Exercise attenuates the fasting-induced transcriptional activation of metabolic genes in skeletal muscle

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Hildebrandt, Audrey L., and P. Darrell Neufer. Exercise attenuates the fasting-induced transcriptional activation of metabolic genes in skeletal muscle. Am J Physiol Endocrinol Metab 278: E1078–E1086, 2000.—Fasting elicits a progressive increase in lipid metabolism within skeletal muscle. To determine the effects of fasting on the transcriptional regulation of genes important for metabolic control in skeletal muscle composed of different fiber types, nuclei from control and fasted (24 and 72 h) rats were subjected to nuclear run-on analysis using an RT-PCR-based technique. Fasting increased (P < 0.05) transcription rate of the muscle-specific uncoupling protein-3 gene (UCP3) 14.3- to 21.1-fold in white gastrocnemius (WG; fast-twitch glycolytic) and 5.5- to 7.5-fold in red gastrocnemius (RG; fast-twitch oxidative) and plantaris (PL; mixed) muscles. No change occurred in soleus (slow-twitch oxidative) muscle. Fasting also increased transcription rate of the lipoprotein lipase (LPL), muscle carnitine palmitoyltransferase I (CPT I), and long-chain acyl-CoA dehydrogenase (LCAD) genes 1.7- to 3.7-fold in WG, RG, and PL muscles. Transcription rate responses were similar after 24 and 72 h of fasting. Surprisingly, increasing metabolic demand during the initial 8 h of starvation (two 2-h bouts of treadmill running) attenuated the 24-h fasting-induced transcriptional activation of UCP3, LPL, CPT I, and LCAD in RG and PL muscles, suggesting the presence of opposing regulatory mechanisms. These data demonstrate that fasting elicits a fiber type-specific coordinate increase in the transcription rate of several genes involved in and/or required for lipid metabolism and indicate that exercise may attenuate the fasting-induced transcriptional activation of specific metabolic genes.

transcription; metabolism; medium-chain acyl-coenzyme A dehydrogenase; hexokinase II; exercise

SKELETAL MUSCLE, BY VIRTUE of its mass and total energy requirement, is the primary tissue responsible for the clearance of dietary glucose and lipids from the circulation and thus plays a key role in maintaining overall metabolic homeostasis (33, 42). The increasing recognition that subtle changes in energy balance, when considered over prolonged periods of time, represent a significant risk factor for the development of such metabolic abnormalities as insulin resistance, hypertriglyceridemia, obesity, and non-insulin-dependent diabetes mellitus has heightened the search for specific cell signaling and regulatory proteins that may influence metabolic control in skeletal muscle (32).

Most of the advances in our understanding of how acute challenges to intermediary metabolism may be sensed and responded to at the molecular level have arisen from work in liver, kidney, and adipose tissue. For example, transition from the fed to the fasted state dramatically activates transcription of a number of genes encoding enzymes with rate-limiting roles in hepatic gluconeogenesis, fatty acid oxidation, and ketogenesis. Aided by molecular studies in transgenic mice and various cell culture systems, detailed characterization of regulatory elements within the promoter regions of these genes has led to the identification of key signaling proteins and transcription factors that respond to various nutritional and/or hormonal manipulations (13, 19, 30).

Skeletal muscle also possesses a remarkable capacity to adapt to changes in metabolic demand, particularly in response to the challenges imposed by changes in contractile activity (45). Progress in deciphering the molecular mechanisms mediating the adaptive changes in gene expression in skeletal muscle have been hampered, however, by the fact that skeletal muscle cells grown in culture lack the important influences of the motor nerve (31), express only the embryonic forms of some proteins (6), and rely almost exclusively on glycolytic metabolism (23), factors that limit their ability to faithfully model critical features of adult myofibers. In the present study, we report a simplified procedure for isolating nuclei from small amounts of skeletal muscle tissue and an RT-PCR-based technique for performing nuclear run-on analysis, the combination of which permits the determination of gene-specific and fiber type-specific changes in transcription rate in rodent skeletal muscle. The purpose of the present study was to examine the extent to which the metabolic challenges imposed by 24–72 h of fasting influence the transcriptional regulation of genes important for metabolic control in skeletal muscle composed of different fiber types. In addition, we tested the hypothesis that overall metabolic demand/energy expenditure in skeletal muscle may directly influence transcriptional regulation by examining the effects of fasting in combination with low-intensity exercise performed during the initial portion of a 24-h fast. We focused particularly on uncoupling protein-3 (UCP3), a recently identified member of the uncoupling protein family that is expressed exclusively in skeletal muscle (10, 25, 43) and that is...
The fiber-type-specific transcriptional responses of several genes with critical roles in fatty acid oxidation, namely lipoprotein lipase (LPL), muscle carnitine palmitoyltransferase I (CPT I), long-chain acyl-CoA dehydrogenase (LCAD), and medium-chain acyl-CoA dehydrogenase (MCAD), were also determined.

**MATERIALS AND METHODS**

Materials. Male Sprague-Dawley rats were bred in-house or were purchased from Charles River Laboratory (Wilmington, MA). All rats were housed individually in a temperature (22°C) and light-controlled room (dark 9:00 AM-9:00 PM) and were given free access to food (Purina Rodent Diet) and water. Radiolabeled compounds were purchased from Amersham Pharmacia Biotech. All other chemicals were of molecular biology grade and were purchased from Boehringer Mannheim, Gibco-BRL, Promega, or Sigma Chemical.

Experimental design. Rats weighed 340–360 g at the time of each experiment. Food was removed from experimental rats at the beginning of the dark cycle (9:00 AM) and was withheld for 24 or 72 h while maintaining free access to water. Control rats continued to have free access to food and water. In a second set of experiments, metabolic demand was increased during fasting (24 h) by having rats complete two 2-h bouts of treadmill exercise (18 min, 5° incline) beginning 1 and 6 h after removal of food (i.e., 10:00 AM and 3:00 PM). Rats were killed at the 24-h mark (~16 h after the last exercise bout) and were compared with additional control and 24-h-fasted rats. At the completion of the experiments, rats were anesthetized (35 mg/kg ip pentobarbital sodium) and placed on a heating pad to maintain body temperature during surgery.

Nuclei isolation. The procedure for isolating nuclei from small amounts of skeletal muscle tissue was developed from our previous work (27) and from techniques described by Hahn and Covault (11). To examine the effects of fasting on skeletal muscles with different metabolic characteristics, nuclei were isolated from soleus (slow-twitch oxidative), plantaris (mixed fast-twitch oxidative/glycolytic), and porcine red (fast-twitch oxidative) and white (fast-twitch glycolytic) gastrocnemius muscle. Skeletal muscles from one experimental tissue. Muscle weights immediately after dissection of red (fast-twitch oxidative) and white (fast-twitch glycolytic) gastrocnemius muscle. Skeletal muscles from one

Materials for nuclear isolation included: 100 mM KCl, 10 mM MgCl2, 4.5 mM DTT, 1.2 mM ATP, 0.6 mM each of CTP, GTP, and UTP, 0.5 mM spermidine, 0.15 mM spermine, and 80 µM RNase inhibitor) for 15 min at 22°C. Nuclei were then subjected to DNase I (20 units, RNase free) digestion in the presence of 1 mM CaCl2 for 15 min at 37°C. This was followed by digestion of nuclear proteins by addition of 10× 100 mM Tris, pH 8.0, 10 mM EDTA, 5% SDS, and 100 µg of protease K and incubation for 30 min at 37°C. To extract the nascent RNA transcripts, 1 ml of TRIzol (GIBCO-BRL), 100 µg of yeast tRNA (to aid in visualization of RNA pellet), and 200 µl of chloroform were added to each sample. Samples were mixed vigorously, incubated on ice for 5 min, and centrifuged at 12,000 g for 15 min at 4°C. Transcribed RNA was precipitated from the aqueous phase by addition of 1 mM CaCl2. RNA transcripts were reextracted by addition of 500 µl of TRIzol and 100 µl of chloroform and were centrifuged for 15 min at 12,000 g and 4°C. RNA was precipitated from the aqueous phase by addition of an equal volume of isopropanol and incubation at ~20°C for 30 min. After centrifugation (12,000 g, 10 min, 4°C), RNA pellets were rinsed twice in cold 70% ethanol (EtOH), transferred to 1.5-ml tubes, dried briefly, and resuspended in 100 µl of 10 mM Tris buffer (pH 8.0). To ensure complete removal of genomic DNA, samples were subjected to a second digestion (37°C, 30 min) with DNase I (10 units) in the presence of 1 mM CaCl2. RNA transcripts were reextracted by addition of 500 µl of TRIzol and 100 µl of chloroform and were centrifuged for 15 min at 12,000 g and 4°C. RNA was precipitated from the aqueous phase by addition of an equal volume of isopropanol and incubation at ~20°C for 30 min. After centrifugation (12,000 g, 10 min, 4°C), RNA pellets were rinsed twice in cold 70% EtOH, dried briefly, and resuspended overnight at 4°C in 2 µl of 10 mM Tris (pH 8.0) and 0.1 mM EDTA.

RT of the nascent RNA was performed using the Superscript II RNAse H–system (GIBCO-BRL) according to the manufacturer’s instructions. Briefly, 18 µl of RNA were mixed with 1.5 µl oligo(dT)12–18 (500 ng/µl), heated to 70°C for 10 min, and rapidly cooled on ice for 5 min. After spinning down condensation, 6 µl of 5× reaction buffer (250 mM Tris, pH 8.3, 375 mM KCl, and 15 mM NaCl), 3 µl of 0.1 M DTT, and 1.5 µl of dNTP mix (10 mM each of dATP, dCTP, dGTP, and dTTP) were added to each sample. After a 2-min preincubation at 42°C, 1.0 µl of Superscript II was added, and samples were incubated at 42°C for 50 min. The enzyme was inactive by incubation at 70°C for 15 min. To account for differences in nuclei content among samples before the run-on reaction, RT products were diluted with nuclease-free H2O based on the relative genomic DNA content of each nuclei preparation (see below). Average volume was set at 150 µl.

To determine the amount of transcript present for a given gene (transcription rate), PCR was performed (in duplicate) on 5 µl of diluted RT product in a reaction mixture (final volume 50 µl) containing 10 mM Tris (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5–2.5 mM MgCl2, 0.3 µl each of PCR-grade dATP, dCTP, dGTP, and dTTP, 500 ng each of the appropriate forward and reverse primers, and 1.5 units of recombinant Taq DNA polymerase (GIBCO-BRL). PCR was performed in a PTC dual block DNA engine (MJ Research) using the general cycle profile: 94°C 2 min (94°C 30 s, annealing temperature 50°C 30 s, 72°C 30 s) × 10 → (94°C 30 s,
Annealing temperature 50 s, 72°C 50 s + 20 s extension (cycle) × remaining number of cycles. PCR primer pairs (Table 1) were designed from rat specific sequence data (Entrez; National Institutes of Health) using DNA analysis computer software (Lazergene; DNASTAR). Annealing temperature, MgCl2 concentration, and PCR cycle number were determined for each primer pair by extensive pretesting to ensure that conditions were optimized and within the linear range for PCR amplification. Control and experimental samples were run in parallel to permit direct relative comparisons. Amplification products were separated by gel (2.5% agarose) electrophoresis, stained with ethidium bromide, visualized, and quantified by ultraviolet exposure using a charge-coupled device integrating camera (Gel Doc; Bio-Rad) and analysis software (Molecular Analyst; Bio-Rad) under nonsaturating conditions.

Isolation and quantification of genomic DNA. To correct for initial differences in nuclei content among samples, genomic DNA was isolated from a portion of each sample of nuclei on the same day as the nuclear run-on reaction. A 20-µl aliquot of nuclei was placed in 380 µl of digestion buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 100 µg of proteinase K) and incubated at 50°C for ~6 h. After adding an additional 380 µl of nuclease-free H2O2, DNA was isolated by extraction with an equal volume of phenol-chloroformisoamyl OH (25:24:1), separated by centrifugation (12,000 g, 4°C), and precipitated from the resulting aqueous phase by addition of 12 vol 3 M NaOAc (pH 8.0, 100 mM NaCl, 1 mM EDTA, and 100 µg of proteinase K), rinsed twice with cold 75% EtOH, and resuspended in 75 µl of RNase-free TE. To separate radio labeled RNA transcripts from unincorporated [32P]UTP, the entire 75 µl was loaded on a freshly prepared spin column (PGC Scientifics) containing G-50 Sephadex (swollen in 10 mM Tris, pH 8.0, 1 mM EDTA, and 100 mM NaCl) and were centrifuged for 4 min at 1,100 g. Total radioactivity within the resulting column effluent was measured in triplicate by liquid scintillation spectrometry. Background activity due to flow through of free [32P]UTP was determined by omission of nuclei in the run-on reaction and was found to represent <1.5% of sample effluent activity.

Statistical analysis. Transcription rate data for all metabolic genes were expressed relative to the transcription rate of the β-actin gene. All data across experimental treatments were expressed relative to data from control rats with mean data set to 1.0. Statistical analyses were performed using a one-way ANOVA with all pairwise multiple comparisons among groups performed using the Student-Newman-Keuls method. The level of significance was set at P < 0.05.

RESULTS

Validation of RT-PCR-based nuclear run-on analysis. Primers specific for the rat β-actin gene were designed and selected (Fig. 1A) to test whether RT-PCR can be used to detect the formation of RNA transcripts produced by nuclear run-on analysis of nuclei isolated from ~400 mg of rat skeletal muscle. An additional intent was to determine whether transcripts produced during the run-on reaction represent unprocessed (intron containing) or processed (spliced) RNA. PCR using a forward primer within exon 4 (FP exon 4) and a reverse primer within exon 5 (RP exon 5) yielded the appropriate 364-bp product from rat genomic DNA (containing intron 4; Fig. 1B, lane 2) and a 277-bp product from total muscle RT-RNA (intron 4 spliced out; Fig. 1B, lane 3). When RNA was isolated from nuclei not subjected to the nuclear run-on reaction (native RNA; Fig. 1B, lane 4), only a very faint 277-bp product was detected (visible with 0.3 s integration), indicating that only a small amount of RNA is initially present in the nuclei preparations. In contrast, a major 277-bp product and a minor 374-bp product were detected from RNA isolated from nuclei after the run-on reaction (Fig. 1B, lanes 5 and 7, duplicate experiments), demonstrating that transcript formation is detected by RT-PCR and that the majority of RNA transcripts were expressed relative to the transcription rate of the β-actin gene.

Table 1. Primers and reaction conditions used for PCR on nuclear RT-RNA

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>AT, °C</th>
<th>[MgCl2], mM</th>
<th>Product Size, bp</th>
</tr>
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<tbody>
<tr>
<td>UCP3</td>
<td>GAACCATCGGCCAGGGAAAGGAGGTCG</td>
<td>GGGGGGACACGTTCAAGTACGGCTTATA</td>
<td>56.0</td>
<td>1.5</td>
<td>250</td>
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<tr>
<td>LPL</td>
<td>CAGCGGCTCCCTTTCTACATGGA</td>
<td>GGGCGGAGGGCCGCTTATA</td>
<td>56.7</td>
<td>2.5</td>
<td>216</td>
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<tr>
<td>CPT I</td>
<td>CAAGCGCCTGTCGAGTATTGTG</td>
<td>ATCTCTGTGGCGCCCTCTG</td>
<td>59.8</td>
<td>2.0</td>
<td>201</td>
</tr>
<tr>
<td>LCAD</td>
<td>GACGACGCGCTGGATCACG</td>
<td>GAGAACGAGGAGGAGGTC</td>
<td>54.9</td>
<td>2.0</td>
<td>380</td>
</tr>
<tr>
<td>MCAD</td>
<td>GCCATCGACTATCGTCCAGA</td>
<td>ATCCGGCCCATCTCTAG</td>
<td>54.7</td>
<td>2.0</td>
<td>343</td>
</tr>
<tr>
<td>HKII</td>
<td>GAAGGCCTGCTTAAGGAGGAGAG</td>
<td>CGTGAAGGCGAACGCTTCTCAG</td>
<td>55.0</td>
<td>2.0</td>
<td>274</td>
</tr>
<tr>
<td>β-Actin</td>
<td>GCGCGGCCATCCTCTAGCAGTTAAC</td>
<td>GGCATCGACGACCCGCTTGG</td>
<td>55.9</td>
<td>2.5</td>
<td>230</td>
</tr>
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</table>

UCP3, uncoupling protein 3; LPL, lipoprotein lipase; CPT I, carnitine palmitoyltransferase I; LCAD, long-chain acyl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; HK II, hexokinase II; [MgCl2], MgCl2 concentration; AT, annealing temperature.
Transcriptional Regulation During Fasting

Fig. 1. Demonstration of RT-PCR-based nuclear run-on analysis: detection of both processed and unprocessed nascent RNA transcripts. A: experimental set-up. To test the utility of RT-PCR-based nuclear run-on analysis, a forward primer (FP) within exon 4 (5′-ATCGTGCGTGACATTAAAGAAG-3′) and two reverse primers (RP), one within intron 4 (5′-ATGAGAAGGGACGAGACTA-3′) and one within exon 5 (5′-CAGCACTGTGTTGGCAT-3′), were selected from the rat β-actin gene. Both RPs were compatible with FP exon 4. Sizes of expected PCR products generated from genomic DNA, heteronuclear RNA (hnRNA), and total cellular RNA are shown. B: results from RT-PCR nuclear run on. Lane 1, 100-bp marker; lane 2, 364-bp PCR product from genomic DNA (gDNA) template using RP exon 5; lane 3, 277-bp PCR product from RT total muscle RNA using RP exon 5; lane 4, faint 209-bp PCR product from RNA isolated from nuclei not subjected to nuclear run-on reaction (native RNA); lane 5, major 277-bp and minor 364-bp PCR products from RNA isolated from nuclei after run-on reaction (processed RNA); lane 6, negative control in which RNA isolated from nuclei after run-on reaction was not RT before PCR; lanes 7 and 8, duplicate experiment of that described for lanes 5 and 6, respectively; lane 9, 209-bp PCR product from gDNA using RP intron 4; lane 10, faint 209-bp PCR product from the same RT-RNA as used in experiment of that described for lane 10 using RT-RNA from lane 7; lane 12, negative control, showing no PCR product from RT total muscle RNA using RP intron 4.

produced are immediately processed (major 277-bp product). As a negative control, a portion of the RNA isolated from nuclei after the run-on reaction was used as a template for PCR without being reverse transcribed; no PCR products were detected (Fig. 1B, lanes 6 and 8), demonstrating that the minor 364-bp product present in lanes 5 and 7 was not due to the presence of residual or contaminating genomic DNA. Varying the run-on reaction time from 30 s to 30 min did not influence the ratio of spliced to unspliced transcript (unpublished data). PCR using the intron-specific reverse primer (RP intron 4, Fig. 1A) generated the appropriate 209-bp product from genomic DNA (Fig. 1B, lane 9) and no product from total muscle RT-RNA (lane 12). When RP intron 4 was used with nuclear run-on RT-RNA as the template, only a faint 209-bp product (Fig. 1B, lanes 10 and 11) was obtained, approximately the same intensity as the 364-bp product obtained using RT exon 5 (Fig. 1B, lanes 5 and 7), providing further evidence that the majority of RNA transcripts produced during the run-on reaction are immediately processed. Our results are similar to a previous report from Rolfe and Sewell (34), also using a PCR-based nuclear run-on procedure, and collectively support recent biochemical and cytological evidence demonstrating that RNA synthesis is intimately linked and coordinated with processing through the presence of processing factors at the carboxy-terminal domain of RNA polymerase II, which catalyzes the capping, splicing, and polyadenylation of nascent transcripts during transcriptional elongation (28).

Effect of fasting on total transcriptional activity. To determine the potential influence of fasting on the overall transcriptional activity in skeletal muscles, a portion of nuclei from each sample was subjected to the nuclear run-on reaction in which cold UTP was replaced with [32P]UTP. Total radioactivity of the isolated nuclear run-on RNA, normalized to genomic DNA, was taken as an index of total transcriptional activity. As shown in Fig. 2, fasting significantly reduced total transcriptional activity by 20–53% in plantaris and white gastrocnemius muscle. The decrease was evident within the first 24 h and was not further depressed after 72 h. Although responses were somewhat variable, fasting also tended (P = 0.051) to decrease total transcriptional activity in red gastrocnemius muscle. Total transcriptional activity in soleus muscle was not affected by fasting.

Fig. 2. Total transcriptional activity in skeletal muscles from control and 24- and 72-h-fasted rats. Aliquots of nuclei isolated from soleus (Sol), plantaris (PL), and red (RG) and white gastrocnemius (WG) muscle were subjected to nuclear run-on analysis in the presence of [32P]UTP as described in MATERIALS AND METHODS. Total RNA transcript formation was determined by liquid scintillation spectrometry, normalized to genomic DNA content to account for variations in nuclei content, and expressed relative to controls. Values are means ± SE. *Significantly (P < 0.05) different from control.
The average transcription rate of the β-actin gene, determined by RT-PCR nuclear run-on analysis, also declined with fasting in plantaris (6.7–14.7%), red gastrocnemius (27–32%), and white gastrocnemius (43–50%, P < 0.05) muscles. Transcription rate of the β-actin gene was not different between fasting and fasting-plus-exercised animals. Analysis of data from all four muscles across treatments revealed that the β-actin transcription rate was significantly correlated (P = 0.0015, r = 0.331) with total transcription rate, reflecting the housekeeping nature of this gene.

Effect of fasting on the transcription rate of specific metabolic genes. Fasting elicited clear and marked changes in the transcription rate of several metabolic genes (relative to β-actin) in the plantaris, red gastrocnemius, and white gastrocnemius muscles while having little effect on transcriptional regulation within the soleus muscle (Fig. 3). As summarized in Fig. 4, fasting elicited a 5.5- to 7.5-fold increase in the transcription rate of the UCP3 gene in both the plantaris and the red gastrocnemius muscles. In white portions of the gastrocnemius muscle, UCP3 transcription rate was increased by 14.3-fold after 24 h and by 21.1-fold after 72 h of fasting. In contrast, the UCP3 transcription rate in the soleus muscle was not significantly elevated after 24 or 72 h of fasting.

With the use of fasting as a metabolic challenge, the intent of the present study was to test the hypothesis that an increased demand for and/or supply of fatty acids in skeletal muscle triggers an adaptive response that includes the coordinate transcriptional activation of genes encoding for proteins required for fatty acid transport and oxidation. In support of this hypothesis, the transcription rate of the LPL, CPT I, and LCAD genes significantly increased by 1.7- to 2.0-fold, 2.1- to 3.0-folds, and 1.7- to 3.7-folds, respectively, over the 24–72 h of fasting (Fig. 4). No differences were found between 24- and 72-h-fasted rats. Compared with LCAD, changes in the transcription rate of the MCAD gene were not as dramatic or consistent with significant activation (2.0- to 2.3-fold) evident only in the white gastrocnemius muscle. Fasting did not influence the transcription rate of the LPL, CPT I, LCAD, or MCAD genes in soleus muscle. To serve as a comparison for fat metabolism genes, the transcription rate of the hexokinase II gene was also determined and found to be significantly increased only after 72 h of fasting in the soleus (2.0-fold), red gastrocnemius (1.8-fold), and white gastrocnemius (1.5-fold) muscles.

Effect of increased metabolic demand on transcriptional regulation during fasting. To determine the potential interaction between substrate supply and metabolic demand on the transcriptional regulation of metabolic genes, a second study was performed, in which metabolic demand was increased during fasting (24 h) by having the rats complete two 2-h bouts of moderate-intensity exercise (18 m/min, 5° incline) performed 1 and 6 h after removal of food. Surprisingly, increasing metabolic demand during fasting attenuated the increases in transcription rate evident with fasting alone (Figs. 5 and 6), a response that appeared to be most apparent in muscles composed predominantly of oxidative fiber types. For example, UCP3 transcription rate in 24-h-fasted-plus-exercised rats was significantly lower than 24-h-fasted rats and was not significantly different from control rats in the red gastrocnemius muscle. Similarly, increasing metabolic demand also attenuated the fasting-induced increase in LPL, CPT I, and LCAD transcription rate in the red gastrocnemius muscle as well as CPT I and LCAD induction in the plantaris muscle. With the exception of LPL, increasing metabolic demand did not affect the fasting-induced transcriptional regulation of any of the genes examined in white gastrocnemius muscle. Fasting plus exercise also did not significantly influence the transcription rate of the β-actin gene relative to fasting alone.

**DISCUSSION**

The primary purpose of the present study was to test the hypothesis that a severe metabolic challenge to skeletal muscle, such as fasting, triggers a coordinate adaptive response in the transcriptional regulation of genes with critical roles in substrate metabolism. The results of the present study demonstrate that fasting induces a coordinate increase in the transcription rate of a number of metabolically related genes, specifically in fast-twitch skeletal muscle. Chief among the responses was a dramatic increase in transcription rate of the UCP3 gene in plantaris, red gastrocnemius, and white gastrocnemius muscles within 24 h after removal of food. Transcriptional activation of several genes required for lipid metabolism also occurred in response to fasting, suggesting a common regulatory mechanism. Surprisingly, however, the added metabolic demand imposed by exercise performed during the initial hours after food removal actually attenuated the transcriptional activation found with 24 h of fasting alone, suggesting the presence of opposing regulatory mechanisms.

**Table 1.** Nuclear run-on analysis of nuclei isolated from the indicated muscles of control, 24-h-fasted, and 72-h-fasted rats. Shown is the negative image of PCR products (ethidium bromide stained) generated from RT-PCR-based nuclear run-on analysis representing the relative transcription rate of the genes for uncoupling protein-3 (UCP3), lipoprotein lipase (LPL), muscle carnitine palmitoyltransferase I (CPT I), long-chain acyl-CoA dehydrogenase (LCAD), medium-chain acyl-CoA dehydrogenase (MCAD), hexokinase II (HKII), and β-actin (β-A). Shown is the analysis of each gene for a single set of samples per muscle.
The discovery of an uncoupling protein whose expression is restricted to skeletal muscle, a major site of energy expenditure, has led to speculation that UCP3 may play a role in regulating nonshivering thermogenesis and whole body energy expenditure (10, 25, 43). Fueling this hypothesis is the fact that the human UCP2 and UCP3 genes map to within 100 kb of one another on chromosome 11 (q13), a region that is coincident with several independently mapped quantitative trait loci for obesity and resting metabolic rate (3, 8, 10, 41). Although UCP3 displays uncoupling activity and increases thermogenesis when overexpressed in yeast (8–10, 15), its physiological role in skeletal muscle has not been firmly established. For example, a number of investigators have found that both food restriction and fasting induce a marked increase in skeletal muscle UCP3 mRNA (2, 35, 40). Our data extend these findings, demonstrating that fasting increases the transcription rate of the UCP3 gene by 5- to 20-fold in both red and white fast-twitch skeletal muscle. Collectively, these findings appear to be counterintuitive, given that skeletal muscle is a recognized site of energy conservation during starvation (24). One possible explanation is that changes in UCP3 expression do not correlate with uncoupling activity because other factors, such as posttranslational modifications, may be required for activation. For example, UCP1 activity in brown adipose tissue is inhibited by purine nucleotide binding and is activated by fatty acids (17). Similar allosteric

Fig. 4. Quantification data of fasting-induced changes in transcription rate represented in Fig. 3. A summary of the image and statistical analysis (n = 9–14 rats/group) of the changes in transcription rate for the indicated genes (defined in Fig. 3) in soleus, PL, RG, and WG muscles of control, 24-h-fasted, and 72-h-fasted rats. All data were normalized to β-actin and were expressed relative to controls (set to 1.0) as means ± SE. Note that, because of the large changes in transcription, the y-axis for UCP3 is larger than for all other genes. *Significantly (P < 0.05) different from control.

Fig. 5. Nuclear run-on analysis of nuclei isolated from the indicated muscles of control, 24-h-fasted (F), or 24-h-fasted + exercised (FE; two 2-h bouts of treadmill exercise performed during the initial 8 h) rats. Shown is the negative image of PCR products (ethidium bromide stained) generated from RT-PCR based nuclear run-on analysis representing the relative transcription rate of the indicated genes (defined in Fig. 3). Analysis of each gene for a single set of samples per muscle is shown.

UCP2 and UCP3 genes map to within 100 kb of one another on chromosome 11 (q13), a region that is coincident with several independently mapped quantitative trait loci for obesity and resting metabolic rate (3, 8, 10, 41). Although UCP3 displays uncoupling activity and increases thermogenesis when overexpressed in yeast (8–10, 15), its physiological role in skeletal muscle has not been firmly established. For example, a number of investigators have found that both food restriction and fasting induce a marked increase in skeletal muscle UCP3 mRNA (2, 35, 40). Our data extend these findings, demonstrating that fasting increases the transcription rate of the UCP3 gene by 5- to 20-fold in both red and white fast-twitch skeletal muscle. Collectively, these findings appear to be counterintuitive, given that skeletal muscle is a recognized site of energy conservation during starvation (24). One possible explanation is that changes in UCP3 expression do not correlate with uncoupling activity because other factors, such as posttranslational modifications, may be required for activation. For example, UCP1 activity in brown adipose tissue is inhibited by purine nucleotide binding and is activated by fatty acids (17). Similar allosteric
regulation has not as yet been ascribed to UCP3, although posttranslational control of UCP3 has recently been implicated in the control of resting metabolic rate in thyroid-treated mice (16). Another possibility is that UCP3 may have other functions apart from its putative role in regulating thermogenesis. Samec et al. (36) have postulated that UCP3 may be involved in regulating the use of lipids as fuel substrates in skeletal muscle. This hypothesis is based on the fact that changes in UCP3 mRNA expression in response to various thermoregulatory and/or nutritional transitions (i.e., cold exposure, fasting, food restriction, refeeding, weaning) do not coincide with corresponding changes in thermogenesis but are more closely linked with the demand for lipid metabolism in skeletal muscle (1, 5, 36, 37, 44). Thus, although UCP3 expression changes dramatically in response to different metabolic challenges, its role in skeletal muscle with respect to mitochondrial function and fatty acid metabolism has yet to be determined.

Fasting coordinately activated transcription of three genes with critical roles in mediating fatty acid metabolism in skeletal muscle (LPL, CPT I, and LCAD). LPL catalyzes the hydrolysis of triglycerides in circulating chylomicrons and very low density lipoproteins, representing the rate-limiting step in the utilization of triglyceride-derived fatty acids. Although regulation of LPL activity in adipose tissue has been shown to be primarily at the translational and posttranslational level, regulation within skeletal muscle is believed to involve both translational and pretranslational control (7). Ladu et al. (21) found in rats that 1 day of fasting significantly increased LPL mRNA levels in soleus and red and white portions of the vastus lateralis muscle. LPL activity did not increase after 1 day of fasting but was significantly increased after 6 days (21). Data from the present study provide the first direct evidence that the increase in skeletal muscle LPL expression in response to fasting is mediated, at least in part, at the level of transcription of the LPL gene. Fasting also elicited increases in transcription of the genes encoding for CPT I and LCAD, two enzymes that are responsible for catalyzing the first steps in the transport of fatty acids across the mitochondrial membrane and subsequent β-oxidation within the mitochondrial matrix, respectively (20, 26). Although no other data are available from skeletal muscle, fasting has been reported to increase CPT I mRNA levels and enzyme activity in liver tissue of rats (26). Interestingly, with the exception of white gastrocnemius muscle, fasting did not significantly influence transcription of the MCAD gene, suggesting the presence of control mechanisms distinct from that of LPL, CPT I, and LCAD.

The soleus muscle in rats is composed primarily of slow-twitch fibers that rely heavily on oxidative metabolism. LPL mRNA has been shown to increase by ∼50% in soleus muscle after 1 day of fasting (21). In contrast, LPL transcription rate in the present study did not change in soleus muscle, suggesting that changes in LPL expression induced by fasting in muscle composed primarily of slow-twitch oxidative fibers may involve posttranscriptional control mechanisms.

In tissues such as liver and kidney, shifts in nutritional and/or hormonal status often evoke on the order of 5- to 20-fold changes in the expression of genes required for fatty acid oxidation and gluconeogenesis (13, 19, 30). In the present study, fasting elicited no more than a two- to fourfold increase in transcription of the LPL, CPT I, LCAD, and MCAD genes, prompting us to consider whether accelerating the demand for fatty acid oxidation in skeletal muscle by combining exercise with fasting would augment the transcriptional induction of these genes relative to fasting alone. To test for this possibility, rats performed two 2-h bouts of moder-
ate-intensity treadmill exercise (separated by 3 h of rest) within the first 8 h of a 24-h fast. In contrast to our hypothesis, the added metabolic demand imposed by exercise did not elicit a persistent, enhanced, or additive effect but, rather, partially attenuated the fasting-induced (24-h) increase in transcription of CPT I and LCAD in plantaris and UCP3, LPL, CPT I, and LCAD in red gastrocnemius muscle (Fig. 6). Interestingly, with the exception of LPL, increasing metabolic demand did not significantly affect the fasting-induced transcriptional induction of UCP3, CPT I, or LCAD in white gastrocnemius muscle, possibly due to lack of recruitment of these muscle fibers during low-intensity exercise. It is important to emphasize that the effects of fasting plus exercise were evaluated 16 h after the last exercise bout to examine the more general effects of increased metabolic demand as opposed to the specific effects of exercise, i.e., the marked transient increases in transcription rates of metabolic genes that occur during the initial 3- to 4-h recovery period after exercise (14, 27, 29). The surprising conclusion that exercise attenuates the transcriptional response to fasting, at least under the limited experimental conditions of the present study, raises the possibility that fasting and exercise may trigger opposing regulatory mechanisms that persist well beyond the cessation of exercise.

The signaling events mediating the transcriptional induction of metabolic genes in skeletal muscle are unknown. One possibility is that increased free fatty acid (FFA) levels associated with fasting or exercise may activate signaling pathways targeted to genes encoding enzymes involved in the lipid oxidation, possibly through activation of the peroxisome proliferator-activated receptor (PPAR) family of transcription factors (22). Brun et al. (4) have recently provided evidence that induction of UCP3 expression in skeletal muscle of newborn mice is linked to the initiation of lipid intake during suckling and, specifically, to the activation of PPAR-α. The suckling period is also characterized by marked increases in transcription of other lipid metabolism genes, including LPL, CPT I, LCAD, and MCAD (12, 18, 26, 39), all of which contain PPAR response elements within their promoter regions (38). Whether similar regulatory mechanisms operate in response to elevated FFA levels during fasting in adult animals, as well as whether such effects may be overridden by exercise, has not been determined. Moreover, the impact of factors such as substrate supply, metabolic demand, and circulating hormonal milieu on the transcriptional regulation of metabolic genes in skeletal muscle, particularly during recovery from exercise, remains virtually undefined.

In summary, the results from the present study demonstrate that fasting induces a marked increase in transcription of the UCP3 gene and a coordinate increase in transcription of several genes required for lipid metabolism in fast-twitch red and white skeletal muscle, likely reflecting the increased reliance of muscle on fatty acid metabolism during starvation. Surprisingly, however, increasing the metabolic demand within skeletal muscle during the initial 8 h of a 24-h fast significantly attenuates the transcriptional activation of several metabolic genes associated with lipid metabolism in red skeletal muscle, raising the possibility that fasting and exercise may trigger opposing regulatory mechanisms.

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