Inadequate protein intake affects skeletal muscle transcript profiles in older humans[1–3]

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ABSTRACT

Background: Inadequate dietary protein intake causes adverse changes in the morphology and function of skeletal muscle. These changes may be reflected in early alterations in muscle messenger RNA levels.

Objective: This study assessed whether inadequate protein intake differentially affects skeletal muscle transcript concentrations and expression profiles in older adults.

Design: Twenty-one older men and women (aged 55–80 y) consumed controlled diets that provided 1.2 g protein · kg^{-1} · d^{-1} (adequate protein) for 1 wk and then were randomly assigned to consume either 0.5 g protein · kg^{-1} · d^{-1} [inadequate protein (IP) group; $n = 11$] or 1.2 g protein · kg^{-1} · d^{-1} (control group; $n = 10$) for a second week. RNA was isolated from fasting-state vastus lateralis biopsy samples obtained at the end of each period, and transcript levels in the IP group were measured by using microarray anlysis. Changes in selected transcript levels were confirmed by real-time polymerase chain reaction in both groups.

Results: Analysis of variance showed 529 differentially expressed transcripts ($P < 0.05$) after inadequate protein intake. Using the false discovery rate (FDR) correction to adjust for multiple comparisons, we observed that 85 transcripts were differentially expressed: 54 were up-regulated and 31 were down-regulated. The differentially expressed transcripts were in functional classes for immune, inflammatory, and stress responses (predominantly up-regulated); contraction, movement, and development (up-regulated); extracellular connective tissue (up-regulated); energy metabolism (down-regulated); protein synthesis (down-regulated); and proliferation (down-regulated). Diet-related differences in the expression of 9 transcripts were cross-validated by using real-time polymerase chain reaction.

Conclusion: The results document changes in skeletal muscle transcript levels induced by short-term inadequate protein intakes in older humans that might precede adverse metabolic, functional, and structural events, including muscle wasting. *Am J Clin Nutr* 2007;85:1344–52.

KEY WORDS Dietary protein, aging, skeletal muscle, transcript profile, microarray, gene expression

INTRODUCTION

In the 2005 report of the Institute of Medicine (1), which reestablished the Recommended Dietary Allowance (RDA) for protein at 0.8 g protein · kg^{-1} · d^{-1} for adults of all ages, the panel underscored the need for research to establish new methods of protein assessment to complement nitrogen balance and isotope kinetic studies. The panel also encouraged further study of the adaptive and accommodative mechanisms used by the body in response to inadequate protein intakes as well as those mechanisms associated with the phenotype of sarcopenia. Sarcopenia is characterized by an age-related decline in skeletal muscle mass, strength, and quality that can lead to decreased mobility, increased risk of injury, and decreased independence (2). These age-associated changes in skeletal muscle can be caused by many factors, including inadequate dietary protein intake. When protein intake is inadequate, the body enters a state of accommodation in which physiologic function is compromised and muscle mass is reduced (3). For example, older women who consumed 0.45 g protein · kg^{-1} · d^{-1} for 10 wk experienced skeletal muscle atrophy that led to a loss of lean body mass, a decline in the cellular immune response to stress, and a decrease in functional muscle capacity over time (4). Older men and women who consumed the RDA for protein for 12 wk experienced decreased fat-free mass and midthigh muscle area, consistent with an accommodation response (5).

Tools developed to assess global changes in transcript levels (ie, transcript profiling with microarrays) may provide an unbiased assessment of biological adaptation and accommodation to changes in nutritional protein status. The transcript profile is reflective of protein function and metabolic modulation of cell biology and, therefore, might be adaptable to protein-status assessment. Microarrays detect simultaneous changes in transcript levels under various external and internal cues. They have been used to show that transcript levels are altered in response to different dietary and exercise manipulations in both animal and human models (6–9) and the differential response of muscle transcript levels to sex, age, and muscle fiber types (10–13). Therefore, we propose that microarrays will reveal a response in transcript levels to short-term consumption of inadequate dietary protein and that the transcript profile will reflect altered cellular activity that precedes functional consequences of accommodation.

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The purpose of this study was to examine the skeletal muscle transcript profile using microarray analysis and use the profile to assess differential transcript levels in response to a short-term inadequate protein diet in older men and women.

SUBJECTS AND METHODS

Subjects and preliminary testing

Twelve postmenopausal women and 12 older men aged 67 ± 7 y ($\bar{x}$ ± SD) were recruited for a 14-d controlled diet study. Before starting the study, each subject completed an evaluation that included a resting-state electrocardiogram, routine clinical blood and urine chemistries, and a written medical history. All subjects had clinically normal heart, liver, and kidney functions and did not have diabetes mellitus. Each subject received written and oral descriptions of the protocol, provided written informed consent, and received a monetary stipend for participating. The study protocol and consent form were approved by the Committee on the Use of Human Research Subjects at Purdue University. One man dropped out of the study, and complete data were unavailable for 2 subjects; the final 21 subjects (11 men and 10 women aged 55–80 y) had an average body mass index (BMI; in kg/m^2) of 26.8 ± 1.4.

Experimental design and dietary control

Each subject was provided a controlled diet for 14 d. An adequate-protein diet containing 1.2 g protein · kg^{-1} · d^{-1} was provided to all participants during days 1–6. On day 7, after the blood and muscle samples were collected, the participants were randomly assigned to either an IP group (adequate protein for 1 wk followed by inadequate protein for 1 wk; $n = 11$) or a control group (2 wk of adequate protein; $n = 10$). The IP group was provided a diet containing 0.2 g protein · kg^{-1} · d^{-1} on day 7 to enhance adaptation to the subsequent inadequate protein intake of 0.5 g protein · kg^{-1} · d^{-1}, which was provided from days 8 to 14. The control group continued to consume a diet of 1.2 g protein · kg^{-1} · d^{-1} from days 7 to 14. The energy and macronutrient intakes of the 2 diet groups at the 2 testing periods are shown in **Table 1**. Each subject's meals were customized to provide sufficient energy for body weight maintenance and a nonprotein energy content of 65% carbohydrate and 35% fat. The energy, macronutrient, and micronutrient components of the diet were calculated by using NUTRITIONIST PRO computer software (First Databank Inc, San Bruno, CA). Animal-based proteins were provided from dairy and egg sources. Muscle tissue–containing foods were excluded from the meals because of their high protein content. Subject instructions, the setting for meal consumption, and the protocol for alcohol, water, and multivitamin and multimineral ingestion were described previously (14).

Analytic methods

Urine

Twenty-four–hour urine collections were obtained on days 4, 5, 6, 11, 12, and 13. The urine samples were collected into containers and refrigerated. Urine collections were portioned and frozen for later testing. Urinary total nitrogen concentrations were measured from aliquots by using a nitrogen analyzer (model FP-528; Leco, St Joseph, MI).

TABLE 1
Dietary intakes of older adults in the control and the inadequate dietary protein intake (IP) groups[1]

Variable and group[2]	Week 1	Week 2
Energy (kcal/d)		
Control	2345 ± 305	2345 ± 305
IP	2287 ± 331	2262 ± 327
Protein (g/d)		
Control	89 ± 16	89 ± 16
IP	91 ± 17	39 ± 7[3]
Carbohydrate (g/d)		
Control	327 ± 43	327 ± 43
IP	313 ± 45	344 ± 51[4]
Fat (g/d)		
Control	76 ± 10	76 ± 10
IP	73 ± 10	81 ± 11[4]
Fiber (g/d)		
Control	23 ± 3	23 ± 3
IP	24 ± 4	19 ± 3[4]
Urinary total nitrogen (g/d)		
Control	11.4 ± 2.4	11.4 ± 2.1
IP	11.9 ± 2.1	5.8 ± 1.5[3]
Blood urea nitrogen (mg/dL)		
Control	17 ± 3	17 ± 3
IP	19 ± 6	11 ± 3[3]

[1] All values are $\bar{x}$ ± SD; $n = 10$ in the control group and $n = 11$ in the IP group. There were no significant differences between groups at week 1.

[2] The control group consumed an adequate amount of protein (1.2 g protein · kg^{-1} · d^{-1}) during weeks 1 and 2; the IP group consumed an adequate amount of protein (1.2 g protein · kg^{-1} · d^{-1}) during week 1 and an inadequate amount of protein (0.5 g protein · kg^{-1} · d^{-1}) during week 2.

[3] Significantly different from the control group within a time period when the group-by-time interaction was significant based on a 2-factor repeated-measures ANOVA, $P < 0.0001$.

[4] Significantly different from week 1 within a treatment group when the group-by-time interaction was significant based on a 2-factor repeated-measures ANOVA, $P < 0.05$.

Blood

Fasting-state blood samples were obtained by venipuncture of an antecubital vein on study days 7 and 14. The blood samples were placed into individual heparin-coated tubes. The tubes were centrifuged at 3000 × *g* for 10 min at 4 °C to obtain the plasma. Blood urea nitrogen concentrations were measured by using the urease/glutamate dehydrogenase coupled enzymatic technique at the Laboratory Corporation of America (Burlington, NC).

Muscle

A sample of muscle was obtained from the vastus lateralis with the use of a percutaneous muscle biopsy technique (15) at the end of weeks 1 and 2. A small portion of the subject's dominant midthigh was anesthetized with ≈4 mL of a 1% lidocaine solution. The muscle sample was collected by using a 6-mm Bergstrom biopsy needle (Microsurgical Instruments, Lake Forest, IL). The extracted muscle tissue was quickly blotted to remove any blood, fat, or connective tissue and then stored in a cryovial and frozen in liquid nitrogen until processed further. The muscle biopsy obtained at week 2 was taken approximately 1 inch (2.54 cm) from the site of the biopsy sample taken at week 1. Muscle biopsy samples were unattainable for 2 subjects in the IP group; complete sets of muscle samples (both weeks 1 and 2) were

available from 10 subjects in the control group and 11 in the IP group.

RNA isolation

Total RNA was isolated from the frozen muscle sample by using Tri-Reagent according to the manufacturer's instructions (Molecular Research Center, Cincinnati, OH). The isolated total RNA was further purified for microarray analysis by using RNeasy Mini Kits according to the manufacturer's protocol (QIAGEN Inc, Valencia, CA). The quantity and integrity of the RNA were determined by using ultraviolet light spectrophotometry and agarose gel electrophoresis, respectively.

Microarray analysis

For the IP group only, the microarray analysis was conducted with the use of RNA isolated from muscle samples taken at the end of the week of adequate dietary protein intake (week 1) and at the end of the week of inadequate protein intake (week 2). The transcript profile of each sample was determined by using the Affymetrix U133A 2.0 GeneChip (22 283 probe sets representing ≈18 400 transcripts) and standard Affymetrix protocols (Affymetrix, Santa Clara, CA) at the Indiana University Center for Medical Genomics (Indianapolis, IN). Briefly, double-stranded complementary DNA (cDNA) was synthesized from RNA by reverse transcription. Biotin-labeled cRNA was synthesized from the cDNA by in vitro transcription. Fragmented biotinylated cRNA was hybridized to the chip, the chip was scanned, and the data were summarized by using Affymetrix Microarray Suite (MAS) 5.0 software.

Real-time polymerase chain reaction validation

Real-time polymerase chain reaction (RT-PCR) was used to cross-validate the microarray results for 9 differentially expressed transcripts. The 9 transcripts chosen for PCR-based cross-validation were selected on the basis of 3 criteria: *1*) the differential expression was greater than 1.9-fold, *2*) multiple probe sets for the transcripts or transcripts encoding multiple isoforms of a protein family consistently showed differential expression (eg, collagen and hemoglobin), or *3*) the transcript encoded a protein that fit into an overrepresented functional class. A differentially expressed transcript did not have to meet all 3 criteria to be used for cross-validation.

For the cross-validation, RNA was isolated from muscle samples of the control group subjects and was reisolated from muscle samples of the IP group. RNA was reverse transcribed to cDNA by using a previously described protocol (16). The cDNA samples were analyzed by RT-PCR with the use of the BioRad My IQ RT-PCR system and the BioRad SYBR Green supermix according to the manufacturer's protocol (BioRand, Hercules, CA). The expression of each of the transcripts was determined by using the threshold cycle value procedure described by Livak and Schmittgen (17). The primer information and cycle conditions for the 9 transcripts of interest and one reference transcript were as follows: *1*) ankyrin repeat domain 2 (GenBank accession number: NM_020349), forward 5'-GGAAGAAACGCAAGCAGAAG-3', reverse 5'-ACCTTCATTTTCCCCTCCAC-3', 40 cycles, annealing temperature (T_a) = 56.0 °C; *2*) collagen, type I, α 1 (K01228), forward 5'-GTGGTGACAAGGGTGAGACAG-3', reverse 5'-GGAGACCGTTGAGTCCATCTT-3', T_a = 53.2 °C; *3*) cytochrome-*c*-oxidase subunit Vb (BC006229), forward 5'-GGTGTTCCCACTGATGAAGAG-3', reverse 5'-GTTGGAGATGGAGGGGACTAA-3', T_a = 54.1 °C; *4*) secreted frizzled-related protein 4 (AW089415), forward 5'-CACAACGGTGGTGGATGTAA-3', reverse 5'- ATCATCCTTGAACGCCACTC-3', T_a = 48.0; *5*) hemoglobin, α 2 (AF349571), forward 5'-CTTCAAGCTCCTAAGCCACT-3', reverse 5'-GGTATTTGGAGGTCAGCAC-3', T_a = 52.0 °C; *6*) lysosomal-associated multispanning membrane protein-5 (NM_006762), forward 5'- CGTAGTCAAGAACCGGGAGA-3', reverse 5'- GTGAGCAGGCACAGGAGATAG-3', T_a = 49.0 °C; *7*) metallothionein 1X (NM_005952), forward 5'-CTGTTGGCTCCTGTGCCTGT-3', reverse 5'-TTGTCTGACGTCCCTTTG-3', T_a = 55.0; *8*) *ras*-related associated with diabetes (NM_004165), forward 5'-GCTGTTTGAAGGTGTCGTG-3', reverse 5'-GCTGTTACGAGCTACGATG-3', T_a = 55.0; *9*) CD163 (Z22969), forward 5'-CAGTGTGTGATGACTCTTGG-3', reverse 5'-GACAATCCCACAAGGAAGAC-3', T_a = 56.0 °C; and *10*) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM-002046), forward 5'-TCACCATCTTCCAGGAGCG-3', reverse 5'-CTGCTTCACCACCTTCTTGA-3', T_a = 54.0 °C.

Statistical analyses and inclusion criteria

Clinical variables

Values are reported as means ± SDs. For the diet, urine, and blood variables, the main effects of group (control compared with IP) and time (week 1 compared with week 2; within-subject effect) and the group-by-time interaction were assessed by 2-factor repeated-measures analysis of variance ($P < 0.05$). The analyses established that group-by-time interactions existed for dietary protein, urinary total nitrogen excretion, and blood urea nitrogen. When the interaction was significant, Tukey's multiple comparison adjustment was used for pairwise comparison across time and between treatment groups. Comparisons between the control and IP groups at weeks 1 and 2 were done by using unpaired *t* tests. Clinical variables were analyzed with the use of JMP Statistical Discovery software (version 3; SAS Institute, Cary, NC).

Microarray analysis of inadequate protein group

The raw data from each of the 22 microarray GeneChips were summarized by using the Affymetrix's Microarray Suite (MAS) 5.0 software. SAS software (version 9.1; SAS Institute, Cary, NC) was used for the statistical analysis of the microarray data.

Step 1: inclusion criteria. The MAS 5.0 software assigns a "present" or "absent" detection call to each transcript on a chip based on Wilcoxon's signed-rank test ("present", $P < 0.05$; "absent", $P > 0.05$). The number of probe sets evaluated for a change in transcript level was reduced to include only those transcripts deemed "present" for ≥8 of 11 chips for ≥1 of the 2 wk of dietary intervention in the IP group. This reduced the list of probe sets from 22 283 to 6832.

Step 2: normalization. The MAS 5.0 expression data were $\log_2$ transformed and a global normalization of each Affymetrix chip was performed by using the 2-stage analysis method described by Wolfinger et al (18). This procedure normalizes each chip to a common median expression level and it controls for chip-to-chip variation.

Step 3: evaluation of differential transcript levels. On a transcript-by-transcript basis, a 2-sample *Z* test was used to assess differences in transcript levels between the weeks of adequate and inadequate dietary protein intakes in the IP group. A nonparametric smoothing function was fit to describe the relation between variance and total intensity of each transcript. The estimated variance was used to test the effect of diet on transcript level.

Step 4: false discovery rate correction. Microarray analysis is characterized by a large number of individual comparisons, which increases the risk of type I errors (false positives). Overcorrection for type I errors (eg, with the Bonferroni correction) increases the risk of type II errors (false negatives). To balance the risk of type I and type II errors, differential expression of transcripts was determined by using the FDR correction (19) and a *P* value of 0.05.

Functional annotation of differentially expressed transcripts

To aid interpretation of the differential expression of transcripts due to inadequate protein consumption, the data were functionally annotated by using DAVID 2.1 Beta (Internet: http://david.abcc.ncifcrf.gov/; 20). DAVID is a web-based application that allows users to access a relational database of functional annotations that are derived primarily from LocusLink at the National Center for Biotechnology Information. This analysis was conducted on the entire set of differentially expressed transcripts (529 transcripts; $P < 0.05$). DAVID analysis was initially conducted by using the medium classification stringency (minimum of 4 transcripts with 4 common functions for a functional group to be established); this yielded 20 functional groups, but almost half of the transcripts were uncategorized. To expand the functional categories available for the uncategorized transcripts, the low classification stringency (a minimum of 3 transcripts with ≥4 common functions) was applied and this categorized the 529 transcripts into 30 functional groups. The remaining uncategorized transcripts were then annotated by using Gene Ontology data available for each probe set through the Affymetrix website (Internet: www.affymetrix.com) and placed into 1 of the 30 functional groups established by the DAVID classification. The functional groups generated were examined and compared. Related functional groups were condensed manually into 22 groups to minimize redundancy in the functional categories. Finally, the 85 transcripts that met the higher FDR correction criterion ($P = 6 \times 10^{-4}$) were highlighted in the 22 functional groups (**Table 2**).

Additional functional characterization was conducted by using GenMapp 2.0 (Internet: www.GenMapp.org). This program is designed for viewing and analyzing genome-scale data on downloadable and user-defined maps representing biological pathways as well as other groupings (eg, nuclear receptors, cellular compartment). The 529 differentially expressed transcripts were fit into the available maps of Gene Ontology classifications by using the MappFinder subroutine. This program identifies overrepresented Gene Ontology functional groups by using a *z* score, and a *P* value is determined by using a nonparametric bootstrapping procedure (21, 22).

Availability of the microarray data set

The Affymetrix GeneChip data have been submitted to the GEO database (Internet: http://www.ncbi.nlm.nih.gov/geo/) for use by the scientific community. In addition, detailed versions of the lists of differentially expressed transcripts and their annotation will be made available on request.

TABLE 2
Functional classification of transcripts that were differentially expressed after 1 wk of inadequate dietary protein intake in older adults[1]

Functional group[2]	Up-regulated	Down-regulated
Immune, inflammation, and stress response	42 (5)	9 (0)
Transport	11 (1)	4 (0)
Cytoskeleton	18 (2)	5 (0)
Extracellular connective tissue	7 (5)	1 (0)
Contraction, movement, and development	28 (13)	13 (3)
Metal binding	4 (0)	5 (0)
Mineral metabolism	1 (1)	6 (2)
Kinases and signaling	17 (3)	12 (1)
Metabolism	8 (1)	6 (1)
Metabolism, carbohydrate	6 (0)	5 (0)
Metabolism, energy	3 (0)	35 (4)
Metabolism, fatty acid and lipid	5 (1)	5 (0)
DNA metabolism	1 (0)	4 (0)
RNA metabolism	6 (1)	5 (1)
Transcription	14 (0)	19 (2)
Protein catabolism	16 (3)	14 (0)
Protein synthesis	10 (1)	25 (0)
Proliferation, negative control	14 (3)	2 (0)
Proliferation, positive control	2 (0)	20 (3)
Binding proteins	10 (2)	11 (1)
Oxygen transport	0 (0)	2 (2)
Unknown function	28 (1)	20 (2)

[1] Differentially expressed transcripts were determined from the microarray analysis of muscle RNA from the inadequate protein group ($n = 11$) by comparing the period of adequate dietary protein (week 1 of the study) with that of the period of inadequate dietary protein (week 2 of the study). Transcripts were classified into functional groups on the basis of gene ontology conducted in DAVID 2.1 (20). The first value represents the number of differentially expressed transcripts identified by using a 2-factor *z* test ($P < 0.05$), whereas the value in parentheses represents the number of differentially expressed transcripts identified after adjustment for multiple comparisons with the false discovery rate criterion ($P = 6 \times 10^{-4}$).

[2] Several transcripts appear in more than one functional group. Multiple occurrences of a transcript are not reflected in the list.

RESULTS

From weeks 1 to 2, there were no changes in energy or macronutrient intakes in the control group. Energy intake did not change, protein intake decreased, and carbohydrate and fat intakes increased at week 2 in the IP group (Table 1). Dietary protein intake at week 2 differed between the control and IP groups ($P < 0.05$).

The urinary total nitrogen excretion and blood urea nitrogen concentration were not different between the control and IP groups at week 1 and did not change from weeks 1 to 2 in the control group. Consistent with the reduced protein intake, urinary total nitrogen excretion and blood urea nitrogen decreased in the IP group (-6.2 ± 1.3 g/d and -8 ± 3 mg/dL, respectively; $P < 0.05$) from weeks 1 to 2 and were significantly lower than values in the control group at week 2 ($P < 0.0001$; Table 1).

Number of expressed and differentially expressed transcripts identified with the use of microarray analysis in the IP group

Of the 22 283 probe sets on the Affymetrix U133A GeneChip, 6832 (31%) met the present or absent inclusion criteria and were examined further for differential expression. Statistical evaluation showed that levels of 529 of the 6832 transcripts were significantly different over time, ie, between the periods of adequate protein (1.2 g protein · kg^{-1} · d^{-1}) and inadequate protein (0.5 g protein · kg^{-1} · d^{-1}) intake ($P < 0.05$). With the use of FDR correction to adjust for multiple comparisons, 85 transcripts were found to be significantly different ($P = 6 \times 10^{-4}$) after consumption of an inadequate protein diet for 1 wk (**Table 3**).

Differential expression by functional categories for the IP group

The distribution of transcripts within functional groups was determined for both the list of 529 and the list of FDR differentially expressed transcripts (Table 2). There are many functional categories based on gene ontology. Because the list of differentially expressed transcripts from the current study was small, especially after the FDR correction, we chose to limit the number of functional categories by pooling similar or related groups. This enabled broader functional trends to be seen in the data set.

In 12 of the 22 functional groups, there was a >2-fold difference in the number of transcripts that were up- or down-regulated: immune and inflammation (more up-regulated transcripts); transport (up-regulated); cytoskeleton (up-regulated); extracellular connective tissue (up-regulated); contraction, movement, and development (up-regulated); mineral metabolism (down-regulated); energy metabolism (down-regulated); DNA metabolism (down-regulated); protein synthesis (down-regulated); negative regulators of proliferation (up-regulated); positive regulators of proliferation (down-regulated); and oxygen transport (down-regulated). These patterns were also reflected in the FDR set of differentially expressed transcripts (Table 2). Processing the differentially expressed transcripts through GenMapp, with the use of the available Gene Ontology maps, resulted in patterns comparable with those observed from the DAVID functional annotation (data not shown). Biological process groups that were overrepresented by z score included positive regulation of cell proliferation (down-regulated during the inadequate protein period; z score = 2.28, P = 0.036), humoral immune responses (up-regulated; z score = 2.78, P = 0.013), and cell-cell signaling (up-regulated; z score = 2.47, P = 0.032). Cellular compartment groups that were overrepresented included lysosome (up-regulated; z score = 2.41, P = 0.027) and extracellular matrix (up-regulated; z score = 2.99, P = 0.011), whereas molecular functions overrepresented include carbohydrate binding (up-regulated; z score = 4.17, P = 0.001) and cytokine activity (up-regulated; z score = 3.02, P = 0.013).

Real-time polymerase chain reaction validation of selected results from the microarray transcript profile

There was 100% concordance of directional trends in transcript levels between the microarray analysis and RT-PCR (**Table 4**) for the 9 candidates selected from the group of 85 differentially expressed transcripts. In general, the RT-PCR analysis showed a greater difference in transcript levels than did the microarray analysis. The largest difference in expression between the microarray and RT-PCR analysis was for the collagen 1, α 1 transcript; it was ≈170% greater with the RT-PCR method than with the microarray analysis (15.2 compared with 5.6, respectively).

The differential transcript levels seen by microarray analysis in the IP group were repeated when RT-PCR was used to analyze the transcript level of 9 candidates from the list of 85 differentially expressed transcripts (Table 4). In addition, 7 of the transcripts examined clearly showed divergent fold changes from weeks 1 to 2 between the control and the IP groups, ie, the change was either more extreme (≥60% higher) or had an opposite directional change between the groups.

DISCUSSION

When faced with a decrease in dietary protein intake, the body will adapt by increasing the efficiency of nitrogen retention and the utilization of amino acids (3, 23–25). However, if protein intake is inadequate the body will not fully adapt but will enter a state of accommodation that is characterized by a reduction in physiologic functions. For example, older women who consumed 56% of the RDA for protein for 10 wk were in marked negative nitrogen balance and experienced profound reductions in lean body mass (2–5% decrease), body cell mass (8% decrease), skeletal muscle mass (14% decrease), muscle strength, muscle function, and cellular immune responses (4, 26, 27). More subtle, but statistically significant, losses of fat-free mass (1.1 ± 0.8 kg decrease) and skeletal muscle size (1.7 ± 0.6 cm^2 decrease) were observed in older men and women who habitually consumed 100% of the RDA for protein for 12 wk (5). In this context the protein intake used in the current study (63% of the RDA) was inadequate and should elicit accommodation in the subjects when fed for an extended period. The 51% decrease in urinary total nitrogen excretion and 42% decrease in blood urea nitrogen concentration observed within 1 wk of switching from adequate to inadequate protein intakes indicate that the subjects adhered to the diets provided and are consistent with a change in protein metabolism.

The molecular details of adaptation and accommodation to inadequate protein intake in humans are not known. We sought to contribute to this knowledge gap by conducting an analysis of the transcript-level responses to dietary protein restriction in skeletal muscle. The transcriptome is a sensitive indicator of changes that occur across multiple levels of biological regulation (ie, in the metabolic space and protein space in addition to the level of transcription regulation; 28). Although the identification of the mechanisms responsible for differential transcript levels caused by inadequate dietary protein intake is not possible from the current study, the results indicate broad changes in the biology of the muscle that are associated with short-term dietary protein inadequacy and that may signal future deficits in physiologic function. This is consistent with previous work of global transcript profiling of muscle under a variety of conditions, eg, aging (29, 30), energy restriction (31, 32), type 2 diabetes (33), and comparisons of type I and type II muscle fibers (13).

A large number of transcripts (7.7% of the "present" transcripts, 1.2% with FDR correction) were responsive over time, ie, to the change in protein intake. In most cases, the directional shift in expression within functional categories is consistent with a priori expectations. For example, because protein intake and

TABLE 3
Transcripts identified as differentially expressed with the use of the false discovery rate correction ($P = 6 \times 10^{-4}$) in muscle of older adults who consumed a diet with inadequate protein for 1 wk

	GenBank ID[1]	Name	Ratio[2]
Immune, stress, and inflammatory response	NM_000745	Cholinergic receptor, nicotinic, alpha polypeptide 5	1.90
	NM_000079	Cholinergic receptor, nicotinic, alpha polypeptide 1	1.56
	BE903880	CD44 antigen	1.89
	Z22969	CD163 antigen	2.37
	NM_017801	Chemokine-like factor super family 6	1.41
Transport	U51478	ATPase, Na^+/K^+ transporting, beta 3 polypeptide	1.45
Cytoskeleton	NM_005720	Actin related protein 2/3 complex, subunit 1B, 41 kDa	1.77
	BC002654	Tubulin, beta 6	1.68
Extracellular connective tissue	K01228	Collagen, type I, alpha 1 (2×)[3]	5.61
	NM_000089	Collagen, type I, alpha 2 (2×)[3]	4.02
	AA292373	Collagen, type VI, alpha 1	1.54
	AU144167	Collagen, type III, alpha 1 (3×)[3]	3.16
	AL575735	Collagen, type V, alpha 2 (2×)[3]	2.20
Contraction, movement, and development	NM_002475	Myosin alkali light chain 1 slow a	0.80
	NM_013292	Myosin light chain 2	0.85
	NM_020349	Ankyrin repeat domain 2	0.89
	NM_003118	Secreted protein, acidic, cysteine-rich	1.92
	NM_002966	S100 calcium binding protein A10	1.49
	NM_004039	Annexin A2	1.56
	NM_002345	Lumican	1.58
	AI826799	EGF-containing fibulin-like extracellular matrix protein 1	1.79
	NM_005979	S100 calcium binding protein A13	1.71
	NM_000138	Fibrillin 1 (Marfan syndrome)	1.66
	BC000055	Follistatin-like 1	1.81
	U37283	Microfibrillar associated protein 5	1.50
	BC005858	Fibronectin 1 (4×)[3]	2.43
	NM_014624	S100 calcium binding protein A6	1.49
	NM_025008	Thrombospondin repeat containing 1	1.54
	BF218922	Chondroitin sulfate proteoglycan 2 (2×)[3]	2.20
Mineral metabolism	AF333388	Metallothionein 1H	0.67
	NM_005952	Metallothionein 1X (2×)[3]	0.65
	BG538564	Ferritin, light polypeptide	1.49
Kinases and signaling	NM_005627	Serum/glucocorticoid regulated kinase	0.71
	NM_004165	*Ras*-related associated with diabetes (2×)[3]	3.09
	AA731713	Dendrin	1.45
	AF245505	Adlican	1.89
Metabolism	M24317	Alcohol dehydrogenase 1C (class I), gamma polypeptide	0.80
	NM_006169	Nicotinamide *N*-methyltransferase (2×)[3]	2.57
Metabolism, energy	BC006229	Cytochrome *c* oxidase subunit Vb	0.78
	NM_001864	Cytochrome *c* oxidase subunit VIIa polypeptide 1	0.78
	NM_004541	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1, 7.5 kDa	0.78
	BC005230	Ubiquinol-cytochrome *c* reductase binding protein	0.76
Metabolism, fatty acid and lipid	AB018580	Aldo-keto reductase family 1, member C3	1.63
RNA metabolism	H96549	TIAI cytotoxic granule-associated RNA binding protein	0.73
	NM_002933	Ribonuclease, RNase A family, 1	1.43
Transcription	NM_003069	SWI/SNF related, matrix associated	0.69
	X72631	Nuclear receptor subfamily 1, group D, member 1	0.77
Protein catabolism	NM_006762	Lysosomal associated multispanning membrane protein 5	1.76
	NM_001909	Cathepsin D	1.47
	AL573058	Complement component 1, r subcomponent	1.54
Protein synthesis	NM_024537	Hypothetical protein FLJ12118	1.24
Proliferation, positive	BC001188	Transferrin receptor (p90, CD71) (2×)[3]	0.78
	NM_013407	Deoxyhypusine synthase	0.76
	NM_020184	Cyclin M4	0.79
Proliferation, negative	NM_001425	Epithelial membrane protein 3	1.67
	AW089415	Secreted frizzled-related protein 4	1.67
	NM_022356	Leucine proline-enriched proteoglycan (leprecan) 1	1.53
Binding proteins	AL117523	Sterile alpha motif domain containing 4	0.80
	NM_004742	BAI1-associated protein 1	1.78
	NM_016303	WW domain binding protein 5	1.53
Oxygen transport	AF349114	Hemoglobin, beta (3×)[3]	0.65
	AF349571	Hemoglobin, alpha 2 (6×)[3]	0.58
Unknown function	NM_017885	Host cell factor C1 regulator 1	0.68
	BE739519	Likely ortholog of mouse hypoxia induced gene 1	0.78
	AL565741	Hypothetical protein LOC90355	1.77

[1] The unique identification number for the sequence.

[2] The ratio of muscle transcript level at week 2 (during inadequate protein intake) to that at week 1 (during adequate protein intake) in the inadequate protein group ($n = 11$). A ratio > 1 represents up-regulation (ie, higher transcript level during week 2 than during week 1); a ratio < 1 represents down-regulation.

[3] Indicates multiple probe sets for the same gene transcript; the number in parentheses after the name of the gene indicates the number of probe sets that had differential expression of the transcript.

TABLE 4
Real-time polymerase chain reaction (RT-PCR) cross-validation of selected differentially expressed transcripts identified previously by microarray analysis[1]

Gene	IP group ($n = 11$)		Control group: fold-change by RT-PCR ($n = 10$)
	Fold-change by microarray	Fold-change by RT-PCR	
Lysosomal-associated multispanning membrane protein 5	↑ 1.5	↑ 2.0	↑ 2.6
Ankyrin repeat domain	↓ 1.1	↓ 1.6	↑ 1.2
Cytochrome *c* oxidase subunit Vb	↓ 1.3	↓ 1.2	↔ 1.0
Hemoglobin, alpha 2	↓ 1.8	↓ 2.3	↑ 1.2
Ras-related associated with diabetes	↑ 3.0	↑ 3.7	↑ 1.6
Metallothionein 1X	↓ 1.6	↓ 1.5	↑ 1.2
Collagen, type I, alpha 1	↑ 5.6	↑ 15.2	↑ 2.0
Frizzled-related protein 4, secreted	↑ 1.7	↑ 2.7	↑ 2.5
CD163 antigen	↑ 2.7	↑ 5.9	↑ 3.4

[1] The inadequate protein (IP) group consumed adequate protein (1.2 g protein · kg^{-1} · d^{-1}) during week 1 and inadequate protein (0.5 g protein · kg^{-1} · d^{-1}) during week 2; the control group consumed adequate protein (1.2 g protein · kg^{-1} · d^{-1}) during weeks 1 and 2. Fold change = (transcript level at week 2/transcript level at week 1).

metabolism influence energy metabolism (34), we expected the transcripts related to metabolism to be down-regulated; 35 of the 38 transcripts (4 of 4 that met the FDR criterion) functionally categorized to energy metabolism were down-regulated after consumption of the inadequate protein diet. This included 6 transcripts for mitochondrial H^+ transporting ATP synthase complex subunits (14–17% reduction), 7 transcripts for cytochrome *c* oxidase subunits (11–22% reduction), and 8 transcripts for NADH dehydrogenase complex members (11–23% reduction). Thus, although the level of down-regulation was modest, the consistency with which the family members were down-regulated suggests that this small change was biologically meaningful.

Dietary protein is also a substrate for cell protein synthesis; thus, we expected the transcripts associated with growth (eg, protein synthesis and cell proliferation) to be down-regulated. This was seen in both the larger set of differentially expressed transcripts and in those that met the FDR criterion. The observations that transcripts related to the positive control of proliferation were reduced (20 of 22 down-regulated, as well as down-regulation of the transcript for the proliferation marker, the transferrin receptor) and those related to the negative control of proliferation increased (14 of 16 up-regulated) suggests a coordinated effort to reduce the biological processes that require a large amount of amino acids. Within this context it is of interest to note that satellite cells, which contribute to muscle repair and regeneration, proliferate in mature muscle. Thus, the decrease in transcripts related to proliferation after inadequate protein intake could indicate a reduced ability for muscle repair. Transcripts related to protein synthesis were down-regulated by inadequate protein intake (25 of 35 differentially regulated transcripts). In particular, the transcripts encoding important muscle proteins were down-regulated by 10–20% with the inadequate protein diet, eg, myosin alkali light chain1 slow a, myosin light chain 2, myosin regulatory light chains MRCL2 and 3, myosin heavy polypeptide 1, tropomyosin 2, troponin T3, and troponin 1. In contrast, the changes in transcripts encoding proteins that control protein catabolism were less clear. Although the Gene Ontology group for the lysosome was overrepresented in this data set, and 3 transcripts related to protein catabolism (cathepsin D, complement component 1 r subunit, and lysosomal associated multispanning membrane protein 5) were up-regulated on the basis of the FDR criterion, in the larger set, gene transcripts related to protein catabolism were as likely to be down-regulated as up-regulated. The lack of a clear up-regulation in transcripts related to protein catabolism could indicate that this process is not strongly influenced on a transcriptional level, but this requires further investigation.

In contrast with the changes in the transcript profile that reinforced our a priori expectations, a number of unexpected changes in functional categories were observed. The largest group of differentially expressed transcripts was in the immune, inflammation, and stress response functional group (42 of 51 up-regulated at $P < 0.05$ level; 5 of 5 up-regulated on the basis of the FDR criterion). This suggests that the switch to inadequate dietary protein intake is a significant physiologic stress on the muscle of older subjects, which may be especially important because the skeletal muscle of older persons is documented to have greater levels of markers of stress and inflammation than that of younger persons (29, 30, 35). On the basis of these data, it is not clear whether the up-regulation of genes in the immune, inflammation, and stress response functional group is due to the activation of immune and inflammatory transcripts directly in the muscle cell or whether this is a result of immune cell infiltration into the muscle. Unfortunately, there was not enough muscle tissue to perform a histologic assessment of immune cell infiltration in this study. Regardless, the "stress" molecular phenotype might also be reflected in the increased expression of transcripts for various kinases and other signaling molecules (17 of 29 up-regulated; 3 of 4 for those meeting the FDR criterion). The overexpression of a wide variety of collagen isoforms (1.5 to 5.6-fold up-regulation) and transcripts for proteins such as fibrillin 1, fibronectin 1, and the collagen-binding protein osteonectin also supports this notion. In contrast, several markers that reflect inflammatory stress when expressed in the liver were down-regulated by the switch to inadequate protein intake, eg, metallothionein isoforms 1H, 1X, 1E, and 2A and ferritin heavy chain. This may have been due to a redistribution of zinc and iron from

the serum and peripheral tissues to the liver during inflammatory stress (ie, muscle mineral concentrations are reduced) (36, 37).

The second biopsy sample was taken from a separate incision an inch or more away from the first biopsy incision. Previous studies have shown that repeated biopsies from the same leg, but using separate incision sites, did not influence stress-related proteins (eg, activation of MAP kinase cascades) (38) or the levels of transcripts known to change with the stress of acute exercise (39). However, we cannot rule out the possibility that the changes over time for some of the transcript levels observed in the present study (especially for the inflammatory and immune responses) were due to the repeated biopsies rather than to a diet effect.

Several important questions regarding diet-induced changes in the transcript profile were not addressed in this study and warrant additional research. For example, the study was not statistically powered to assess any sex-related effects on these outcomes. Also, further research is required to assess whether the diet-related responses observed in muscle transcript levels are unique to older people or whether they are a common feature of the response to inadequate dietary protein intake across age groups. Ultimately, there may also be value to using a microarray approach in conjunction with more traditional methods, such as nitrogen balance studies and amino acid kinetic analysis, to help define dietary protein requirements. However, before this can be done, additional studies that include a wider range and more protein intake levels are needed to determine whether the changes observed in functional groups (eg, the up-regulation of the immune, inflammation, and stress profile) are alleviated with successful adaptation or whether they continue and are an early marker of accommodation. Finally, it is critical that future studies have longer prestudy stabilization periods to defined diets. This will minimize changes in muscle gene expression due to shifts from habitual intake. Such changes could explain the changes in transcript levels that were observed in the control group with the use of RT-PCR analysis.

The results of this study show that older persons who consume inadequate dietary protein for 1 wk experience a wide variety of changes in transcript levels in skeletal muscle. These responses are consistent with the existence of metabolic and physiologic changes that may precede accommodation, including inflammatory stress, reduced metabolism and oxygen transport, reduced protein synthesis, and muscle wasting.

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