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Transcriptome and translational signaling following endurance exercise in trained skeletal muscle: impact of dietary protein

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Rowlands DS, Thomson JS, Timmons BW, Raymond F, Fuerholz A, Mansourian R, Zwahlen MC, Métairon S, Glover E, Stellingwerff T, Kussmann M, Tarnopolsky MA. Transcriptome and translational signaling following endurance exercise in trained skeletal muscle: impact of dietary protein. *Physiol Genomics* 43: 1004–1020, 2011. First published July 5, 2011; doi:10.1152/physiolgenomics.00073.2011.—Postexercise protein feeding regulates the skeletal muscle adaptive response to endurance exercise, but the transcriptome guiding these adaptations in well-trained human skeletal muscle is uncharacterized. In a crossover design, eight cyclists ingested beverages containing protein, carbohydrate and fat (PTN: 0.4, 1.2, 0.2 g/kg, respectively) or isocaloric carbohydrate and fat (CON: 1.6, 0.2 g/kg) at 0 and 1 h following 100 min of cycling. Biopsies of the vastus lateralis were collected at 3 and 48 h following to determine the early and late transcriptome and regulatory signaling responses via microarray and immunoblot. The top gene ontology enriched by PTN were: muscle contraction, extracellular matrix - signaling and structure, and nucleoside, nucleotide, and nucleic acid metabolism (3 and 48 h); developmental processes, immunity, and defense (3 h); glycolysis, lipid and fatty acid metabolism (48 h). The transcriptome was also enriched within axonal guidance, actin cytoskeletal, Ca^{2+}, cAMP, MAPK, and PPAR canonical pathways linking protein nutrition to exercise-stimulated signaling regulating extracellular matrix, slow-myofibril, and metabolic gene expression. At 3 h, PTN attenuated AMPKα1^{Thr172} phosphorylation but increased mTORC1^{Ser2448}, rps6$^{Ser240/244}$, and 4E-BP1-γ phosphorylation, suggesting increased translation initiation, while at 48 h AMPKα1^{Thr172} phosphorylation and *PPARG* and *PPARGC1A* expression increased, supporting the late metabolic transcriptome, relative to CON. To conclude, protein feeding following endurance exercise affects signaling associated with cell energy status and translation initiation and the transcriptome involved in skeletal muscle development, slow-myofibril remodeling, immunity and defense, and energy metabolism. Further research should determine the time course and posttranscriptional regulation of this transcriptome and the phenotype responding to chronic postexercise protein feeding.

nutrigenomics; mitochondrial biogenesis; fatty acid oxidation; extracellular matrix; AMP-activated protein kinase

IT HAS BEEN PROPOSED THAT human physical endurance and dietary protein intake were fundamental factors determining human evolutionary fitness (10) and, more recently, that dietary protein intake may play an import role in promoting aspects of skeletal muscle adaptation to exercise (23, 15). Estimated average energy expended in physical activity by a typical Paleolithic hunter-gatherer [91 kJ·kg^{-1}·day^{-1} (17)] was within the range of modern endurance athletes and largely involved in procurement of a protein-rich diet (~30% daily energy, 30–70% animal source) consumed within the hours following hunting, tracking, or gathering (10, 17). The human musculoskeletal homeostatic system and endurance capacity evolved over millennia (10), and the endurance-trained state is arguably the natural expression condition of skeletal muscle (9). This information suggests that exercise-induced disturbance to homeostasis coupled with a protein-rich diet following endurance exercise are the normal environmental cues for adaptive remodeling in trained skeletal muscle. Accordingly, a connection between postexercise protein feeding and improved endurance phenotype has been recently demonstrated (51, 57).

The stress of endurance exercise on the worked muscle can include disruption of cellular energy homeostasis, oxidation of DNA, proteins, and lipids, inflammation, and muscle tensile stress and damage (19, 39). These stimuli are rapidly sensed, transduced, and integrated through signaling pathways into a coordinated transcriptional response regulated by posttranscriptional events (e.g., mRNA splicing and stability), leading to the synthesis of specific proteins needed to ameliorate subsequent threats to cellular homeostasis (19, 70). In principle, chronic exposure should lead to the accumulation of specific proteins representing the adapted skeletal muscle phenotype contributing to enhanced endurance capacity (23). Recently, gene microarray analysis has provided an unbiased interrogation of the transcriptome response to endurance exercise and has uncovered novel pathways for discovery. Even after a single bout of endurance exercise, there is a coordinated upregulation of many of the mRNA transcripts encoding for components of the extracellular matrix and mitochondria in human skeletal muscle (35). Additionally, overexpression of genes involved in energy metabolism, mitochondrial biogenesis (46, 54, 69), inflammation and stress response (64, 69), membrane transport, (54), cellular signaling (64), contractile processes (54), and the cytoskeleton (59) has been reported. Combined, the microarray data suggest gene expression reprogramming is a major mechanism guiding phenotypic adaptation to exercise.

In addition to regulated gene expression, nutrition and exercise stimuli act together to control posttranscriptional regulation of protein synthesis. It is now well established that amino acids and carbohydrate (via insulin) regulate translation initiation via mammalian targets of rapamycin complex I

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(mTORC1) (32). Moreover, mTORC1 is involved in activation of mitochondrial biogenesis (13), and long-term feeding of branched-chain amino acids to mice led to mTORC1 activation, improved mitochondrial oxidative capacity, exercise endurance, and survival (15). These findings suggest that the consumption of protein in the early postexercise period may potentiate exercise-induced adaptive signaling in humans. However, to the best of our knowledge the global expression profile specifically induced in response to protein feeding following endurance exercise has not yet been explored; this rationale forms the principle objective of the current study based on microarray analysis of the skeletal muscle transcriptome.

To complement the transcriptome, we determined the protein nutrition effect on translation initiation via mTORC1 pathway activity. Consistent with classically reported adaptive responses to endurance exercise (25), we hypothesized that postexercise protein nutrition alters the expression programs regulating tissue structure (e.g., extracellular matrix and cytoskeletal remodeling), slow-fiber transformation, mitochondrial biogenesis, and fatty acid oxidation toward the established endurance-exercise phenotype. We report on these and other protein-induced ontology providing novel in vivo insight into the nutrient-stimulated genomic and translational control of the molecular response in trained skeletal muscle to an acute bout of intense endurance exercise.

MATERIALS AND METHODS

Subjects

Eight endurance-trained male cyclists, aged 32.8 ± 6.4 yr (mean ± SD), standing 178.8 ± 3.6 cm, and weighing 76.7 ± 5.2 kg, completed the study. Maximal oxygen uptake (VO_{2max}) was 4.6 ± 0.6 l/min with a corresponding peak power output (W_{max}) 349 ± 34 W. The study was approved by the McMaster University Hamilton Health Sciences Human Research Ethics Board. All subjects provided informed written consent prior to participation.

Experimental Design

The design was a single blind, randomized, crossover comprising two experimental periods. During the periods, exercise and diet were controlled and the intervention consisted of the ingestion of an isocaloric protein-enriched or control beverage, with outcome measures obtained from blood and skeletal muscle tissue collected following a bout of intense cycling (Fig. 1).

Preliminary testing. One to two weeks prior to the start of the first experimental period, VO_{2max} and W_{max} were ascertained using cycle ergometry (Lode Excalibur Sport V2, Groningen, Netherlands) and on-line assessment of external respiration (Moxus, AEI Technologies, Pittsburgh, PA) via an incremental test to exhaustion (51).

Baseline. At 0900 following an overnight fast, and 7 days prior to the first experimental period, a baseline muscle biopsy was collected. All physical activity and diet were standardized 7 days prior and throughout experimental periods, as subjects recorded all diet and training for 5 days (*day −7* to *day −1*), which was repeated during the second period. Exercise familiarization was conducted at 1700, 2 days prior to baseline biopsy, where participants cycled for 90 min (30 min warm-up at 50% of W_{max}, followed by 5 min intervals of 70 and 50% W_{max}, respectively, for 45 min, and a 15 min cool-down at 30% W_{max}) and then consumed packaged meals only for the remaining period based on estimated daily energy requirement calculated from the diet diary; these meals were the same as that prior to the experimental day (see below). Participants ingested water ad libitum during exercise. The following day, participants were asked to refrain from exercise.

Experimental periods. On the experimental day (*day 1*), a catheter was placed in an antecubital vein and kept patent with isotonic saline. Participants ingested one granola bar with 250 ml water 15 min prior to exercise to simulate typical pre-exercise conditions.

Exercise comprised 105 min of intense interval cycling: 30 min warm-up building from 30 to 50% of W_{max}, then four blocks intervals, with 2 min recovery periods at 50% W_{max} between intervals, and a 6 min recovery period at 50% W_{max} between blocks. *Blocks 1* and *2* comprised 4 × 2 min of intervals at 90% W_{max}, while *blocks 3* and *4* consisted of intervals at 80% W_{max}. During exercise, participants received an artificially sweetened electrolyte solution to maintain hydration and were fan-cooled to minimize thermal distress.

Following exercise, the treatment or control nutritional intervention was consumed immediately and again 1 h later (Fig. 1). Muscle biopsy samples were collected at 3 and 48 h (0800–0900, fasted) postexercise from the vastus lateralis as described previously (20). Biopsy sample times were chosen to examine the acute and longer-term genomic and translational signaling responses modeled from the seminal postendurance exercise transcriptome analysis of Mahoney et al. (35). Blood was collected into EDTA-evacuated tubes before exercise, at 0, 30, 60, 120, and 180 min postexercise, then centrifuged at 3,000 rpm for 10–15 min followed by aspiration. Muscle and blood were stored at −80°C until analysis.

Nutritional Intervention and Dietary Control

The intervention comprised 1.9 g/kg (dry weight) of powder made into 500 ml water. Each of the two servings were isocaloric and provided 0.2 g/kg fat (freeze-dried canola oil) with either: 1.2 g/kg carbohydrate (1:1 maltodextrin-fructose) and 0.4 g/kg protein (2:1 milk protein concentrate-whey isolate), abbreviated (PTN); or 1.6 g/kg carbohydrate (CON). The PTN formulation provided carbohydrate to induce hyperinsulinemia and maximize the glycogen synthe-

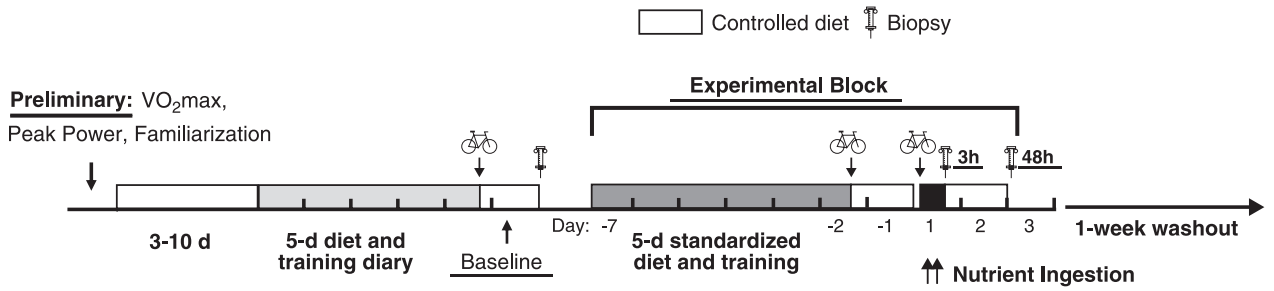

Fig. 1. Experimental design: the preliminary testing, the 5 days of standardized diet and exercise, and the 40 h controlled exercise and diet prior to the baseline muscle biopsy are shown. The experimental block comprised of 5 days of standardized diet and training, the 40-h controlled lead-in, a 100-min cycle ride, the nutrition intervention and muscle biopsies at 0 and 1 h, and 3- and 48-h postexercise, respectively.

sis rate after exercise (27) and protein to provide excess amino acid signal and substrate for high protein synthesis rates for ≥6 h (62).

Participants also ingested a preweighed controlled diet starting on *day −1*, and for the entire period of *day 1* until the completion of *day 2*, which provided energy estimated to balance requirement (3,459 ± 662 kcal/day; as 55% carbohydrate, 16% protein, and 29% fat). In both experimental conditions, the alternate nutritional powder (PTN or CON) was ingested 5 and 9 h postexercise, along with other food to balance total daily nutrient intake between treatments and, by design, isolate the specific timing effects of the postexercise protein nutrition.

Illumina Microarrays

Total RNA extraction, labeled-cRNA synthesis, hybridization, and microarray selection. Muscle tissue (15–20 mg) for gene microarray analysis was disrupted and homogenized in 900 μl of lysis buffer using a FastPrep instrument and lysing tubes containing ceramic beads (MP Biomedicals, Irvine, CA). From 450 μl of the lysate (corresponding to 5–10 mg of tissue) total RNA was extracted and purified with the RNAdvance tissue kit (Agencourt, Beckman Coulter Genomics, Danvers, MA) and quality checked with the Bioanalyzer 2100 (RNA integrity ≥8). All cRNA targets were synthesized, labeled, and purified via an automated procedure (50). All samples were analyzed with HumanRef-8 v2.0 Expression BeadChips (Illumina, San Diego, CA), which comprise probes to interrogate 22,184 transcripts.

Processing and statistical analysis. Scanning was performed using the BeadArray Reader and signal intensity quantified in GenomeStudio (Illumina). The microarray output was deposited in National Center for Biotechnology Information's Gene Expression Omnibus (GSE27285). Homoscedasticity was obtained using the Box-Cox power transformation; a constant was chosen to realize positive values before log2 transformation to normality and to stabilize the variance related to mean expression. All arrays were then quantile normalized. Treatment and time-affected differential expression were estimated using a mixed-model analysis of variance (ANOVA) for repeated measures (Partek, St. Louis, MO). Model fixed effect parameters were sequence, treatment, time, and the interactions treatment*sequence, treatment*subject, treatment*time (PTN effect), sequence*time (carryover), sequence*treatment*time (period), and subject*time (biological variability); subject was nested within sequence [subject(sequence)]. A moderated F test was then applied using the Global Error Assessment to take into account sample size leading to a more robust analysis. For the array analysis, we acknowledge that developments in statistical analysis are on-going and that effect size and biological variance-standardized likelihood-based gene selection criteria may be inferentially superior. In the absence of a satisfactory validated approach we utilized the traditional null hypothesis-based gene selection criteria ($P < 0.05$). In addition to the fold change, we calculated the Cohen effect size (ES) from composite standard deviation obtained from the MSE. The analysis returned sufficient power to detect a moderate effect size of 0.9–0.95. A transcript selection based on a lower P value would have reduced the probability of selecting false positive transcripts, but at the expense of inferential depth from the dataset by raising the ES threshold to large. To illustrate: at power 80% and an alpha 0.01, the ES threshold for significance would be 1.29; at an alpha 0.001, ES would be 1.65. Bioinformatic evaluation of the research question was based primarily on the protein nutrition effect: the differential expression profile in response to the addition of protein to the postexercise nutritional milieu (protein minus control at 3 and 48 h, respectively: PTN-CON3h, PTN-CON48h). Secondary information was provided from the reference environment: expression relative to baseline (Pre) in the control (CON-Pre3h, CON-Pre48h) and protein conditions (PTN-Pre3h, PTN-Pre48h); however, the full analysis is not presented here for brevity.

Bioinformatics. The global primary classification analysis based on evolutionary relationships was provided in Panther 6.1 (Protein ANalysis THrough Evolutionary Relationships, http://www.pantherdb.org). The Panther classification analysis output provided the molecular function and biological processes ontology for evaluation of the principle biology differentially affected by treatment and time. A skeletal muscle tissue reference list from all transcripts significantly (16,357 transcripts, $P < 0.01$) expressed at least once was used as background. We assigned a filtering criteria for Panther classifications of $P < 0.05$ and an enrichment level 1.5-fold considered biologically relevant. To provide further functional clustering, classifications were grouped into Family ontology. Families were loaded into Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com) software for targeted network and canonical pathway analysis. The IPA analysis criteria comprised Ingenuity Expert Information + KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) + GO databases. We filtered by selecting species=human and tissue=skeletal muscle (relaxed filter); immune cells were included during analysis of immune and defense because of the role leucocytes have in skeletal muscle function including cellular communication, inflammation, and phagocytosis following stress (47). Within each ontology, we examined IPA network, function, and canonical pathway outcomes and selected the top pathways assigned a focus score of 3.0 or higher, equivalent to 99.9% confidence (focus score is derived from the negative logarithm of the P value). Supplementary analysis of the full gene selection was conducted in KEGG, and an independent confirmatory analysis of the normalized gene sets was conducted in Gene Set Enrichment Analysis (GSEA, http://www.broadinstitute.org/gsea).

Quantitative PCR

Candidate nutrient responsive gene expression (prior to microarray from separate RNA extraction) was quantified by TaqMan real-time RT-PCR (qPCR) (20). Included were genes associated with cell energy homeostasis (*PPARGC1A, PDK4, FOXO1A*), growth and differentiation (*FOXO1A*; insulin-like growth factor 1, *IGF1*), inflammation (nuclear factor of kappa B, *NFKB*), lipid and cholesterol biosynthesis (serum response element binding protein 2, *SREBP2*), cell and membrane stress (DNA damage inducible transcript 3, *DDIT3*), calcium signaling (Down syndrome critical region gene 1, *DSCR1*), and mitochondrial branched-chain amino acid metabolism (branched chain keto-acid dehydrogenase kinase, *BCKDK*).

Phosphorylation Status of mTORC1 Pathway-associated Signaling Proteins by Immunoblot

Approximately 30 mg of wet muscle tissue was homogenized in sucrose-mannitol buffer and protease inhibitor cocktail (AEBSF, Aprotinin, Leupeptin, Bestatin, Pepstatin A, E-64), and the homogenate protein concentration determined by the Bradford assay. Protein (20 μg) was boiled in Laemmli buffer, then resolved, and separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto PVDF membrane. Primary antibodies were: phospho-Akt (Ser473, 1:1,000, #9271, Cell Signaling, Danvers, MA), total-Akt (1:1,000, #9272, Cell Signaling), phospho-AMPKα (Thr172, 1:1,000) (#4188, Cell Signaling), total-AMPKα (1:1,000, #07-363; Millipore, Billerica, MA), phospho-p38MAPK (Thr180/Tyr182, 1:1,000, #9212, Cell Signaling), total-p38MAPK (1:1,000, #9215, Cell Signaling), phospho-ERK1/2 (Thr202/Tyr204, 1:1,000, #9101, Cell Signaling), total-ERK (1:1,000, #9102, Cell Signaling), phospho-mTOR (Ser2448, 1:1,000, #2971, Cell Signaling), total-mTOR (1:1,000, #2972, Cell Signaling), phospho-S6 ribosomal protein (Ser240/244, 1:1,000, #4838, Cell Signaling), total-S6 ribosomal protein (1:2,000, #2217, Cell Signaling), total-4E-BP1 (1:1,000, #9452, Cell Signaling), phospho-$p70^{S6K}$ (Thr389, 1:400, #11,759, Santa Cruz Biotechnology, Santa Cruz, CA), total $p70^{S6K}$ (1:400, #230, Santa Cruz). Loading standards were anti-actin (1:10,000) (#612657; BD Biosciences, Mississauga, ON, Canada) and anti-tubulin (1:1,000) (#2125 Cell Sig-

naling). Membranes were probed with a horseradish peroxidase-conjugated secondary antibody (Amersham, Piscataway, NJ), visualized with chemiluminescence (ECL Plus, Amersham) and quantified with densitometry (*ImageJ* v1.34s software, http://rsbweb.nih.gov/ij/).

Muscle Glycogen, Plasma Insulin, and Glucose

Pro- and macro-glycogen fractions were extracted (1), and the resulting glycogen concentration determined from analysis of the glycosyl units via a fluorometric method (43). Plasma insulin and glucose concentration was determined by enzyme-linked immunoabsorbant assay (IS130D Insulin ELISA kit; Calbiotech, Spring Valley, CA) and fluorometry (43), respectively.

Statistical Analysis

The effect of treatment and time on all dependent variables except the Illumina microarray (described above) was estimated from mixed models (Proc Mixed, SAS Version 9.1; SAS Institute, Cary, NC). Data were log transformed prior to analysis. Uncertainty was presented as 95% confidence limits or *P* value. Inference was by effect size and magnitude-based likelihood, where effect size thresholds were qualified as trivial 0.0–0.2, small 0.2–0.6, moderate 0.6–1.2, large 1.2–2.0, very large 2.0–4.0, extremely large >4.0 (26, 51). The relationship between the magnitude of the log2 differential expression estimated by qPCR vs. microarray was provided by Pearson correlation.

RESULTS

Microarray Analysis

The microarray analysis revealed a large number of genes regulated in response to exercise and protein nutrition. At both time points, gene expression count was greater with PTN compared with CON, and activity likely associated with the exercise response, was higher at 3 h than at 48 h (Fig. 2). The top 100 expressed genes affected by PTN obtained from the GSEA are shown as a heat map in Fig. 3; this gene selection was verified against the GEA ANOVA selection with very large ($r^2 = 0.93$) and extremely large ($r^2 = 0.77$) correlations for the PTN treatment contrasts PTN-CON3h and PTN-CON48h, respectively. The Panther Classification gene ontology analysis is summarized in an Excel spreadsheet in Data Supplement 1, while the full ontology listing is provided in Data Supplement 2.[1] The analysis defined the transcriptome by biological process and molecular function, providing the basis for biological interpretation and subsequent inference. Accordingly, the transcriptome was summarized into four key areas described below.

Muscle Development and Remodeling

Developmental processes, specifically mesoderm development, was overrepresented with PTN, including genes involved in angiogenesis, and muscle and skeletal development (for summary see Table 1 and for full ontology detail Data Supplements 1 and 2). Genes grouped in muscle development and angiogenesis were mostly downregulated with PTN at 3 h, with the exception of the upregulation of *MYLPF* and the myogenic enhancing factors *MYOG* and *GDF7* (Table 1). Expression of other myogenic enhancers and development factors also affected by PTN included *GDF1* downregulated at 3 h, upregulated at 48 h, and *MYOD1* and *MYF5* upregulated at 48 h (Table 1). Moreover, at 3 h, expression of the muscle growth suppressor myostatin (*GDF8*) was downregulated with PTN, while expression of the muscle morphogenic factor wingless-type MMTV integration site family, member 5A *(WNT5A)* was upregulated (Data Supplement 2). Interrogation of the developmental processes ontology with IPA identified axonal guidance signaling as the top canonical pathway affected by PTN (Table 3). In this pathway we identified differential expression within semaphorin, netrin, integrin, and ephrin receptor-mediated signaling pathways (not shown).

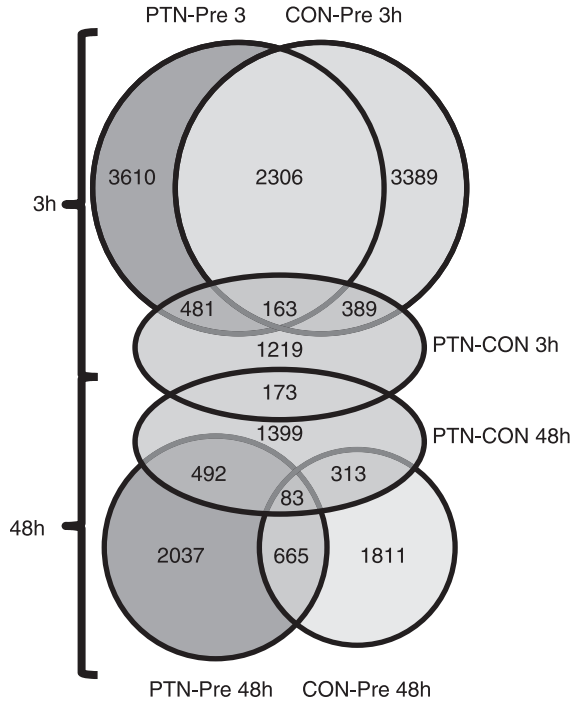

Fig. 2. Venn diagram showing the number of significantly selected genes from the GEA ANOVA within and common to the 6 differential comparisons used in the analysis of the transcriptome response to the effect of protein nutrition and exercise. PTN, protein; CON, control.

Overrepresented cell adhesion and cell structure biological processes (3 h), and extracellular matrix molecular function ontologies (3 and 48 h) provide evidence for PTN-affected expression of cell surface and structure related gene expression (see Data Supplement 1 worksheet 2 for ontology summary, and Data Supplement 2 for gene summaries and statistical detail). Gene expression in the latter was mainly upregulated relative to baseline, but PTN mostly downregulated this program, relative to CON, with representative ontology including extracellular matrix structural protein (e.g., collagens) and extracellular matrix glycoprotein (e.g., fibulin, elastin microfibril interface). Concerning structure, tubulin and intermediate filament (vimentin, keratin, nestin) molecular function ontology were overrepresented by PTN at 3 h.

Associated with skeletal muscle contractile function, PTN regulated the cytoskeletal molecular-function ontology at 3 h, including up- and downregulated genes within the actin-binding motor protein ontology. At 48 h, PTN exclusively upregulated eight transcripts for actin binding motor protein (Data Supplements 1 and 2). Other myofilament transcript expression was differentially affected by PTN, revealing a temporal ex-

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

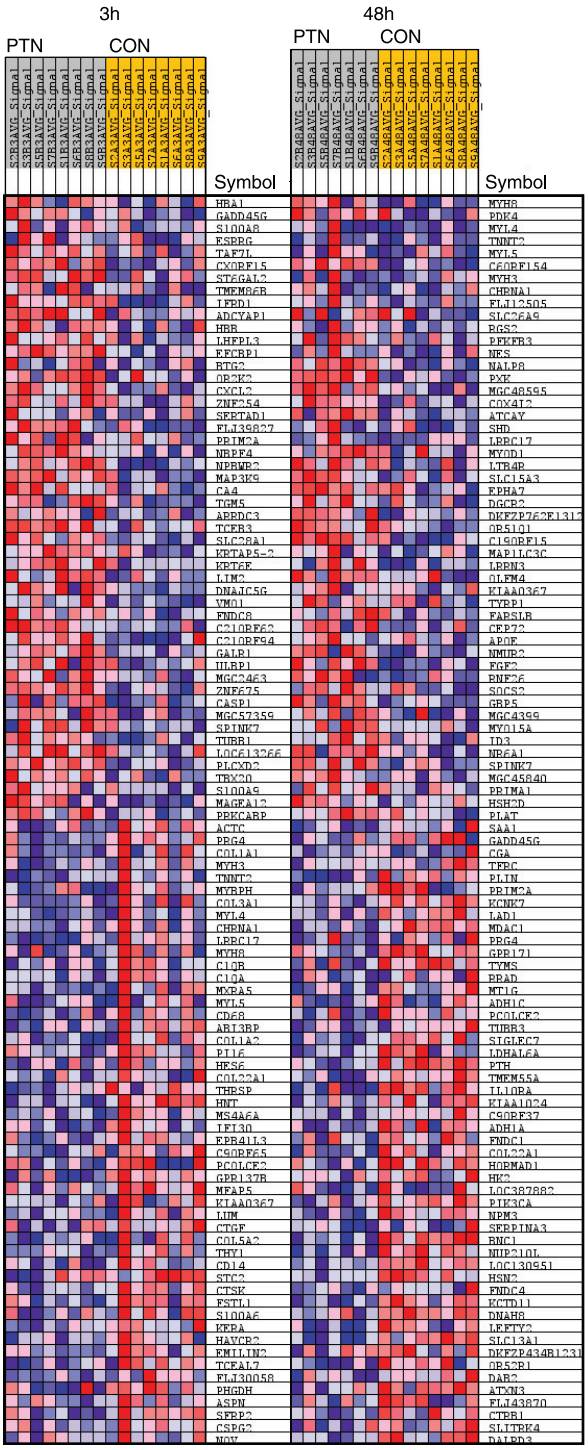

Fig. 3. Heat map of the top 100 genes by expression magnitude obtained from the GSEA for the protein and the control diet conditions at 3 and 48 h postexercise.

pression pattern within the muscle contraction gene ontology of mainly downregulation at 3 h and upregulation 48 h (Table 2). Interrogation of the signal transduction ontology in IPA confirmed cytoskeletal, extracellular matrix receptor, and mechanotransduction-sensitive canonical pathways was affected by protein (Table 3). Finally, transporter expression was overrepresented with protein (Data Supplement 1), of which upregulation of voltage-gated potassium ion channels is highlighted because of relevance to muscle contractile function (Table 2).

Acute Immune and Inflammatory Response

Protein feeding affected the immunity and defense ontology at 3 h, with differentially regulated classifications complement, major histocompatibility complex (MHCII), and macrophage-mediated immunity (Data Supplement 1). Interrogation of the ontology in IPA presented dendritic cell maturation as the top canonical pathway (Table 3), with cell-to-cell signaling and interaction and the inflammatory response as the two top functions, within which functional gene clusters were associated with leukocyte activation, adhesion, and recruitment. Three heat shock protein 70 (HSP) Family chaperone genes were upregulated (Data Supplement 2).

Transcriptional Regulation and Posttranslational Processing

Nucleoside, nucleotide, and nucleic acid metabolism and protein modification ontology were underrepresented in response to PTN (Data Supplement 1, worksheet 1). In this study, we focused on genes involved in protein modification and protein breakdown to inform on nutritional regulation of protein turnover (Fig. 4). Protein feeding underrepresented but upregulated expression of apoptotic inhibition genes at 3 h; however, proapoptotic caspases were also upregulated (Data Supplement 1 and 2; Fig. 4). Cathepsins (lysosomal proteolysis) were up- and downregulated, matrix metallopeptidases upregulated, and metallopeptidase inhibitors downregulated with protein nutrition at 3 h (Fig. 4). Genes involved in ubiquitin-specific proteolysis, including ubiquitin-protein E2 and E3 ligases and proteins were generally downregulated by exercise (Data Supplement 2) and PTN at 3 h (Fig. 4; Data Supplement 2).

Metabolic Transcriptome

Carbohydrate and lipid metabolism ontology were over- or underrepresented by exercise relative to baseline at both 3 and 48 h, but only at 48 h was PTN differentiated ontology detected (Data Supplement 1, worksheet 1). To summarize, the pattern of gene expression regulating glycolysis suggested mostly downregulated carbohydrate metabolism. Plasma membrane and mitochondrial fatty acid transporter and electron-transport chain protein complex transcripts were mostly upregulated, and there was mixed up- and downregulation of genes involved in fatty acid handling, lipid and steroid metabolism (Fig. 5).

Lipid, fatty acid, and steroid metabolism ontology was investigated further within the analysis of the late response transcriptome because of the AMPK phosphorylation and qPCR data, and because of the biological relevance to endurance exercise adaptation (25). Furthermore, a top node in the IPA network analysis was peroxisome proliferator-activated receptor gamma (*PPARG*), with *PPARG* moderately downregulated, then largely upregulated with PTN at 3 h (Data Supplement 2), respectively; the PPARγ/δ canonical pathways were detected in the IPA analysis (Table 3) with genes upregulated by PTN downstream of PPARD (LPN1, PPARGC1A, PDK4, CD36) and PPARG (CPT2, ACOX7, ACS, FABP5, RARB) involved in lipid transport and metabolism; and finally, the carbohydrate-catabolic and lipid-metabolic processes were

Table 1. *Effect of postexercise protein feeding on the expression transcripts within the developmental processes gene ontology at 3 and 48 h postexercise*

Gene Name (Symbol)[a]	Accession[b]	Effect Size[c] 3 h	Effect Size[c] 48 h
Angiogenesis			
Angiopoietin-like 2 (ANGPTL2)	012098	−1.35	
Brain-specific angiogenesis inhibitor 1 (BAI1)	001702	1.11	
CD248 antigen, endosialin (CD248)	020404	−1.30	
Collagen, type XVIII, alpha 1 (COL18A1), TV 1	030582	−1.12	
Fibroblast growth factor 1 (acidic) (FGF1), TV 1	000800	−1.19	
c-fos induced growth factor (FIGF)	004469	1.00	
Neuropilin 2 (NRP2), TV 6	201264	−1.12	
Sema domain, (semaphorin) 3C (SEMA3C)	006379	−1.05	
Tenomodulin (TNMD)	022144	−1.48	
Muscle Development			
Cdon homolog (mouse) (CDON)	016952	−0.99	
Cysteine-rich protein 1 (intestinal) (CRIP1)	001311	−2.27	
eEF1A2 binding protein (DKFZp434B1231)	178275	−2.36	
Dystrophin (DMD), TV Dp40	004019	−1.60	
Dual specificity phosphatase 4 (DUSP4), TV 2	057158	−1.61	
Growth differentiation factor 7 (GDF7)	182828	1.31	
Muscle blind-like 2 (Drosophila) (MBNL2), TV 1	144778	−1.06	
Myosin regulatory light chain MRCL3 (MRCL3)	006471	−1.00	4.31
Myosin, heavy polypeptide 1, skeletal muscle adult (MYH1)	005963	−1.21	2.87
Myosin, heavy polypeptide 3, skeletal muscle embryonic (MYH3)	002470	−6.80	2.55
Myosin, heavy polypeptide 8, skeletal muscle perinatal (MYH8)	002472	−4.57	10.26
Myosin, light polypeptide 5, regulatory (MYL5)	002477	−3.00	2.49
Fast skeletal myosin light chain 2 (MYLPF)	013292	1.09	
Myogenin (myogenic factor 4) (MYOG)	002479	1.29	
Vesicle-associated membrane protein 1 (VAMP1), TV 3	016830	−1.57	
Periostin, osteoblast specific factor (POSTN)	006475	−1.48	1.25
Skeletal Development			
Amelogenin, Y-linked (AMELY)	001143		1.26
Bone gamma-carboxyglutamate protein (osteocalcin) (BGLAP)	199173		1.26
Calcitonin/calcitonin-related polypeptide, alpha (CALCA), TV 3	001033953		−1.10
Decorin (DCN), TV B	133504	−1.76	−1.53
EGF-containing fibulin-like ECM protein 1 (EFEMP1), TV 1	004105	−2.04	−1.88
Fibulin 1 (FBLN1), TV C	001996	−1.14	−1.23
Fibulin 2 (FBLN2), TV 1	001004019	−1.02	−1.43
FYVE, RhoGEF and PH domain containing 5 (FGD5)	152536		−1.30
Latent transforming growth factor beta binding protein 4 (LTBP4)	003573		−1.41
msh homeo box homolog 1 (Drosophila) (MSX1)	002448		1.41
Parathyroid hormone (PTH)	000315		−1.50
Ribosomal protein S6 kinase, 90 kDa, 1 (RPS6KA1), TV 1	002953		1.11
Syndecan 2 (SDC2)	002998	−1.03	−1.25
Short stature homeobox 2 (SHOX2), TV SHOX2a	006884		−1.62
SMAD specific E3 ubiquitin protein ligase 1 (SMURF1), TV 2	181349		1.42
Tumor necrosis factor (ligand) (TNFSF11), TV 1	003701	1.28	−1.86
Other Mesoderm Development			
ADAM metallopeptidase domain 17 (ADAM17)	003183		1.13
ADAM metallopeptidase domain 19 (ADAM19), TV 2	033274		−1.19
Asporin (LRR class 1) (ASPN)	017680	−2.35	
B-cell CLL/lymphoma 9-like (BCL9L)	18255	0.99	
Boc homolog (mouse) (BOC)	033254		−1.07
Butyrophilin, subfamily 1, member A1 (BTN1A1)	001732	1.07	
Butyrophilin, subfamily 3, member A3 (BTN3A3), TV 1	006994	1.11	
Calcitonin-related polypeptide, beta (CALCB)	000728		−1.63
CD248 antigen, endosialin (CD248)	020404	−1.30	−1.84
Collagen, type V, alpha 1 (COL5A1)	000093	−1.62	
Collagen, type X, alpha 1 (COL10A1)	000493	−1.07	
Collagen, type XI, alpha 2 (COL11A2), TV 2	080681	−1.26	
Cysteine-rich protein 2 (CRIP2)	001312		1.06
Dual specificity phosphatase 2 (DUSP2)	004418		1.28
eEF1A2 binding protein (DKFZp434B1231)	178275	−2.36	
EPH receptor A10 (EPHA10), TV 2	173641		−1.28
EPH receptor A2 (EPHA2)	004431	1.26	

Continued

Table 1.—*Continued*

Gene Name (Symbol)[a]	Accession[b]	Effect Size[c] 3 h	48 h
EPH receptor A7 (EPHA7)	004440		1.67
Fibrillin 1 (Marfan syndrome) (FBN1)	000138	−1.05	
Fibroblast growth factor 2 (basic) (FGF2)	002006		1.51
Fibulin 1 (FBLN1), TV D	006486	−1.14	−1.23
Fibulin 2 (FBLN2), TV 1	001004019	−1.02	
Forkhead box H1 (FOXH1)	003923		−1.11
Forkhead box I1 (FOXI1), TV 1	012188		1.25
FOXD4-like 2 (FOXD4L2)	199135	1.35	
fyn-related kinase (FRK)	002031		1.11
GLI-Kruppel family member (GLI3)	000168	−1.17	
Growth differentiation factor 1 (GDF1)	001492	−1.25	1.25
Hemopoietic cell kinase (HCK)	002110		−1.25
Matrix Gla protein (MGP)	000900	−1.72	
Myeloproliferative leukemia virus oncogene (MPL)	005373		1.14
Myogenic differentiation 1 (MYOD1)	002478		1.60
Myogenic factor 5 (MYF5)	005593		1.07
NCK adaptor protein 2 (NCK2), TV 1	003581		1.26
Nuclear factor of activated T-cells 5, (NFAT5), TV 5	173215	1.01	
Transcneuropilin 1 (NRP1), TV 1	003873		1.17
Nuclear factor (erythroid-derived 2), 45 kDa (NFE2)	006163	1.00	
odz, odd Oz/ten-m homolog 1(Drosophila) (ODZ1)	014253	1.21	
Oncomodulin (OCM)	006188		−1.65
Paired immunoglobin-like type 2 receptor α (PILRA), TV 2	178272		−1.09
Paired immunoglobin-like type 2 receptor β (PILRB), TV 2	175047	1.06	
Periostin, osteoblast specific factor (POSTN)	006475	−1.57	1.25
Polyhomeotic-like 2 (Drosophila) (PHC2), TV 1	198040		1.15
preB-cell leukemia transcription factor 3 (PBX3)	006195		−1.18
preB-cell leukemia transcription factor 4 (PBX4)	025245		1.26
Proprotein convertase subtilisin/kexin type 5 (PCSK5)	006200		−1.09
Proprotein convertase subtilisin/kexin type 6 (PCSK6), TV 6	138325	−0.99	
Runt-related transcription factor 1 (RUNX1)	001754	−1.21	
Sema domain (semaphorin) 3F (SEMA3F)	004186		1.32
Sema domain (semaphorin) 6D (SEMA6D), TV 6	024966		1.19
Sprouty homolog 1 (SPRY1), TV 1	005841		−1.15
Sprouty homolog 4 (Drosophila) (SPRY4)	030964	1.00	1.48
T-box 5 (TBX5), TV 2	080718	1.05	
Trefoil factor 3 (intestinal) (TFF3)	003226	−1.25	
Transforming growth factor, beta-induced, 68 kDa (TGFBI)	000358	−1.36	
Tumor necrosis factor superfamily, member 11 (TNFSF11)	003701	1.28	−1.86
Tropomyosin 1 (alpha) (TPM1), TV 5	000366		1.13
Vesicle-associated membrane protein 2 (VAMP2)	014232		−1.16

Data are the significantly selected genes in the respective Panther classifications within the gene ontology Family developmental processes at 3 and 48 h. [a]Angiogenesis, muscle development, skeletal development are Child to mesoderm development (Father); mesoderm development is Father to developmental processes (Family). TV, transcript variant. [b]NCBI Reference Sequence. [c]Confidence limits for effect size for 3 h were 0.98–0.99; for 48 h 1.06–1.07. Fold effect and *P* value are provided in Data Supplement 3.

listed within the top 20 enriched processes in the independent GSEA analysis the effect of protein at 48 h.

Relative to baseline (Pre), the expression of the coactivator *PPARGC1A* was extremely upregulated at 3 h in control, but not affected by PTN; however, at 48 h *PPARGC1A* was moderately upregulated with PTN (Data Supplement 2). To provide further confirmatory evidence of protein nutrition-mediated expression within the energy metabolism related late transcriptome, the gene selections were mapped against 2,307 *PPARGC1A*-responsive genes (A. Safdar, 2011, personal communication). The significant gene selection revealed within the lipid, fatty acid, and steroid metabolism ontology was confirmed and included within Fig. 5. We also analyzed for other DNA-binding proteins active on the *PPARGC1A* promoter (24). Genes upregulated with PTN were estrogen-related receptor gamma (*ESRRG*; effect size very large) transcript variant 3 at 3 h, and at 48 h histone deacetylase 8 (*HDAC8*; moderate), cAMP-response element binding protein 1 (*CREB1*; large), and *MYOD1* (large). Additionally, DNA (cytosine-5)-methyltransferase 3 beta (*DNMT3B*) was moderately downregulated at 48 h. Signaling pathway activity related to the *PPARGC1A* promoter was indicated for AMPK via the Western blot analysis (Fig. 6) and cAMP-mediated/PKA and Ca^{2+}/calmodulin kinase via IPA canonical pathways (Table 3).

Candidate and Confirmatory Gene Expression

A statistical summary of the analysis of candidate protein nutrition responsive gene expression by qPCR is provided in Table 4. At 3 h, moderate to extremely large increases in *DDIT3*, *PPARGC1A*, and *FOXO1A* were observed, in the control condition at 3 h relative to Pre. Moderate increases PTN vs. CON were seen in *PPARGC1A*, *PDK4, DDIT3,* and *FOXO1A* at 3 h, while at 48 h, only *PDK4* and *PPARGC1A*

Table 2. *Effect of postexercise protein feeding on the expression of transcripts within the muscle contraction gene ontology at 3 and 48 h postexercise*

Gene Name (Symbol)[a]	Accession[b]	Effect Size 3 h	Effect Size 48 h
Adenosine A2a receptor (ADORA2A)	000675	−1.16	
Adenosine A2b receptor (ADORA2B)	000676		−1.12
alphakinase 3 (ALPK3)	020778		−1.49
Calcium channel, voltage-dependent, beta 3 subunit (CACNB3)	000725	1.14	
Calcium channel, voltage-dependent, beta 4 subunit (CACNB4), TV2	000726	−0.99	
Cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle) (CHRNA1)	000079	−3.51	1.84
Cholinergic receptor, nicotinic, gamma (CHRNG)	005199		1.07
Complement component 3a receptor 1 (C3AR1)	004054	−1.37	
Creatine kinase, mitochondrial 1B (CKMT1B)	020990	1.15	
Dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive) (DYSF)	003494		−1.18
Dystrobrevin, alpha (DTNA), TV 7	001392		−1.33
eEF1A2 binding protein (DKFZp434B1231)	178275	−2.36	−2.81
Endothelin receptor type B (EDNRB), TV 2	003991	1.21	
Fast skeletal myosin light chain 2 (MYLPF)	013292	1.09	
fer-1-like 3, myoferlin (C. elegans) (FER1L3), TV 1	013451	−1.35	
Hypothetical protein FLJ39502 (FLJ39502)	173648		1.54
Hypothetical protein LOC340156 (LOC340156)	001012418		−1.46
Leukotriene B4 receptor (LTB4R)	181657		1.45
LIM domain 7 (LMO7)	005358		−1.24
Myosin binding protein C, slow type (MYBPC1), TV 3	206820	−1.00	
Myosin binding protein H (MYBPH)	004997	−5.59	−3.50
Myosin binding protein H-like (MYBPHL)	001010985	−1.17	
Myosin light chain 1 slow a (MLC1SA)	002475	−1.42	1.82
Myosin regulatory light chain MRCL3 (MRCL3)	006471	−1.00	4.31
Myosin, heavy polypeptide 1, skeletal muscle, adult (MYH1)	005963	−1.21	2.87
Myosin, heavy polypeptide 3, skeletal muscle, embryonic (MYH3)	002470	−6.80	2.55
Myosin, heavy polypeptide 8, skeletal muscle, perinatal (MYH8)	002472	−4.57	10.26
Myosin, light polypeptide 1, alkali; skeletal, fast (MYL1), TV 1f	079420	1.03	
Myosin, light polypeptide 4, alkali; atrial, embryonic (MYL4), TV 2	002476	−3.79	3.22
Myosin, light polypeptide 5, regulatory (MYL5)	002477	−3.00	2.49
Nebulin-related anchoring protein (NRAP), TV 2	198060		2.01
Oncomodulin (OCM)	006188		−1.65
Oxytocin, prepro- (neurophysin I) (OXT)	000915	1.16	
Phosphorylase kinase, gamma 1 (muscle) (PHKG1)	006213		−1.15
Potassium large conductance calcium- activated channel, subfamily M, beta member 1 (KCNMB1) titin (TTN), TV novex-3	004137		1.30
Transgelin (TAGLN), TV 1	133379	−1.59	
Tropomyosin 1 (alpha) (TPM1), TV 5	001001522	1.18	
Troponin T type 2 (cardiac) (TNNT2), TV 4	000366		1.13
	001001432	−4.82	3.57

Data are the significantly selected genes within the Panther classification muscle contraction at 3 and 48 h. [a]Genes listed under Panther Classification analysis outcome are gene ontology Father. Transcript variant = TV. [b]NCBI Reference Sequence. [c]Confidence limits for effect size for 3 h were 0.98–0.99. Fold effect and *P* value are provided in Data Supplement 3.

returned evidence for upregulation. To confirm the magnitude of expression between qPCR and the microarray, mean qPCR fold change for all comparisons was very highly correlated with the mean fold change from the Illumina microarray ($r^2 = 0.99$).

AMPKα1 Phosphorylation and mTORC1 Pathway Regulation of Translation Initiation

Evaluation of the effect of protein feeding on phosphorylation activity within the mTORC1 pathway is shown in Fig. 6. At 3 h in response to PTN, large and very large (effect size) respective increases in RPS6 (3.23-fold change relative to control; 95% fold confidence limits ×/÷1.40, $P < 0.001$) and 4E-BP1 (2.58; ×/÷1.20, $P < 0.001$) phosphorylation were observed, which was associated upstream with a trivial increase in $p70^{S6K}$ (1.25; ×/÷1.57, $P = 0.31$) and AKT/PKB (0.95; ×/÷1.32, $P = 0.70$) phosphorylation, unclear outcome for p38-MAPK (1.49; ×/÷1.99, $P = 0.24$) and ERK1/2-p42 (0.77; ×/÷2.52, $P = 0.56$), but a moderate increase in mTORC1 (1.30; ×/÷1.30, $P = 0.05$) (Fig. 6). By 48 h, RPS6 phosphorylation was negligible (1.16; ×/÷1.51, $P = 0.46$), but 4E-BP1 phosphorylation was reduced (0.74; ×/÷1.28, $P = 0.02$) and AMPKα1 phosphorylation increased (1.65; ×/÷1.42, $P = 0.008$).

Plasma Insulin and Glucose and Muscle Glycogen Resynthesis

Protein feeding had trivial impact on circulating insulin concentrations over the 3-h period postexercise (overall mean concentration in control: 30 ± 16 μUI/ml; mean effect of protein: 2.0% 95% CL ± 15%, $P = 0.83$) and glucose (5.4 ± 0.9 mmol/l, 4.9 ± 7.0%, $P = 0.27$). The quantity of glycogen in dried muscle at baseline was 902 ± 133 mmol/kg dry wt. Protein feeding had no effect on glycogen concentrations at 3 h (overall mean concentration in control: 406 ± 217 mmol/kg dry wt; mean effect of protein −3.0%; ± 18.0%, $P = 0.79$) or 48 h (964 ± 325 mmol/kg dry wt; 2.2%; ± 18.0%, $P = 0.84$).

Table 3. *The top IPA canonical pathways affected by postexercise protein feeding at 3 and 48 h following exercise following interrogation of Panther Family gene ontology selections*

Ontology Family	Top Canonical Pathways[a]	Biology
Developmental processes	axonal guidance signaling[3 h, 48 h]	assembly and organization; attraction and adhesion; signaling through extracellular matrix and surface receptors
Muscle contraction	actin cytoskeleton signaling[3 h, 48 h]	signaling controlling rearrangement of the actin cytoskeleton and actinomyosin assembly
	calcium signaling[48 h]	cell growth and development, slow fiber development, contractile proteins
Signal transduction	ephrin receptor signaling[3 h, 48 h] relaxin signaling[48 h]	growth, development, adhesion, angiogenesis
	cAMP-mediated signaling[48 h] PKA signaling[48 h]	cell growth and development
	ERK-MAPK signaling[48 h]	mechanotransduction
Immunity and defense	dendritic cell maturation[3 h]	inflammatory response, downregulation of endocytic/phagocytic activity; MHC-specific antigen presentation; T-cell activation
Carbohydrate	glycolysis[48 h]	glucose catabolism; β-oxidation; fatty acid transport; mitochondrial electron transport chain
Lipid and steroid metabolism	fatty acid metabolism[48 h]	
	PPAR signaling (γ/δ)[48 h]	

[a]Canonical pathway differentially detected in response to the PTN intervention versus CON at 3 h and or both 48 h, respectively.

DISCUSSION

The informatics analysis of this microarray provides the nutrigenomic signature induced by protein ingestion following intense endurance exercise. The early transcriptome assayed at 3 h following exercise and PTN was characterized by majority downregulated gene expression in the overrepresented ontology muscle contraction, mesoderm development, extracellular matrix signaling and structure, proteolytic processes, and signal transduction ontology, and underrepresented nucleoside, nucleotide and nucleic acid metabolism, suggesting an attenuated transcriptome response to exercise-induced stress, which was associated with attenuated decline in AMPKα1 phosphorylation. The later transcriptome (48 h) assayed at rest following an overnight fast, suggested upregulated mitochondrial fatty acid transporter and mitochondrial electron transporter expression, slow-myofibril remodeling, and downregulated glycolytic enzyme expression with PTN. Other nutrition regulated molecular events (e.g., mRNA stability, epigenetic regulation) in addition to the transcriptome will also highly likely influence the final expressed protein phenotype. However, from a functional genomics viewpoint the represented transcriptome and signal transduction pathway analysis (Fig. 7) provides the blueprint for the skeletal muscle biology affected by PTN that constitute the processes of recovery from and adaptive remodeling to endurance exercise stress.

Skeletal Muscle Tissue Structural Remodeling and Slow Myofibril Development is Regulated by Protein Nutrition

Skeletal muscle remodeling of contractile protein, cytoskeletal, and extracellular matrix are central to endurance training adaptation (19, 58, 69). Therefore, it was noteworthy that these ontology were overrepresented in response to postexercise protein co-ingestion. With respect to muscle contraction, protein nutrition induced a temporally differentiated expression of myosin isoforms at 3 and 48 h, characterized by mostly downregulation at 3 h, but large (effect size) upregulation at 48 h. This time-course response was especially evident in the myogenic control factors, suggesting that during early recovery protein feeding may acutely direct the transcriptome away from developmental processes. This unexpected pattern is further supported by the downregulation of the transient isoforms of embryonic and perinatal myosin heavy and light chain, and cardiac troponin at 3 h, but may nevertheless suggest new fiber development (remodeling) (30).

Myogenic (*MYOG, MYOD*, muscle respiratory factor 4) and metabolic (*HK, PDK4*) gene expression in response to endurance exercise without postexercise nutrition has previously been shown to be highest 8–24 h following exercise (68). Therefore, full appreciation of the time course of myofibril gene expression and other developmental processes affected by PTN may be missing from the present analysis given the 3 and 48 h biopsy time points. Furthermore, the late gene expression (48 h) may represent, in part, the downstream response to the molecular program induced by the early transcriptome dominated by acute exercise and elevated signaling from amino acids and insulin. The late response, in contrast, is a comparatively different signaling environment being resting and fasted, which suggests that the transcriptome was still affected by the exercise and protein-feeding intervention for at least the following 48 h.

Nevertheless, analysis of expression within the calcium signaling canonical pathway pointed to a role for protein nutrition in upregulating slow-twitch myofibrillar development following endurance exercise. Previously, calcium-calmodulin signaling and activation of calcineurin was shown to affect gene expression through the transcription factors CREB, NFAT, and MEF2, leading to increased slow fiber-type MHC, MYL, troponin, and SERCA isoform expression with endurance training (22), a pattern consistent with the present protein nutrition-mediated response and the typical phenotypic profile of endurance athletes. In support, *NFATc* and *CREB1* expression was upregulated with exercise and protein nutrition, and other evidence for expression with the calcium signaling pathway was provided from enrichment within the calmodulin-related protein and the select calcium binding protein ontologies. Additionally, *WNT5A* expression suggests myogenic gene induction (*MYF5*, *MYOD*) and slow-fiber specific transcription via the noncanonical pathway involving calmodulin-mediated kinase II, calcineurin, and NFAT (67). Collectively, the muscle contraction transcriptome supports a role for protein nutrition in myofibril remodeling towards the slow-fiber phenotype following endurance exercise.

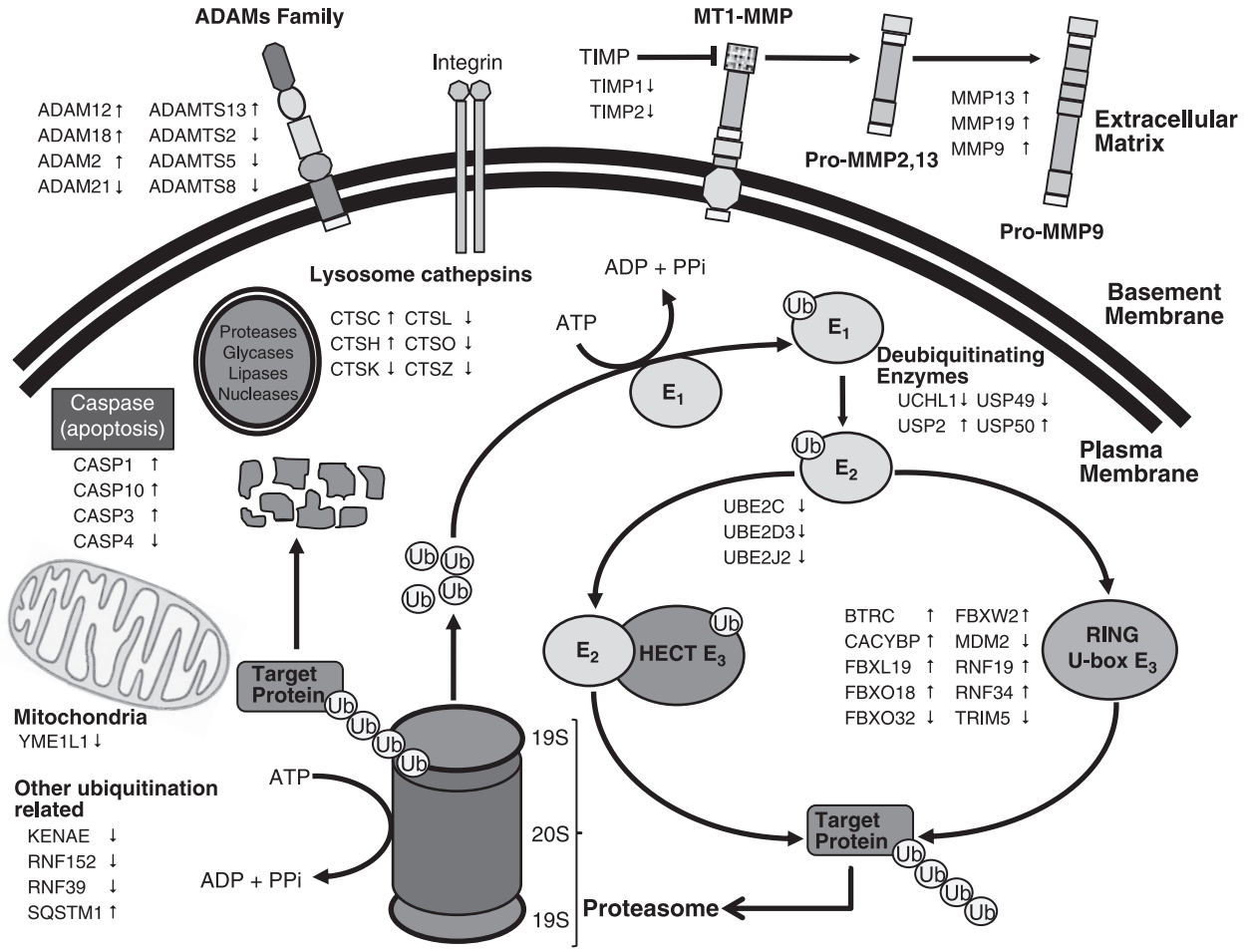

Fig. 4. Effect of protein vs. control nutrition following intense endurance exercise on gene expression involved in protein modification and breakdown at 3 h postexercise. Summarized are genes classified under the molecular function ontologies: cysteine peptidase, metallopeptidase, and ubiquitin-specific protease activity. Gene symbols are listed in proximity to the pathway component to which they belong. Gene expression up ↑/down ↓; ADAM2, 12, 18, 21 and ADAMTS2, 5, 8, 13, a disintegrin and metalloprotease domain family and subfamily a disintegrin and metalloprotease with thrombospondin motifs; timp1, 2, metallopeptidase inhibitor 1 and 2; MMP9, 13, 19, matrix metallopeptidase; CTSC, CTSH, CTSK, CTSL, CTSO, CTSZ, cathepsin family; UCHL1, ubiquitin carboxyl terminal esterase L1; USP2, 49, 50, ubiquitin-specific protease 2, 49, 50; CASP1, 4, 10, initiator caspases and CASP3 affector caspase; BTRC, CACYBP, FBXL19, FBXO18, FBXO32, FBXW2, MDM2, RNF19, RNF34, TRIM5, E3 ubiquitin ligase complex; UBE2C, UBE2D3, EBE2J2, E2 ubiquitin-conjugating enzyme family; YME1L1 Mitochondrial escape 1 like 1; RNF152, ring finger protein 152; RNF39, ring finger protein 32; SQSTM1 Sequestome 1.

With respect to cytoskeletal function, protein nutrition affected microfilament expression, suggesting regulation of cell structure motility and vesicle and organelle trafficking (16). Intermediate filaments contribute to sarcomere integrity and provide mechano-chemical links between the sarcolemma, nuclei, and mitochondria (11). Therefore, increased expression of desmin and the cytokeratins may offer protection against sarcolemma disruption (55). In the current study, protein nutrition mostly upregulated keratin and keratin-associated protein gene expression (9 of 12 genes) and downregulated vimentin and nestin at 3 h. The degree of sarcomerogenesis depends on the extent of muscle damage (48), and vimentin and nestin have been described as markers of skeletal muscle injury (61). Therefore, the intermediate filament expression profile suggests that sarcomere damage was lower with protein nutrition and that protein nutrition regulated intermediate filament remodeling. By 48 h, the cytoskeletal program comprised entirely upregulated actin-binding motor protein transcription. Coupled with the myosin gene expression profile at this time point, these data suggest a temporally dependent cytoskeletal filament remodeling program, in that the early response is primarily associated with cell structural integrity, while the late response is concerned with remodeling of contractile proteins. Further research is required to determine the relevance of this expression program on myofibril and cell structural remodeling using chronic exercise-feeding intervention models that also include measures of posttranscriptional regulation of mRNA, and (proteomic) measures of specific protein synthesis and breakdown.

Protein nutrition also affected the extracellular matrix transcriptome, which supports known endurance training adaptation. Protein affected vascular (angiogenesis) and neuronal development (Data Supplement 1; sensory perception, synaptic transmission ontology), with the former central to increased tissue capillary growth (58) and the latter to motor neuron development (21). Angiogenesis and neuronal development share common guidance cues through the ex-

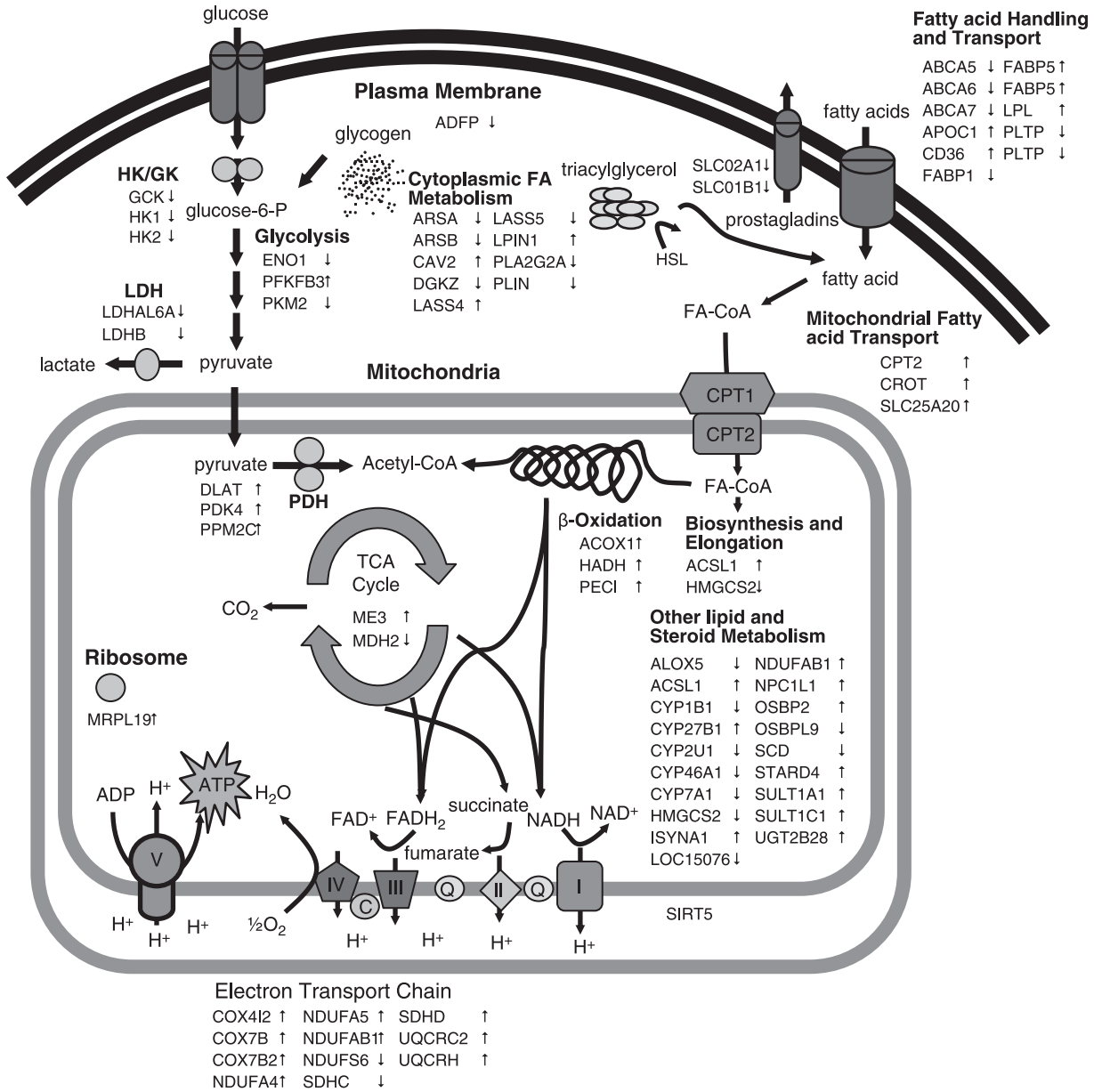

Fig. 5. Effect of protein vs. control nutrition following intense endurance exercise on gene expression involved in the regulation of carbohydrate (glycolysis) and fatty acid transport, lipid and steroid metabolism in the skeletal muscle at 48h post exercise. Gene symbols are listed in proximity to the pathway component to which they belong. Gene expression up ↑/down ↓; ABCA5-ABCA7, ATP-binding cassette sub-family A members 5–7; ACOX1, acyl-CoA oxidase 1 palmitoyl; ACSL1, acyl-CoA synthetase long-chain family member 1; ADFP, adipose differentiation-related protein (perilipin-22); ALOX5, arachidonate 5-lipoxygenase; APOC1, apolipoprotein C-I; ARSA and ARSB, arylsulfatase A and B; CAV2, caveolin 2; CD36, thrombospondin receptor; COX4I2, COX7B, COX7B2, cytochrome c oxidase subunit IV, VIIb, VIIb2; CPT2, carnitine palmitoyltransferase 2; CROT, carnitine O-octanoyltransferase; CYP1B1, CYP27B1, CYP2U1, CYP46A1, CYP7A1, cytochrome P450 family; DGKZ, diacylglycerol kinase zeta; DLAT, dihydrolipoamide S-acetyltransferase; ENO1, enolase 1; FABP1 and FABP5, fatty acid binding protein 1 and 5; FA-CoA, fatty acyl coenzyme A; GCK, glucokinase (hexokinase 4); HADH, hydroxyacyl-CoA dehydrogenase; HK1 and 2, hexokinase 1 and 2; HMGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial); ISYNA1, inositol-3-phosphate synthase 1; LASS4 and LASS5, LAG1 homolog ceramide synthase 4 and 5; LDHAL6A and LDHB, lactate dehydrogenase A-like 6A and lactate dehydrogenase B; LOC15076, hypothetical protein LOC150763; LPIN1, lipin 1; LPL, lipoprotein lipase; MDH2, malate dehydrogenase 2 NAD (mitochondrial); ME3, malic enzyme 3 NADP(+)-dependent, mitochondrial; MRPL19, mitochondrial ribosomal protein L19; NDUFA4, NDUFA5, NDUFAB1, NDUFS6, NADH dehydrogenase (ubiquinone); NPC1L1, Niemann-Pick disease type C1 gene-like 1; OSBP2 and OSBPL9, oxysterol binding protein 2, and protein-like 9; PDK4, pyruvate dehydrogenase kinase, isozyme 4; PECI, peroxisomal D3,D2-enoyl-CoA isomerase; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; PKM2, pyruvate kinase muscle isoform; PLA2G2A, phospholipase A2 group IIA; PLIN, perilipin; PLTP, phospholipid transfer protein; PPM2C, pyruvate dehydrogenase phosphatase catalytic subunit 1; SCD, stearoyl-CoA desaturase; SDHC-SDHD, succinate dehydrogenase complex, subunit C and D; SLCO1B1 and SLCO2A1, solute carrier organic anion transporter family; SLC25A20, solute carrier family 25 (carnitine/acylcarnitine translocase); STARD4, StAR-related lipid transfer (START) domain containing 4; SULT1A1 and SULT1C1, sulfotransferase family cytosolic 1A and 1C; UGT2B28, UDP glucuronosyltransferase 2 family polypeptide B28; UQCRC2 and UQCRH, ubiquinol-cytochrome c reductase core protein and hinge protein.

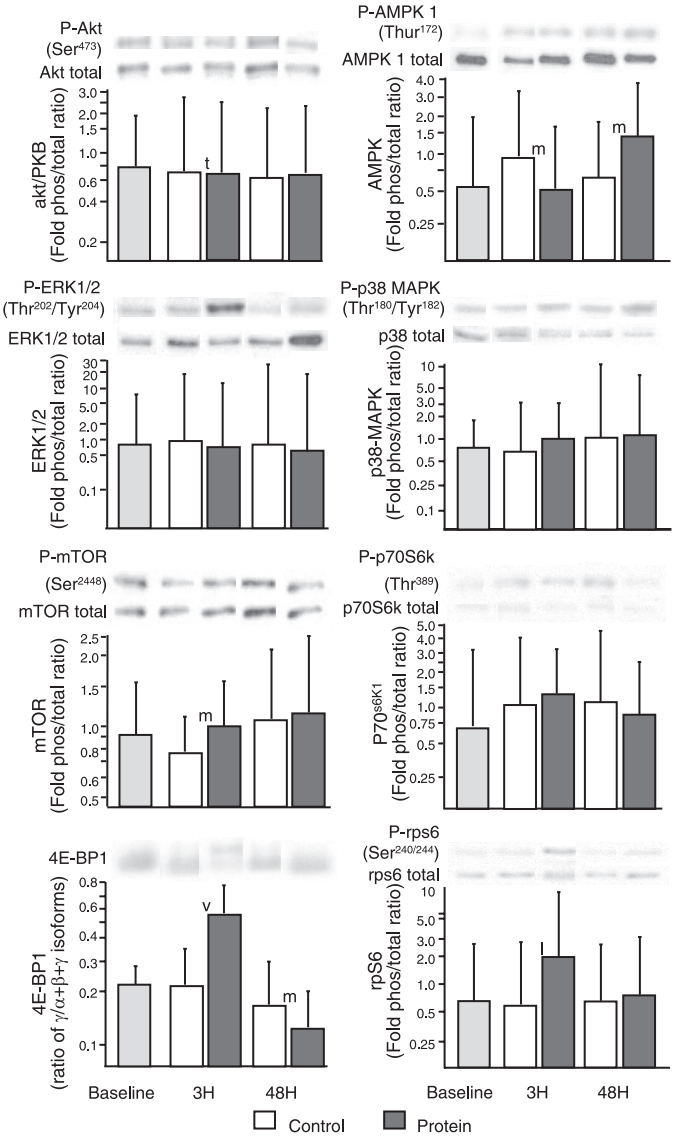

Fig. 6. The phosphorylation status of mTORC1 pathway-associated signaling proteins at 3 and 48 h following exercise in response to protein vs. control nutrition postexercise. Data are the back-transformed least-squares means ± SD of the phosphorylated/total protein, and *y*-axes are natural log scale according to the statistical analysis. The symbols located above the bars define the magnitude of the protein-feed differential phosphorylation; the definition is located in the footer of Table 4. Shown above the respective comparisons are bands cut from representative immunoblots.

tracellular matrix (53) and interconnection with cytoskeletal remodeling through cell adhesion molecules (16). Indeed, we observed that protein nutrition also enriched cell adhesion and expression involved in extracellular matrix and cell surface receptors. Other microarray studies also show enriched expression activity in axonal guidance and actin cytoskeletal signaling processes in untrained men following endurance exercise (35), short-term endurance training interventions (58), and with chronic endurance training (54) triggering the expression profile for tissue structural remodeling. To the best of our knowledge, here we provide the first data to show that nutrition further influences global mRNA expression of these skeletal muscle developmental processes, suggesting that protein modulates the exercise-induced structural transcriptome. The mechanism might be lower relative stress on cell and tissue structural integrity, secondary to elevated extracellular amino acid concentrations (57), which may also partly explain attenuation of circulating muscle damage markers (e.g., creatine kinase) observed elsewhere (51, 57).

Postexercise protein feeding also affected the protein modification and breakdown transcriptome at 3 h (Fig. 4). Extracellular matrix metalloprotease genes were upregulated by PTN, suggesting increased breakdown of extracellular matrix, which might aid tissue reabsorption and enhanced remodeling, cell-matrix interactions, and proteolytic cleavage of transmembrane proteins into the extracellular domain (52). Lysosomal protease (cathepsins) expression was also mostly downregulated suggesting decreased lysosomal protein degradation and autophagy (8). Differential expression of genes encoding ubiquitination targeting proteins (E2, E3) and apoptosis suggest that protein nutrition may also influence the activity of this proteolytic process (4). Nonlysosomal and Ca^{2+}-independent proteolysis (ubiquitination) are primarily responsible for myofibrillar protein breakdown and therefore skeletal muscle remodeling (56). Elevated proteolytic gene expression in the 1–4 h following endurance exercise has previously been reported independent of training status (12, 35). The postexercise PTN transcriptome suggests reduced protein degradation could be associated with the present increase in mTORC1 phosphorylation at 3 h (Fig. 6), which together suggests lower cell stress via reduced autophagy (42) and increased protein synthesis (32). Together, the proteolytic transcriptome and mTORC1 signaling supports a model of muscle remodeling outlined by Tipton (60), where both increased muscle protein synthesis and targeted muscle-protein breakdown represent an important component of adaptive remodeling. Actual measures of protein-enzyme activity, regulatory control of protolytic processes, and quantitative evidence for remodeling of candidate proteins are required to confirm this hypothesis.

Protein Nutrition Modulates the Skeletal Muscle Immune and Inflammatory Transcriptome Early in Recovery

Prolonged strenuous endurance exercise induces marked systemic immunosuppression, which appears to be offset with carbohydrate ingestion (41). Protein ingestion around the exercise period may also affect immune function (14), but the comparative body of research is relatively sparse. In the present study, downregulated expression in complement- and macrophage-mediated immunity and upregulated HSP70 chaperone gene expression provide new evidence that protein ingestion following intense endurance exercise modulates aspects of the skeletal muscle immune response and protection of cell proteins from stress (34). Downregulation of complement component 1 and upregulation of the complement cascade inactivator (*CFI*) suggests attenuated classical immune pathway activity, while the overrepresented MHCII- and macrophage-mediated immunity ontology suggests effects on macrophages and other antigen-presenting cells. Macrophages modulate the immune response by producing chemokines and cytokines and enacting phagocytosis of damaged tissue following strenuous endurance

Table 4. *Statistical summary of the magnitude of the effect of protein feeding at 3 and 48 h following exercise on gene expression quantified by qPCR*

	Differential Comparison																	
	CON-Pre3 h			CON-Pre48 h			PTN-Pre3 h			PTN-Pre48 h			PTN-CON3 h			PTN-CON48 h		
Transcript	ES	CL	P	ES	CL	P	ES	CL	P	ES	CL	P	ES	CL	P	ES	CL	P
PPARGC1A	**37**[a]	**8**	**0.000**	−0.31	1.22	0.501	**50**[a]	**9**	**0.000**	0.41	1.47	0.441	**0.78**[l]	**1.56**	**0.180**	**0.83**[l]	**1.58**	**0.160**
FOXO1A	**3.4**[a]	**1.6**	**0.000**	0.22	0.86	0.475	**5.8**[a]	**2.0**	**0.000**	**0.42**[l]	**0.93**	**0.207**	**0.73**[v]	**0.94**	**0.030**	0.17	0.84	0.540
BCODK	**−0.46**[l]	**0.69**	**0.081**	−0.09	0.77	0.766	−0.30	0.72	0.277	−0.29	0.73	0.301	0.23	0.90	0.480	−0.21	0.73	0.460
PDK4	**0.71**[l]	**1.45**	**0.196**	**−0.66**[l]	**0.98**	**0.086**	**2.1**[v]	**1.8**	**0.004**	−0.02	1.28	0.957	**1.0**[l]	**1.5**	**0.070**	**0.97**[l]	**1.46**	**0.080**
SREBF2	0.38	0.85	0.266	−0.05	0.63	0.846	0.53	0.91	0.152	0	78	1.000	0.10	0.82	0.760	0.06	0.88	0.860
DSCR1	**1.3**[l]	**1.8**	**0.084**	−0.44	1.17	0.354	**1.6**[v]	**1.90**	**0.038**	−0.09	1.34	0.863	0.21	1.33	0.690	0.46	1.43	0.420
NFkB	**0.91**[l]	**1.62**	**0.143**	0.20	1.34	0.703	**1.7**[v]	**1.9**	**0.019**	0.69	1.58	0.250	0.55	1.56	0.360	0.44	1.59	0.460
IGF-1	0.01	0.43	0.960	−0.02	0.28	0.851	0.05	0.41	0.755	0.14	0.39	0.378	**0.04**[t]	**0.33**	**0.780**	0.18	0.39	0.250
DDIT3	**4.4**[a]	**2.6**	**0.000**	0.07	1.58	0.905	**8.1**[a]	**3.5**	**0.000**	−0.38	1.28	0.447	**1.2**[l]	**1.9**	**0.090**	−0.44	1.37	0.400
SIRT3	**−0.46**[l]	**0.80**	**0.159**	**−0.50**[l]	**0.79**	**0.117**	−0.31	0.88	0.373	−0.33	0.85	0.344	0.23	1.00	0.560	0.28	1.01	0.480

Effect size (ES) is the mean difference divided by the composite SD for the respective transcript; CL, ±95% confidence limit for the effect size. Data are rounded to 2 significant figures. Magnitude-based inference and likelihood (26). The likelihood of at least a small ES was calculated from the t-distribution as follows and identified within the table in boldface and with the likelihood abbreviation as a superscript: <1.0%, almost certainly not; 1.0–5%, very unlikely; 5–25%, unlikely; 25–75%, possible; 75–95%, likely (superscript abbreviation, l); 95–99.5%, very likely (v); >99.5%, almost certain (a). Otherwise, outcomes are unclear (no superscript) when >5% likelihood outcome is both greater and less than small, or trivial (t) when the probability was >75% and not otherwise unclear. PTN, protein; CON, control.

exercise (47). Dendritic cells are also important antigen presenting cells, which stimulate B and T cells and thereby the initial immune response (6). The complement cascade is also part of the acute phase inflammatory response and attracts neutrophils and macrophages to the site of injury and mediates removal of cellular debris with phagocytes (18). Therefore, reduced complement and macrophage-mediated gene expression suggests lower muscle damage in first few hours postexercise with protein ingestion, which is also consistent with the moderated proteolytic transcriptome.

Regulation of Energy Transfer Metabolic Pathways: Attenuated Glycolytic Enzyme but Increased Fatty Acid Transport and Mitochondrial Electron Transporter Gene Expression

The present data demonstrate that dietary protein ingestion following endurance exercise stimulates a metabolic-mitochondrial transcriptome consistent with the adaptive response to endurance training (25). The protein-affected expression profile was evident only in the latter biopsy (48 h) and

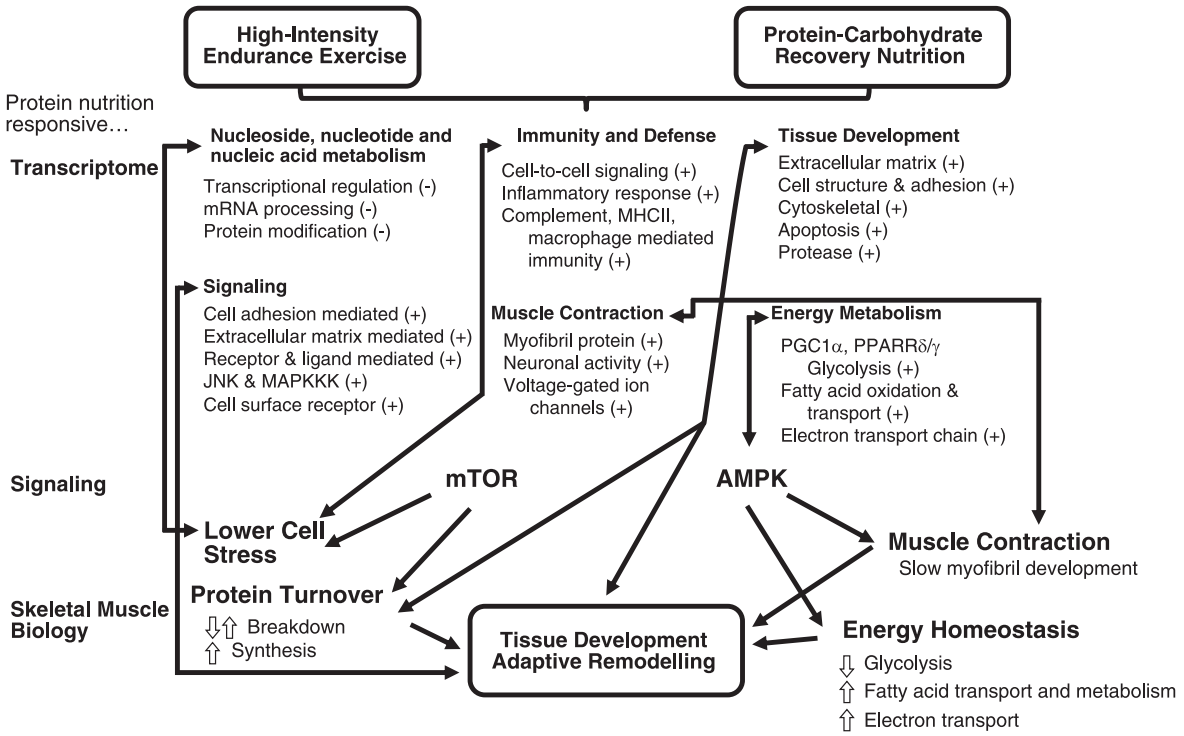

Fig. 7. The transcriptome, signaling pathways, and the potential skeletal muscle biology regulated by protein vs. control nutrition following high-intensity endurance exercise. Shown is a summary of the over (+) and under (−) represented gene ontology derived from the analysis, the clear signaling pathways activated by protein nutrition, and the genomic-functional interrelationships.

comprised an expression pattern consistent with downregulated glycolysis, but upregulated mitochondrial fatty acid transport and electron chain components. The transcriptome driving the metabolic-mitochondrial program may be associated with increased PPARD/G activation as indicated by the IPA canonical pathway analysis and evidence for increased *PPARGC1A* expression and promoter activity, while increased AMPKα1 phosphorylation provides a candidate signaling mechanism. AMPK activation increases expression of genes involved in skeletal muscle oxidative capacity and mitochondrial biogenesis via PPARGC1A phosphorylation and expression (29). In turn, PPARGC1A is required for AMPK action on gene expression (29) and induces transcription of genes associated with lipid metabolism (24). The reason for increased AMPKα1 phosphorylation at 48 h is unknown; however, replication of diet and exercise between conditions for 0–48 h postexercise suggests that the signaling and expression environment induced by the intervention lead to altered expression at 48 h. Such a time course is unremarkable in light of reports of altered gene expression between 24 and 48 h following exercise (35, 68). AMPK phosphorylation is frequently associated with cell energy stress acting to inhibit mTORC1 activity thereby maintaining cellular amino acid concentration by increasing autophagy and reducing translation initiation and cell growth (32). A high-carbohydrate meal after endurance exercise lowered AMPKα1/α2 phosphorylation compared with a low-carbohydrate meal (65). Therefore, the reduction of AMPKα1 phosphorylation at 3 h to baseline pre-exercise values suggests that protein-carbohydrate-lipid co-ingestion postexercise may ameliorate acute energy stress, relative to isocaloric carbohydrate-lipid, which may, in part, account for the observed generalized attenuation of the acute exercise-related transcriptome with PTN. Future research should confirm the effect of dietary protein and amino acids on AMPK signaling and metabolic adaptation in skeletal muscle to endurance exercise.

Other candidate signaling mechanisms integrating the protein nutrition signal with the metabolic-mitochondrial transcriptome included cAMP (37) and Ca^{2+} signaling (Table 3), which may activate transducers of regulated CREB1 (e.g., calmodulin-mediated protein kinases) associated with mitochondrial biogenesis (66). Additionally, p38MAPK increases *PPARGC1A* expression (2) and PPARGC1A activity and stability (49). However, we saw no evidence for a nutrition effect on p38MAPK, but it is possible that the 3 h sample may have missed a p38MAPK or ERK1/2 signal, as it has been previously reported that these kinases rapidly peaked and returned to baseline within 30–60 min following exercise (63). These points leave the impact of postendurance-exercise protein ingestion on MAPK-stimulated PPARGC1A activity unresolved. Finally, postexercise dietary protein ingestion may influence PPARGC1A activity through an epigenetic mechanism as suggested by reduced *DNMT3B* expression at 48 h. DNMT3B methylates the *PPARGC1A* promoter reducing expression activity (7). Future research using sequencing methods should examine the hypothesis that elevated blood or cellular amino acid concentrations following protein ingestion could increase *PPARGC1A* expression via an epigenetic mechanism.

Protein Feeding Increases mTORC1 Pathway Activity Early in Recovery from Endurance Exercise

Our mTORC1 pathway investigation at 3 h suggested increased translation initiation, which is consistent with protein feeding following endurance exercise in animals (38). Along with posttranscriptional regulation (not examined in the current study), nutritional regulation of translation initiation is likely an important determinant of final protein expression. While it is established that mTORC1 pathway activity is increased after resistance exercise and protein feeding (31), the present data are the first to confirm that the protein component of postexercise nutrition is responsible for increased mTORC1 activity following endurance exercise in humans. Previously, Ivy et al. (28) also reported increased mTORC1 and RPS6 phosphorylation with protein-carbohydrate feeding in men, but the control was water-placebo, unlike our control, which was isocaloric carbohydrate-fat. The authors also reported no significant change in the phosphorylation of the RPS6 kinase $p70^{S6K}$. Therefore, unlike increased $p70^{S6K}$ phosphorylation induced with PTN in the immediate few hours following resistance exercise (33), the present trivial $p70^{S6K}$ signal appears insufficient to account for the large increase in RPS6 phosphorylation, suggesting other upstream signaling processes to be involved or that sample timing might be a factor: rps6 phosphorylation was observed to be elevated for longer than $p70^{S6K}$ (3).

In addition to the negative regulatory action of AMPKα1 on mTORC1 via tuberin (TSC2) and Rheb protein phosphorylation (32), 4E-BP1 was moderately downregulated in the protein condition at 48 h (Fig. 6). It would be intriguing to suggest downregulation of translation at this time induced by protein co-ingestion 48 h earlier, but for the fact that p70S6k and rps6 were not clearly affected. p70S6k/rps6 and 4E-BP1 are the best-characterized mTORC1 substrates regulating translation (32), but phosphorylation of rps6 can also occur on Ser235/236 by p90rsk via ERK1/2 (44). ERK1/2 also promotes phosphorylation of elongation initiation factor 4E (eIF4E) through activation of the mitogen-activated protein kinase-interacting kinases 1 and 2 (MNK1/2) (41). MNK1/2 are also activated by the non-mTORC1 associated p38MAPK pathway, which results in enhanced phosphorylation of eIF4E (32). Neither ERK1/2 or p38MAPK was affected at 3 or 48 h, which leads to the conclusion that translation initiation at 48 h was likely to be little different from baseline. The consequence maybe significant for the relative extent of transfer of the transcriptome into nascent polypeptides at 3 and 48 h, with the 3 h program being synthesized at a higher rate than the program at 48 h. Atherton et al. (5) reported concordance of normalized p70S6k and 4E-BP1 activation and increased myofibril fractional muscle protein synthesis rate (FSR) to 90 min following 48 g of whey protein ingestion; thereafter, the signaling association from 180 to 360 min became less clear with the drop in FSR greater than the drop in activation. Signaling was more closely related to plasma leucine concentration, which was confirmed recently by Pennings et al. (45). Given the 48 h biopsy was taken fasted the FSR was almost certainly lower than at 3 h, which might imply quantitatively greater expression of the acute vs. late proteins. However, the significance of the temporal association between muscle protein synthesis rate and the time-affected transcriptome on the skeletal muscle

functional protein phenotype is largely unexplored and should be considered in future investigation.

Finally, the regulation of mTORC1 signaling and autophagy in vitro involves an amino acid transporter system. Cellular uptake of L-glutamine and subsequent rapid efflux in the presence of essential amino acids is the rate-limiting step activating mTORC1 (40). L-Glutamine uptake is regulated by solute carrier member SLC1A5 and bidirectional transport of extracellular leucine and intracellular glutamine through SLC7A5/SLC3A2 (40). The present analysis yielded some evidence for an impact of exercise on upregulated large neutral amino acid (*SLC7A5, SLC3A2* transcript variant 1*, SLC7A8* transcript variant 1) and cystine/glutamate transporter (*SLC7A11*) expression at 3 h relative to the baseline (i.e., comparison CON-Pre3h), but there was no PTN vs. CON differential. Therefore, our in vivo data suggest that endurance exercise upregulates a similar amino acid transporter system according to Nicklin et al. (40), but protein nutrition is required to increase mTORC1^{Ser2448} phosphorylation, suggesting that other amino acid-linked signaling mechanisms are involved.

Summary

In this paper, we confirm our a priori hypotheses that the addition of protein to postexercise carbohydrate-lipid nutrition differentially altered the transcriptome involved in tissue structure and remodeling through regulation of extracellular matrix, cytoskeletal, and contractile protein gene expression, with the latter profile favoring the slow-fiber phenotype. In addition, we found that acutely, protein co-ingestion modulated aspects of the skeletal muscle immune and inflammatory response, the proteolytic transcriptome, and heat shock protein expression suggesting nutrition-modulated expression activity involved in cellular clean-up and protein stabilization. From a metabolic perspective, protein regulated the expression of genes involved in energy metabolism and mitochondrial function, favoring downregulated glycolysis and upregulated fatty acid transport and electron transport. Finally, our phosphoprotein observations suggest an early increase in translation initiation and a later association between AMPK phosphorylation in the protein nutrition-mediated metabolic transcriptome. Together, the gene expression program favoring attenuation of the exercise-induced stress-related response, regulation of developmental processes, and accentuation of the metabolic-mitochondrial program suggests that protein co-ingestion following endurance exercise may have conferred a fitness advantage in the evolutionary context and, in the modern era, may facilitate recovery and training adaptation in the endurance athlete. Further research should determine the time course and post-transcriptional regulation of this transcriptome, and chronic postexercise protein feeding studies should proceed to determine if the resulting transcriptome and signaling events are associated with functionally meaningful changes to skeletal-muscle phenotype.

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