1.18	0.055	1.16	0.115
	LDHA	1.13	0.277	−1.15	0.202
	PGK1	1.02	0.768	1.05	0.564
Hypoxic response	*HIF1A*	1.03	0.865	−1.03	0.803
Insulin signaling pathway	*PPARGC1A*	1.26	0.304	**5.95**	**0.005**
	PRKAA2	1.12	0.891	−1.06	0.571
Oxidative phosphorylation	*COX4I1*	1.07	0.544	**1.36**	**0.003**
	COX4I2	−1.17	0.069	**−2.02**	**0.000**
Phosphocreatine biosynthetic process	*CKM*	1.05	0.345	**1.27**	**0.013**
Phosphotransferase activity	*AK1*	1.09	0.434	1.10	0.437

T0, at rest pre-exercise; T1, immediately postexercise; T2, 4 h postexercise. Boldface indicates significant fold change.

creased significantly following a 10 mo period of training (1.3-fold, $P = 0.020$). Graphical representations of fold changes following training are shown relative to untrained values in Fig. 3*A*.

Relationship Between Skeletal Muscle mRNA Expression and Physiological Variables

We investigated the relationships between mRNA abundance and two measured physiological variables: VHR_{max} and $[La]T_1$. These analyses were limited to the six genes that had significant fold differences between T_0 and T_2 (*CKM, COX4I1, COX4I2, PDK4, PPARGC1A, SLC2A4*). Linear regression and correlation analyses were performed for individual $2^{-\Delta\Delta CT}$ values and VHR_{max} and $[La]T_1$ at each time point (T_0, T_1, T_2) for *group A*.

In *group A*, significant relationships between mRNA level and VHR_{max} and $[La]T_1$ were detected for two genes, *PPARGC1A* and *COX4I1*. Both genes had significant relationships with VHR_{max} 4 h postexercise: *COX4I1* ($R^2 = 0.607$, $P = 0.023$) and *PPARGC1A* ($R^2 = 0.730$, $P = 0.007$)

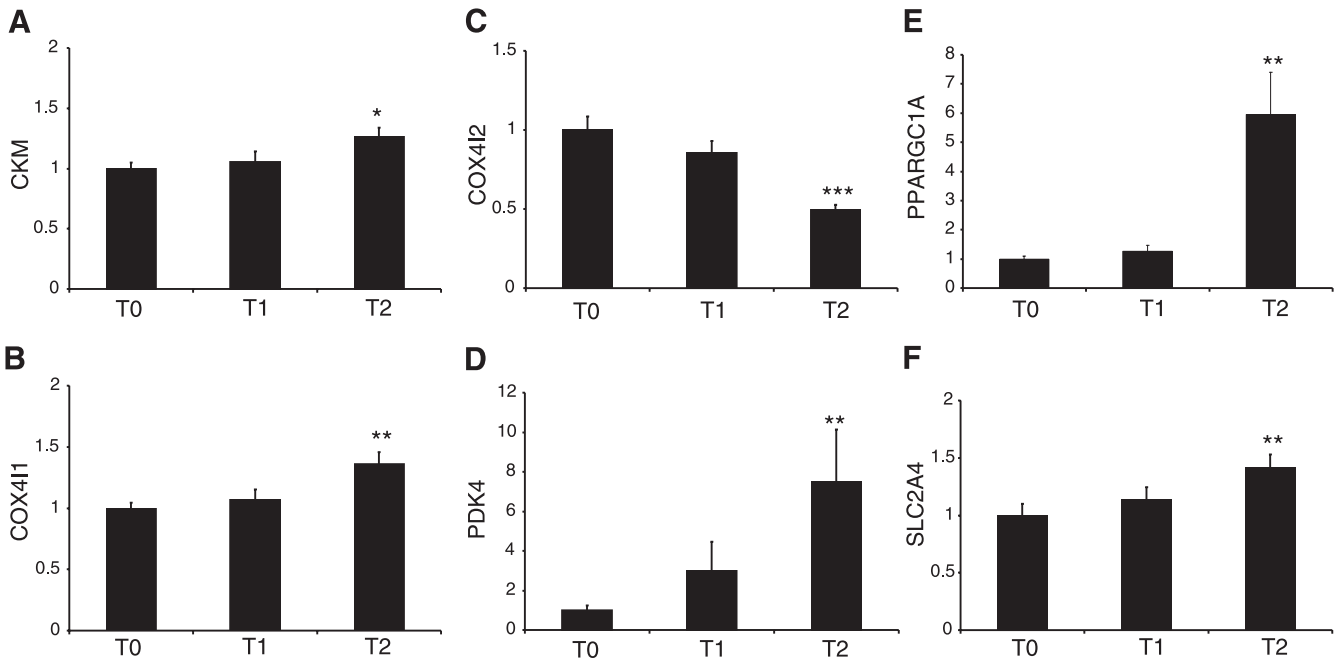

Fig. 2. Relative mean fold changes in mRNA expression of candidate genes determined by real-time qRT-PCR in untrained horses. Influence of a single bout of endurance exercise in Thoroughbred horses from *group C* at T_0, T_1, and T_2. Data represent significant mean fold changes in mRNA expression for 6 genes *CKM* (*A*), *COX4I1* (*B*), *COX4I2* (*C*), *PDK4* (*D*), *PPARGC1A* (*E*), *SLC2A4* (*F*) between the sampling time points. qRT-PCR data was normalized to the reference gene (*TTN*), relative to resting gene expression (T_0), which represents a fold change of 1. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ determined by paired Student's *t*-tests.

Table 4. *Gene expression changes between time points following a period of training (group B) and following treadmill exercise in trained horses (group C)*

	Rest		Trained - Exercise			
	UT vs. T		T_0 vs. T_1		T_0 vs. T_2	
Gene Symbol	Fold Change	*P* Value	Fold Change	*P* Value	Fold Change	*P* Value
PDK4	−1.41	0.226	1.24	0.282	**2.33**	**0.046**
PPARGC1A	1.08	0.755	−1.08	0.473	**3.37**	**0.000**
COX4I1	**1.28**	**0.020**	−1.04	0.184	1.09	0.768
COX4I2	1.12	0.260	1.24	0.134	**−1.53**	**0.005**

T, trained; UT, untrained. Boldface indicates significant fold change.

(Fig. 4). The strongest correlation was between $[La]T_1$ and *PPARGC1A* mRNA 4 h postexercise ($R^2 = 0.939$, $P < 0.001$). Significant associations between *COX4I1* mRNA and $[La]T_1$ before exercise ($R^2 = 0.588$, $P = 0.027$) and 4 h postexercise ($R^2 = 0.577$, $P = 0.029$) were also observed (Fig. 4).

We also performed these analyses in the trained cohort (*group C*). Resting (T_0) *PPARGC1A* mRNA had a significant inverse relationship with $[La]T_1$ ($R^2 = 0.464$, $P = 0.043$) (Fig. 4).

DISCUSSION

While the phenotypic adaptations to elite athleticism in Thoroughbred horses are well described, the molecular mechanisms underlying these characteristics are poorly understood and there are few documented studies describing gene and protein expression in equine skeletal muscle following exercise (15, 25, 35). Here we report for the first time that in equine skeletal muscle a single bout of treadmill exercise alters expression of metabolic genes. To understand the relevance of these findings to overall athletic capability we investigated expression relationships with physiological performance measures and compared the findings with gene expression observations following a period of training.

Lack of Evidence for HIF-activated Response in Exercising Equine Skeletal Muscle

Local hypoxia is an important stimulus for structural and functional changes in skeletal muscle (4). As horses routinely develop a marked arterial hypoxemia during intense exercise (55) we investigated the effect of exercise on HIF-1α-dependent gene expression. HIF-1α protein accumulation is dependent on posttranscriptional mechanisms (4), and therefore it was not surprising that we did not observe a significant alteration in *HIF1A* (encoding HIF-1α) transcript expression. Prolonged exercise training under hypoxic conditions may, however, increase *HIF1A* mRNA levels in human skeletal muscle (65). The observed increase in HIF-1α protein (Supplemental Fig. S1) and elevated lactate concentrations postexercise are suggestive of stimuli for activation of glycolysis. Surprisingly, we observed no significant change in gene expression for the panel of

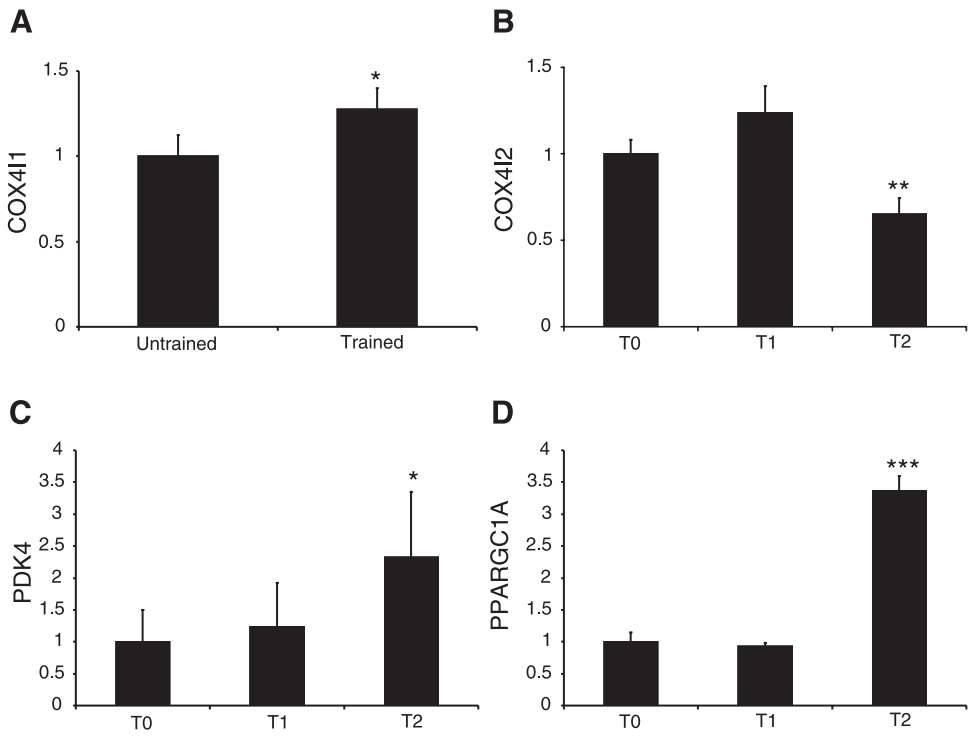

Fig. 3. Relative mean fold changes in mRNA expression of candidate genes determined by real-time qRT-PCR in trained horses. A significant mean fold change in gene expression was determined for *COX4I1* in *group B* following a 10 mo training period (T_0) (*A*). Influence of a single bout of endurance exercise in Thoroughbred horses from *group C* at T_0, T_1, and T_2. Significant changes in mRNA expression were identified for *COX4I2* (*B*), *PDK4* (*C*), and *PPARGC1A* (*D*) between T_0 vs. T_2 following a single bout of endurance exercise in trained Thoroughbred horses from *group C* (rest, T_0; immediately postexercise, T_1; 4 h postexercise, T_2). qRT-PCR data was normalized to the reference gene (*TTN*), relative to resting gene expression (T_0), which represents a fold change of 1. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ determined by paired Student's *t*-tests.

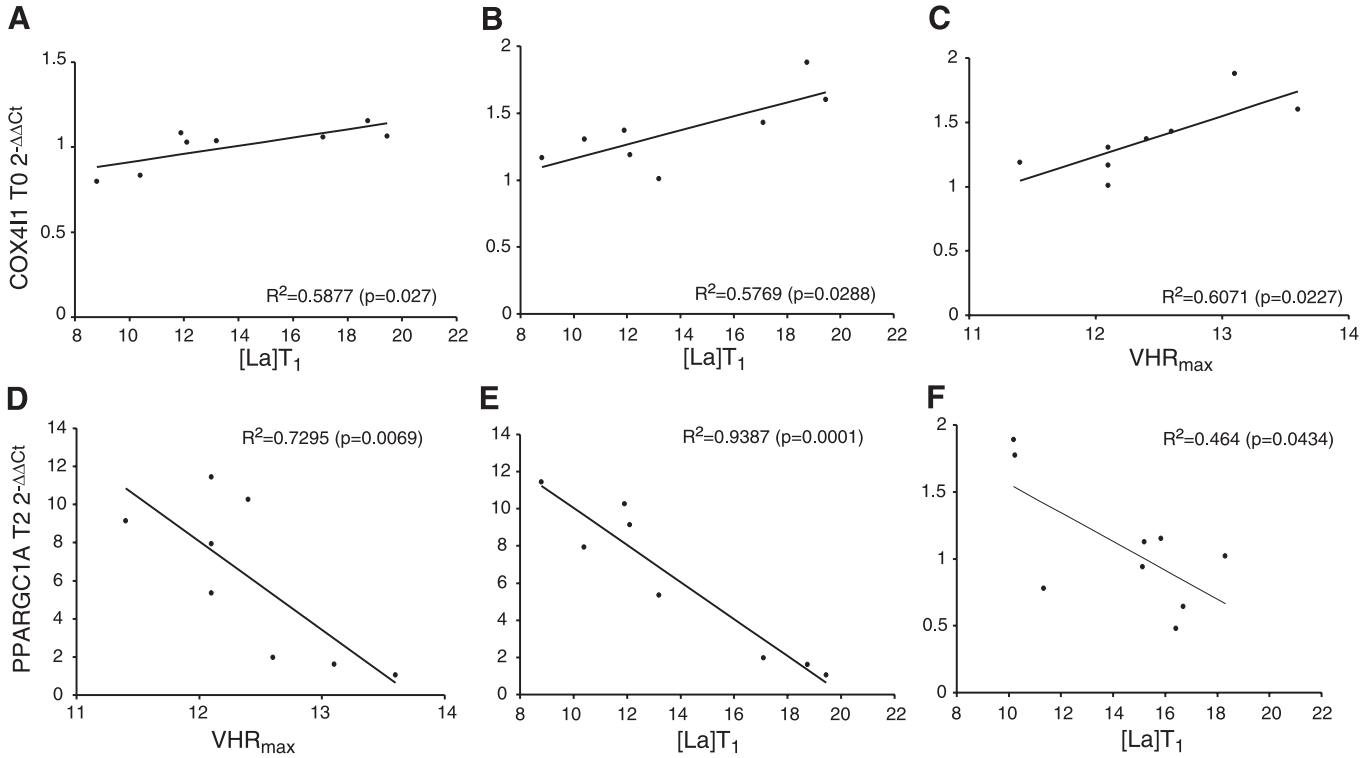

Fig. 4. Candidate gene expression relationships with measured physiological variables. Relationships between velocity at maximum heart rate (VHR_{max}) and peak postexercise lactate concentration ($[La]T_1$) and mRNA gene expression ($2^{-\Delta\Delta Ct}$) for *COX4I1* and *PPARGC1A* in *group A* (*A–E*) and *group C* (*F*). Regression and correlation analyses for *COX4I1* T_0 vs. $[La]T_1$ (*A*), *COX4I1* T_2 vs. $[La]T_1$ (*B*), *COX4I1* T_2 vs. VHR_{max} (*C*), *PPARGC1A* T_2 vs. VHR_{max} (*D*), *PPARGC1A* T_2 vs. $[La]T_1$ (*E*), *PPARGC1A* T_0 vs. $[La]T_1$ (*F*).

glycolytic enzymes despite elevated lactate concentrations indicating increased glycolytic flux. This is both in agreement and conflict with human exercise studies (54, 67) and may be a function of the duration and intensity of the exercise and the time at which samples were collected.

As well as a role in the regulation of glycolysis, HIF-1α regulates the oxidative enzyme COX4 (cytochrome c oxidase, subunit 4) in an oxygen dependent manner by alternately recruiting the isoforms COX4-1 (*COX4I1*) and COX4-2 (*COX4I2*) (18). In normal oxygen concentrations gene expression of *COX4I1* is increased and *COX4I2* is repressed. Alternatively, under conditions of reduced oxygen, HIF-1α activity is increased along with a number of downstream genes including *COX4I2* and a mitochondrial protease gene *LONP1*, required for COX4I1 protein degradation. The COX4 subunit switching provides a mechanism to maximize the efficiency of respiration under different oxygen concentrations (18). We found no alteration in the expression of *LONP1* and a significant decrease in *COX4I2* mRNA 4 h postexercise in both the untrained (−2.0-fold, $P < 0.001$) and trained (−1.5-fold, $P = 0.005$) cohorts, a pattern consistent with sufficient oxygen availability. This was supported by the significant increase in *COX4I1* expression at the same time point (1.4-fold, $P = 0.003$). The exercise duration and intensity was indicative of the horses exercising at $\dot{V}O_{2max}$. Shorter duration (i.e., <70 s) and higher intensity (supramaximal) exercise in trained individuals may be necessary to illicit a HIF-mediated response resulting in COX4 subunit switching.

Basal COX4I1 Activity Increases With Training and Postexercise Expression is Positively Related to Athletic Ability

While *COX4I1* transcripts increased significantly postexercise in the untrained cohort, significant increases in *COX4I1* expression were not observed in the trained cohort. The positive relationship between *COX4I1* and overall athletic ability (measured as VHR_{max}) in the untrained cohort suggested the importance of local transcriptional changes contributing to the system-wide phenotype. This was supported by our observation of a significant increase in basal *COX4I1* (1.3-fold, $P = 0.020$) following the 10 mo training period. There is a direct relationship between mitochondrial COX4 abundance and mitochondrial density (17), and the increase in *COX4I1* mRNA transcripts following training may reflect a long-term adaptive response resulting in increased mitochondrial capacity. It is well established that training improves oxidative capacity, and recently it has been demonstrated that endurance training increases basal oxidative flux enhancing muscle insulin sensitivity via increased fatty acid oxidation in the resting state, while not affecting energy production (7). In horses, *COX4I1* mRNA responses may therefore reach a plateau during training following the accumulation of mitochondria that enable improved basal oxidative activity.

Significant Relationship Between PGC-1α and Plasma Lactate Accumulation

PGC-1α plays a key role in cellular energy metabolism and homeostasis (40) and is induced under multiple physical exercise regimes including either single endurance or long-term

training regimes in humans and rodents (6, 39). PGC-1α regulates genes involved in mitochondrial biogenesis (68) resulting in an enhanced capacity for oxidative phosphorylation (38), and a linear relationship with COX4 has been described (8). In this study we also observed a relationship ($r^2 = 0.556$, $P = 0.034$) between *PPARGC1A* and *COX4I2* gene expression immediately postexercise in the untrained cohort, but this is not evident for *COX4I1,* or at any other time point. The significance of this is unclear. In untrained horses *PPARGC1A* mRNA transcripts had a marked increase in expression (6.0-fold, $P = 0.005$) 4 h postexercise, while in trained horses the response was effectively reduced by half (3.4-fold, $P = 0.0001$). This is in contrast to studies in human subjects where prolonged low resistance training resulted in a marked increase in skeletal muscle *PPARGC1A* mRNA expression (39). This may reflect the age variation between the untrained (4 yr old) and trained (2 yr old) sample cohorts, which is recognized as a limitation to these comparisons. Nonetheless, skeletal muscle adaptations in horses have previously been shown to plateau after a certain period of training (63). While these data require further validation in comparable cohorts, our data provide some indication that molecular responses that may influence aerobic respiration during exercise may be blunted in trained equine skeletal muscle, while longer-term adaptations are reflected in basal properties. Therefore, exercise following a sustained period of training may serve to maintain, rather than enhance, mitochondrial function.

We observed highly significant inverse linear relationships with *PPARGC1A* mRNA and both VHR_{max} and $[La]T_1$ 4 h postexercise. The trend indicated higher *PPARGC1A* expression associated with lower speeds and less lactate accumulation. There is good evidence to indicate that PGC-1α may influence lactate uptake into skeletal muscle by mediation through the monocarboxylate transporter, MCT1 (9). While we have not investigated *MCT1* gene expression our data seem to indicate lower lactate accumulation in the bloodstream, which may reflect greater skeletal muscle lactate uptake, associated with higher expression of PGC-1α. Interestingly, in the trained cohort these exercising gene expression relationships were not maintained, but basal mRNA levels were significantly associated with peak postexercise plasma lactate concentrations.

Targeted Selection for Fatty Acid Metabolism

PGC-1α is a key regulator of insulin sensitivity by controlling glucose transport. In an inactive or fasting state glucose uptake into muscle is mediated by SLC2A3 (59), while during exercise, this process is regulated by SLC2A4 (GLUT4) (27). We observed a significant increase in *SLC2A4* [solute carrier family 2 (facilitated glucose transporter), member 4] gene expression 4 h postexercise and a significant linear relationship ($r^2 = 0.566$, $P = 0.031$, data not shown) with *PPARGC1A*. The utilization of glucose is also tightly regulated by the assembly of the pyruvate dehydrogenase complex (PDC), which is controlled by pyruvate dehydrogenase kinase (PDK). PDK blocks the formation of the PDC resulting in the β-oxidation of fatty acids to acetyl-CoA as the substrate for oxidative phosphorylation. We observed a 7.5-fold increase in *PDK4* mRNA 4 h postexercise in the untrained cohort of horses. While there was also a significant increase in *PDK4* in the trained cohort, it was twofold less than the untrained group. The increase in *PDK4* mRNA during recovery from exercise is consistent with prolonged inhibition of the PDC to decrease glucose oxidation and increase mitochondrial fatty acid oxidation (66). In a population genomics-based genome scan we recently identified a number of key genomic regions that have been targets for selection in Thoroughbreds that contain genes relevant to exercise-related traits. In particular metabolic genes, including *PDK4* and *COX4I1*, with biological functions in insulin signaling and fatty acid metabolism, were enriched in positively selected regions (19).

As well as directly influencing the mitochondrial phenotype, PGC-1α has been reported to be involved in muscle fiber type switching from fast-twitch (type II) to slow-twitch (type I) fibers (28). Unusually for mammalian species horses have a much greater proportion of type II fibers (41) and in particular type IIx fibers that have a maximal velocity of shortening that is three times higher than that of IIa fibers (43) but a very low oxidative capacity. The untrained cohort in our study had a significantly higher proportion of fast-twitch muscle fibers (Fig. 1*A*). While muscle fiber types were not quantified in the trained cohort, it has been established that training in Thoroughbred horses induces the conversion of MyHC-IIx to MyHC-IIa isoforms leading to increased resistance to fatigue (44).

In summary, we have presented the first study to identify temporally altered metabolic gene transcripts in equine skeletal muscle following exercise. In particular genes responsible for fatty acid metabolism, oxidative metabolism, and mitochondrial function are differentially regulated. The most notable finding was the strikingly similar pattern observed for the four tested genes in the trained cohort; compared with the untrained group there was a markedly reduced (*PPARGC1A, PDK4, COXI2*) or absent (*COX4I1*) response following exercise. While our results indicate that local mRNA adaptations contribute in part to the overall athletic phenotype, it is likely that many genes and their products will contribute additively to effect an adapted physiology.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES

1. **Adhihetty PJ, Irrcher I, Joseph AM, Ljubicic V, Hood DA.** Plasticity of skeletal muscle mitochondria in response to contractile activity. *Exp Physiol* 88, 99–107, 2003.
2. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** Basic local alignment search tool. *J Mol Biol* 215: 403–410, 1990.
3. **Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ.** Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl Acids Res* 25: 3389–3402, 1997.
4. **Ameln H, Gustafsson T, Sundberg CJ, Okamoto K, Jansson E, Poellinger L, Makino Y.** Physiological activation of hypoxia inducible factor-1 in human skeletal muscle. *FASEB J* 19: 1009–1011, 2005.
5. **Arany Z.** PGC-1 coactivators and skeletal muscle adaptations in health and disease. *Curr Opin Genetics Dev* 18: 426–434, 2008.
6. **Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, Kelly DP, Holloszy JO.** Adaptations of skeletal muscle to exercise: rapid

increase in the transcriptional coactivator PGC-1. *FASEB J* 16: 1879–1886, 2002.
7. **Befroy DE, Petersen KF, Dufour S, Mason GF, Rothman DL, Shulman GI.** Increased substrate oxidation and mitochondrial uncoupling in skeletal muscle of endurance-trained individuals. *Proc Natl Acad Sci USA* 105: 16701–16706, 2008.
8. **Benton CR, Nickerson JG, Lally J, Han XX, Holloway GP, Glatz JF, Luiken JJ, Graham TE, Heikkila JJ, Bonen A.** Modest PGC-1alpha overexpression in muscle in vivo is sufficient to increase insulin sensitivity and palmitate oxidation in subsarcolemmal, not intermyofibrillar, mitochondria. *J Biol Chem* 283: 4228–4240, 2008.
9. **Benton CR, Yoshida Y, Lally J, Han XX, Hatta H, Bonen A.** PGC-1alpha increases skeletal muscle lactate uptake by increasing the expression of MCT1 but not MCT2 or MCT4. *Physiol Genomics* 35: 45–54, 2008.
10. **Carter SL, Rennie C, Tarnopolsky MA.** Substrate utilization during endurance exercise in men and women after endurance training. *Am J Physiol Endocrinol Metab* 280: E898–E907, 2001.
11. **Constantinopol M, Jones JH, Weibel ER, Taylor CR, Lindholm A, Karas RH.** Oxygen transport during exercise in large mammals. II. Oxygen uptake by the pulmonary gas exchanger. *J Appl Physiol* 67: 871–878, 1989.
12. **Dempsey JA, Wagner PD.** Exercise-induced arterial hypoxemia. *J Appl Physiol* 87: 1997–2006, 1999.
13. **Dingboom EG, Dijkstra G, Enzerink E, van Oudheusden HC, Weijs WA.** Postnatal muscle fibre composition of the gluteus medius muscle of Dutch Warmblood foals: maturation and the influence of exercise. *Equine Vet J* 95–100, 1999.
14. **Eaton MD, Evans DL, Hodgson DR, Rose RJ.** Maximal accumulated oxygen deficit in thoroughbred horses. *J Appl Physiol* 78: 1564–1568, 1995.
15. **Eizema K, van den Burg M, Kiri A, Dingboom EG, van Oudheusden H, Goldspink G, Weijs WA.** Differential expression of equine myosin heavy-chain mRNA and protein isoforms in a limb muscle. *J Histochem Cytochem* 51: 1207–1216, 2003.
16. **Erickson BK, Seaman J, Kubo K, Hiraga A, Kai M, Yamaya Y, Wagner PD.** Mechanism of reduction in alveolar-arterial PO2 difference by helium breathing in the exercising horse. *J Appl Physiol* 76: 2794–2801, 1994.
17. **Flück M.** Functional, structural and molecular plasticity of mammalian skeletal muscle in response to exercise stimuli. *J Exp Biol* 209: 2239–2248, 2006.
18. **Fukuda R, Zhang H, Kim JW, Shimoda L, Dang CV, Semenza GL.** HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. *Cell* 129: 111–122, 2007.
19. **Gu J, Orr N, Park SD, Katz LM, Sulimova G, MacHugh DE, Hill EW.** A genome scan for positive selection in Thoroughbred horses. *PLos 1* 4: e5767, 2009.
20. **Hinchcliff KW, Geor RJ.** *The Horse as an Athlete: a Physiological Overview. Equine Exercise Physiology: The Science of Exercise in the Athletic Horse*. Edinburgh: Saunders/Elsevier, 2008, p. 463.
21. **Hoppeler H, Flück M.** Plasticity of skeletal muscle mitochondria: structure and function. *Med Sci Sports Exerc* 35: 95–104, 2003.
22. **Hoppeler H, Vogt M.** Muscle tissue adaptations to hypoxia. *J Exp Biol* 204: 3133–3139, 2001.
23. **Irrcher I, Adhihetty PJ, Joseph AM, Ljubicic V, Hood DA.** Regulation of mitochondrial biogenesis in muscle by endurance exercise. *Sports Med* 33: 783–793, 2003.
24. **Jones JH, Longworth KE, Lindholm A, Conley KE, Karas RH, Kayar SR, Taylor CR.** Oxygen transport during exercise in large mammals. *I* Adaptive variation in oxygen demand. *J Appl Physiol* 67: 862–870, 1989.
25. **Jose-Cunilleras E, Hayes KA, Toribio RE, Mathes LE, Hinchcliff KW.** Expression of equine glucose transporter type 4 in skeletal muscle after glycogen-depleting exercise. *Am J Vet Res* 66: 379–385, 2005.
26. **Katz LM, Bayly WM, Hines MT, Sides RH.** Ventilatory responses of ponies and horses to exercise. *Equine Comp Physiol* 2: 229–240, 2005.
27. **Kraniou GN, Cameron-Smith D, Hargreaves M.** Acute exercise and GLUT4 expression in human skeletal muscle: influence of exercise intensity. *J Appl Physiol* 101: 934–937, 2006.
28. **Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R, Spiegelman BM.** Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* 418: 797–801, 2002.
29. **Lindholm A, Piehl K.** Fibre composition, enzyme activity and concentrations of metabolites and electrolytes in muscles of standardbred horses. *Acta Vet Scand* 15: 287–309, 1974.
30. **Livak KJ, Schmittgen TD.** Analysis of relative gene expression data using real-time quantitative PCR and the $2[-\Delta\Delta C(T)]$ method. *Methods* 25: 402–408, 2001.
31. **Long YC, Zierath JR.** Influence of AMP-activated protein kinase and calcineurin on metabolic networks in skeletal muscle. *Am J Physiol Endocrinol Metab* 295: E545–E552, 2008.
32. **Mahoney DJ, Carey K, Fu MH, Snow R, Cameron-Smith D, Parise G, Tarnopolsky MA.** Real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise. *Physiol Genomics* 18: 226–231, 2004.
33. **Mahoney DJ, Parise G, Melov S, Safdar A, Tarnopolsky MA.** Analysis of global mRNA expression in human skeletal muscle during recovery from endurance exercise. *FASEB J* 19: 1498–1500, 2005.
34. **Martineau LC Gardiner PF.** Insight into skeletal muscle mechanotransduction: MAPK activation is quantitatively related to tension. *J Appl Physiol* 91: 693–702, 2001.
35. **Mucher E, Jayr L, Rossignol F, Amiot F, Gidrol X, Barrey E.** Gene expression profiling in equine muscle tissues using mouse cDNA microarrays. *Equine Vet J* 359–364, 2006.
36. **Norrbom J, Sundberg CJ, Ameln H, Kraus WE, Jansson E, Gustafsson T.** PGC-1alpha mRNA expression is influenced by metabolic perturbation in exercising human skeletal muscle. *J Appl Physiol* 96: 189–194, 2004.
37. **O'Gorman GM, Park SD, Hill EW, Meade KG, Mitchell LC, Agaba M, Gibson JP, Hanotte O, Naessens J, Kemp SJ, MacHugh DE.** Cytokine mRNA profiling of peripheral blood mononuclear cells from trypanotolerant and trypanosusceptible cattle infected with Trypanosoma congolense. *Physiol Genomics* 28: 53–61, 2006.
38. **O'Hagan KA, Cocchiglia S, Zhdanov AV, Tambawala MM, Cummins EP, Monfared M, Agbor TA, Garvey JF, Papkovsky DB, Taylor CT, Allan BB.** PGC-1 alpha is coupled to HIF-1 alpha-dependent gene expression by increasing mitochondrial oxygen consumption in skeletal muscle cells. *Proc Natl Acad Sci USA* 106: 2188–2193, 2009.
39. **Pilegaard H, Saltin B, Neufer PD.** Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. *J Physiol* 546: 851–858, 2003.
40. **Puigserver P, Spiegelman BM.** Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. *Endocr Rev* 24: 78–90, 2003.
41. **Quiroz-Rothe E, Rivero JL.** Co-ordinated expression of contractile and non-contractile features of control equine muscle fibre types characterised by immunostaining of myosin heavy chains. *Histochem Cell Biol* 116: 299–312, 2001.
42. **Ren JM, Marshall BA, Gulve EA, Gao J, Johnson DW, Holloszy JO, Mueckler M.** Evidence from transgenic mice that glucose transport is rate-limiting for glycogen deposition and glycolysis in skeletal muscle. *J Biol Chem* 268: 16113–16115, 1993.
43. **Rivero J Piercy RJ.** *Muscle Physiology: Responses to Exercise and Training*. Edinburgh: Saunders Elsevier, 2008, p. 30–80.
44. **Rivero JL, Ruz A, Marti-Korff S, Estepa JC, Aguilera-Tejero E, Werkman J, Sobotta M, Lindner A.** Effects of intensity and duration of exercise on muscular responses to training of thoroughbred racehorses. *J Appl Physiol* 102: 1871–1882, 2007.
45. **Rivero JL, Serrano AL, Barrey E, Valette JP, Jouglin M.** Analysis of myosin heavy chains at the protein level in horse skeletal muscle. *J Muscle Res Cell Motil* 20: 211–221, 1999.
46. **Rivero JL, Talmadge RJ, Edgerton VR.** Correlation between myofibrillar ATPase activity and myosin heavy chain composition in equine skeletal muscle and the influence of training. *Anat Rec* 246: 195–207, 1996.
47. **Rivero JL, Talmadge RJ, Edgerton VR.** Myosin heavy chain isoforms in adult equine skeletal muscle: an immunohistochemical and electrophoretic study. *Anat Rec* 246: 185–194, 1996.
48. **Rivero JL, Talmadge RJ, Edgerton VR.** A sensitive electrophoretic method for the quantification of myosin heavy chain isoforms in horse skeletal muscle: histochemical and immunocytochemical verifications. *Electrophoresis* 18: 1967–1972, 1997.
49. **Robert C, Valette JP, Degueurce C, Denoix JM.** Correlation between surface electromyography and kinematics of the hindlimb of horses at trot on a treadmill. *Cells Tissues Organs* 165: 113–122, 1999.

50. **Rockl KS, Witczak CA, Goodyear LJ.** Signaling mechanisms in skeletal muscle: acute responses and chronic adaptations to exercise. *IUBMB Life* 60: 145–153, 2008.
51. **Roneus N, and Essen-Gustavsson B.** Skeletal muscle characteristics and metabolic response to exercise in young standardbreds. *Am J Vet Res* 58: 167–170, 1997.
52. **Rose RJ, Hendrickson DK, Knight PK.** Clinical exercise testing in the normal thoroughbred racehorse. *Australian Vet J* 67: 345–348, 1990.
53. **Sahlin KTM, Söderlund K.** Energy supply, and muscle fatigue in humans. *Acta Physiol Scand* 162: 261–266, 1998.
54. **Schmutz S, Däpp C, Wittwer M, Vogt M, Hoppeler H, Flück M.** Endurance training modulates the muscular transcriptome response to acute exercise. *Pflügers Arch* 451: 678–687, 2006.
55. **Seaman J, Erickson BK, Kubo K, Hiraga A, Kai M, Yamaya Y, Wagner PD.** Exercise induced ventilation/perfusion inequality in the horse. *Equine Vet J* 27: 104–109, 1995.
56. **Semenza GL.** Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. *Annu Rev Cell Dev Biol* 15: 551–578, 1999.
57. **Snow DH, Valberg SJ.** *Muscle Anatomy, Physiology and Adaptations to Exercise and Training*. Philadelphia: Saunders, 1994, p. 145–179.
58. **Spudich G, Fernandez-Suarez XM, Birney E.** Genome browsing with Ensembl: a practical overview. *Brief Funct Genomic Proteomic* 6: 202–219, 2007.
59. **Stuart CA, Wen G, Williamson ME, Jiang J, Gilkison CR, Blackwell SJ, Nagamani M, Ferrando AA.** Altered GLUT1 and GLUT3 gene expression and subcellular redistribution of GLUT4: protein in muscle from patients with acanthosis nigricans and severe insulin resistance. *Metabolism* 50: 771–777, 2001.
60. **Sugden MC, Holness MJ.** Mechanisms underlying regulation of the expression and activities of the mammalian pyruvate dehydrogenase kinases. *Arch Physiol Biochem* 112: 139–149, 2006.
61. **Taylor CT.** Mitochondria and cellular oxygen sensing in the HIF pathway. *Biochem J* 409: 19–26, 2008.
62. **Tichopad A, Dilger M, Schwarz G, Pfaffl MW.** Standardized determination of real-time PCR efficiency from a single reaction set-up. *Nucl Acids Res* 31: e122, 2003.
63. **Tyler CM, Golland LC, Evans DL, Hodgson DR, Rose RJ.** Skeletal muscle adaptations to prolonged training, overtraining and detraining in horses. *Pflügers Arch* 436: 391–397, 1998.
64. **Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F.** Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3: RESEARCH0034, 2002.
65. **Vogt M, Puntschart A, Geiser J, Zuleger C, Billeter R, Hoppeler H.** Molecular adaptations in human skeletal muscle to endurance training under simulated hypoxic conditions. *J Appl Physiol* 91: 173–182, 2001.
66. **Wende AR, Huss JM, Schaeffer PJ, Giguere V, Kelly DP.** PGC-1alpha coactivates PDK4 gene expression via the orphan nuclear receptor ERRalpha: a mechanism for transcriptional control of muscle glucose metabolism. *Mol Cell Biol* 25: 10684–10694, 2005.
67. **Wittwer M, Billeter R, Hoppeler H, Flück M.** Regulatory gene expression in skeletal muscle of highly endurance-trained humans. *Acta Physiol Scand* 180: 217–227, 2004.
68. **Wright DC, Han DH, Garcia-Roves PM, Geiger PC, Jones TE, Holloszy JO.** Exercise-induced mitochondrial biogenesis begins before the increase in muscle PGC-1alpha expression. *J Biol Chem* 282: 194–199, 2007.
69. **Yang Y, Creer A, Jemiolo B, Trappe S.** Time course of myogenic and metabolic gene expression in response to acute exercise in human skeletal muscle. *J Appl Physiol* 98: 1745–1752, 2005.