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Essential Amino Acids Increase MicroRNA-499, -208b, and -23a and Downregulate Myostatin and Myocyte Enhancer Factor 2C mRNA Expression in Human Skeletal Muscle[1,2]

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Abstract

Essential amino acids (EAA) stimulate muscle protein synthesis in humans. However, little is known about whether microRNAs (miRNA) and genes associated with muscle growth are expressed differently following EAA ingestion. Our purpose in this experiment was to determine whether miRNA and growth-related mRNA expressed in skeletal muscle are up- or downregulated in humans following the ingestion of EAA. We hypothesized that EAA would alter miRNA expression in skeletal muscle as well as select growth-related genes. Muscle biopsies were obtained from the vastus lateralis of 7 young adult participants (3 male, 4 female) before and 3 h after ingesting 10 g of EAA. Muscle samples were analyzed for muscle miRNA (miR-499, -208b, -23a, -1, -133a, and -206) and muscle-growth related genes [MyoD1, myogenin, myostatin, myocyte enhancer factor C (MEF2C), follistatin-like-1 (FSTL1), histone deacytylase 4, and serum response factor mRNA] before and after EAA ingestion using real-time PCR. Following EAA ingestion, miR-499, -208b, -23a, -1, and pri-miR-206 expression increased ($P < 0.05$). The muscle-growth genes MyoD1 and FSTL1 mRNA expression increased ($P < 0.05$), and myostatin and MEF2C mRNA were downregulated following EAA ingestion ($P < 0.05$). We conclude that miRNA and growth-related genes expressed in skeletal muscle are rapidly altered within hours following EAA ingestion. Further work is needed to determine whether these miRNA are post-transcriptional regulators of growth-related genes following an anabolic stimulus. J. Nutr. 139: 2279–2284, 2009.

Introduction

We have previously reported that essential amino acids (EAA)[8] potently stimulate muscle protein synthesis in humans within hours of ingestion (1) This is primarily attributed to enhanced translation initiation and elongation (2). It is clear that expression levels of mRNAs are sensitive to changes in nutrient status of the muscle, because specific genes are rapidly up- or downregulated in human skeletal muscle during an insulin infusion (3), fasting (4), or a high-glycemic meal (4,5). Recent evidence has suggested that the expression of MyoD1 mRNA is increased and myostatin mRNA is decreased 2.5 h after a mixed meal (15% protein, 70% carbohydrates, 15% fat) in older men and women (6). However, it is not known what upstream regulators are driving the expression patterns of these growth-related genes in human skeletal muscle in response to an anabolic stimulus such as nutrition.

Recent developments in the muscle biology field have linked microRNAs (miRNA) with a novel regulatory mechanism of muscle growth (7). miRNA are small RNA fragments that can silence the translation of mRNA by post-transcriptional repression or degradation (8) and cover a vast array of transcriptional control such that ~60% of the mammalian genome is regulated in part by miRNA (9). miRNA are initially transcribed as long RNA strands (primary transcript), but through a series of steps are cleaved to the size of ~22 nucleotides by 2 key enzymes: Drosha and Dicer. It is the mature form that binds to a 7–9 nucleotide complementary sequence within the 3′ untranslated region of mRNA.

The miRNA (miR) -1 and -133a are highly expressed in striated muscle tissue, whereas miR-206 is skeletal muscle tissue

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[8] Abbreviations used: EAA, essential amino acids; FSTL1, follistatin-like protein 1; HDAC4, histone deacytylase 4; miRNA, microRNA; miR-1, microRNA 1; miR-23a, microRNA 23a; miR-133a, microRNA 133a; miR-206, microRNA 206; miR-208b, microRNA 208b; miR-499, microRNA 499; MEF2C, myocyte enhancer factor 2C; pri-miR-1-2, primary microRNA 1-2; pri-miR-133a-1, primary microRNA 133a-1; pri-miR-206, primary microRNA 206; SRF, serum response factor.

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specific (10–12). miR-1 can arise from 2 different primary transcripts, pri-miR-1–1 and pri-miR-1–2, each from a separate gene (7). Similarly, miR-133a can be produced from the transcripts pri-miR-133a-1 and pri-miR-133a-2 (7). Some reports show that mature miRNA expression may not correspond to the expression patterns of the primary transcript, thus adding another layer of complexity to miRNA biosynthesis (13,14). Although much attention has been given to these classical miRNA for control of muscle development, recent characterization of miR-499, -208b, and -23a suggests that these miRNA also have an important role in muscle growth because of their involvement in myogenesis (7,15) and slow myosin heavy chain gene regulation (7,16–18). The muscle miRNA are dysregulated in muscle diseases such as muscular dystrophy (19–21) and take part in altering cell size during cardiac hypertrophy (22–26) and skeletal muscle hypertrophy (13,27) and atrophy (18). There are several verified and predicted gene targets of these miRNA, many of which are transcription factors and signaling molecules associated with regulation of muscle growth (22,24,28–33). It is also apparent that miRNA are turned over quite rapidly (i.e. hours) in skeletal muscle following exercise (14,34). For instance, we showed a rapid downregulation of the primary transcript, pri-miR-1–2, and its mature transcript, miR-1, within 6 h following the combination of resistance exercise and amino acid ingestion in humans (14). These data suggest that miRNA may play a role in regulating the translation of gene transcripts following acute anabolic stimuli (e.g. amino acid ingestion, exercise).

Therefore, our purpose in this experiment was to determine whether miRNA expressed in skeletal muscle and some of their predicted targets (i.e. muscle-growth related mRNA) are up- or downregulated following the ingestion of 10 g of EAA in young, healthy humans. We hypothesized that EAA would alter miRNA expression in muscle (miR-499, -208b, -23a, -1, -133a, -206) as well as muscle growth-related genes [MyoD1, myogenin, myostatin, myocyte enhancer factor 2C (MEF2C), follistatin-like-1 (FSTL1), histone deacytylase 4 (HDAC4), and serum response factor (SRF)].

Materials and Methods

Participants. We studied 7 young participants (3 male, 4 female; age, 29 ± 2 y; height, 167 ± 6 cm; weight, 71 ± 6 kg; BMI, 25 ± 1 kg/m^2). The participants were not engaged in any regular exercise training at the time of the enrollment, although they were physically active and healthy. Screening of participants was performed with clinical history, physical exam, and standard hospital screening laboratory tests. All participants gave informed written consent before participating in the study, which was approved by the Institutional Review Board of the University of Texas Medical Branch (in compliance with the Declaration of Helsinki).

Experimental design. All participants were admitted to the general clinical research center of the University of Texas Medical Branch the day prior to the study. The participants were then fed a standard dinner and a snack at 2200 h. All participants were studied following an overnight fast under basal conditions, refrained from exercise for 24 h prior to study participation, and were studied at the exact same time (i.e. EAA ingestion period 1200–1500 h).

A baseline muscle biopsy was obtained from the lateral portion of the vastus lateralis using a 5-mm Bergström biopsy needle under sterile procedures and local anesthesia (1% lidocaine). Immediately after the baseline biopsy, participants ingested a solution (0.5 L) that contained 10 g of EAA (a dose that induces a maximal stimulation in human muscle protein synthesis) mixed in a noncaloric, noncaffeinated carbonated beverage. The composition of the EAA mixture was the following: L-histidine (0.8 g), L-isoleucine (0.8 g), L-leucine (3.5 g), L-lysine (1.2 g), L-methionine (3.0 g), L-phenylalanine (1.4 g), L-threonine (1.0 g), and L-valine (1.0 g) (Ajinomoto/Sigma Aldrich). Blood samples were periodically taken after EAA ingestion (15, 30, 45, 60, 90, 120, 150, and 180 min). Three hours following the ingestion of the amino acid mixture, another muscle biopsy was taken. We chose a muscle biopsy sampling period of 3 h, because MyoD1 and myostatin mRNA are considerably altered during this time following nutrient ingestion (6). The baseline and 3 h muscle biopsy were sampled from separate incisions on the same leg with the incision sites ~7 cm from each other. Muscle tissue was immediately blotted and frozen in liquid nitrogen and stored at −80°C until analysis.

Amino acid, insulin, glucose, and FFA concentrations. Concentrations of the branch-chained amino acids (leucine, isoleucine, and valine) were measured in the plasma using GC-MS as previously described (35,36). Insulin concentrations were measured via a RIA commercial kit (Diagnostic Products). Plasma glucose was determined by the glucose oxidase method using a YSI 2700 analyzer (Yellow Springs Instruments) immediately after each blood draw. The plasma FFA concentration was determined enzymatically according to the manufacturer's instructions (Wako NEFA; Wako Chemicals).

RNA extraction. Total RNA was isolated by homogenizing 30–40 mg tissue with a homogenizing dispenser (T10 Basic Ultra Turrax, IKA) in a solution containing 1.0 mL of Tri reagent. The RNA was separated into an aqueous phase using 0.2 mL of chloroform and precipitated from the aqueous phase using 0.50 mL of isopropanol. Extracted RNA was washed with 1 mL of 75% ethanol, dried, and then suspended in a known amount (1.5 μL/mg tissue) of nuclease-free water. RNA concentration and integrity were assessed using the Agilent 2100 BioAnalyser (Agilent Technologies). The mean RNA integrity number was 8.7 ± 0.1 (1–10 scale, 10 highest) and a 1.30 ± 0.02 28S:18S ratio. RNA was DNase treated using a commercially available kit (DNA-free, Ambion).

cDNA synthesis. One microgram of total RNA was reverse transcribed into cDNA according to the manufacturer's directions (iScript, Bio-Rad). Briefly, a 20-μL reaction mixture consisting of 1 μg of total RNA, 4 μL of 5× iScript Reaction mix, 1 μL of iScript Reverse Transcriptase, and a known amount of nuclease-free water was placed into the thermocycler (IQ5 Real-Time PCR cycler, Bio-Rad) with the following temperature/time protocol: 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. All isolated RNA and cDNA samples were stored at −80°C until further analysis.

PCR primers. Primer pairs were customized using Beacon Designer 5.0 software (Premier Biosoft Int.) in which they were designed to avoid homology (BLAST analysis) and secondary structures. Primers were purchased from Invitrogen and were considered optimal if they produced primer efficiencies between ~90 and 100% and a single DNA product of predicted size as identified with a melt analysis and DNA agarose gel. Forward and reverse primers for MyoD1, myogenin, myostatin, pri-miR-1–2, pri-miR-133a-1, and pri-miR-206 have been published previously (14,37). The remaining custom-designed DNA sequences and accession numbers are the following: MEF2C (NM_002397), fwd: TCTGGACGAGTCTGGTTAC, rev: AGGAGGAGGAGGAAGAAGG; FSTL1 (NM_007085), fwd: AGAGGAGGAGATGACCAGATATG, rev: CGCTGAAGTGGAGAAGATGC; HDAC4 (NM_006037), fwd: ACGCCTCTGTTCAACTTG, rev: AACTCCCACCAACACATAC; SRF (NM_003131), fwd: AACCTCCACCATCCAAAC, rev: GCTGACACTAGCAGACAC.

Semiquantitative real-time PCR. Determination of relative mRNA expression was performed with real-time PCR using the iQ5 Multicolor Real-Time PCR cycler (Bio-Rad). cDNA was analyzed using SYBR Green fluorescence (iQ SYBR Green Supermix, Bio-Rad). Each reaction contained Sybr green, a mixture of forward and reverse primers, cDNA template, and a known amount of sterile water. The total volume of the reaction tube was 25 μL. All samples were analyzed in duplicate. An initial cycle for 5 min at 95°C was used to denature the cDNA. This was followed with 40 PCR cycles consisting of denaturation at 95°C for 20s

and primer annealing and extension at 55°C for 30 s. After all PCR analyses, a melt analysis followed. The geometric means of glyceraldehyde-3-phosphate dehydrogenase and β 2 microglobulin were used to normalize the genes of interest as recommended by Vandesompele et al. (38). Glyceraldehyde-3-phosphate dehydrogenase and β 2 microglobulin did not differ across time and therefore were considered suitable for normalization. Relative fold changes were determined from the cycle threshold values using the 2^{-Ct} as described by Livak and Schmittgen (39).

miRNA expression. miRNA from RNA samples (5 ng) were detected using specific Taqman miRNA assay kits (Applied Biosystems) for miR-1, -133a, -206, -499, -208b, and -23a. The assays were conducted as recommended by the manufacturer. In short, RT reactions were performed using specific miRNA primers at 37°C for 30 min then 10 min at 95°C. This was followed by real-time PCR using the RT product and specific PCR miRNA primers at 95°C for 15 s then 60°C for 30 s (~40 cycles). All samples were normalized to an internal control (ribosomal 5S), a small nuclear RNA shown to be a suitable gene for normalization miRNA data in skeletal muscle (40).

Statistical methods. All values are expressed as mean ± SEM. To determine differences in our dependent variables measured in the postabsorptive state (basal) and 3 h following EAA ingestion, paired sample *t* tests were used. A repeated-measures ANOVA was used to analyze plasma amino acids, insulin, glucose, and FFA. When main effects existed, Bonferroni post hoc comparisons were conducted. Significance was set at $P < 0.05$.

Results

Plasma concentrations. The plasma concentrations of leucine and valine were significantly elevated from 15–120 min following EAA ingestion ($P < 0.05$) and isoleucine was elevated above basal up to 90 min ($P < 0.05$) (**Table 1**). Insulin levels increased from 15 to 60 min ($P < 0.05$) but afterwards returned to baseline. Glucose concentrations were slightly lower at 60–180 min following EAA ingestion ($P < 0.05$). FFA concentrations decreased at 60 min but were elevated at 180 min following EAA ingestion ($P < 0.05$).

miRNA expressed in skeletal muscle. Next, we determined whether EAA ingestion would acutely alter the expression levels of the classical muscle miRNA, miR-1, -133a, and -206, their primary transcripts, as well as new members of the miRNA muscle family (miR-499, -208b, and -23a) within skeletal muscle. Following the ingestion of EAA, we found that the expression of miR-499 (**Fig. 1***A*) and -208b (Fig. 1*B*) increased by ~40% ($P < 0.05$) and miR-23a (Fig. 1*C*) and miR-1 (Fig. 1*D*) increased by ~30% compared with basal expression levels ($P < 0.05$). The expression levels of pri-miR-1–2, pri-miR-133a-1, miR-133a, and miR-206 were unchanged following EAA ingestion. However, pri-miR-206 mRNA expression levels increased by ~200% (data not shown).

Muscle growth-related genes. We then followed this up by determining the expression of muscle genes associated with skeletal muscle growth, many of which are predicted targets of our selected miRNA. As a result, we found that EAA ingestion increased mRNA expression of MyoD1 (**Fig. 2***A*; $P < 0.05$) by ~100%, decreased myostatin (Fig. 2*B*; $P < 0.05$) and MEF2C (Fig. 2*C*; $P < 0.05$) mRNA expression by ~75 and ~50%, respectively, and increased FSTL1 (Fig. 2*D*; $P < 0.05$) mRNA expression by ~50% following EAA ingestion. EAA did not affect myogenin, HDAC4, or SRF mRNA expression 3 h after ingestion (data not shown).

Discussion

The unique finding of this study was that the expression of several miRNA and growth-related genes were altered following the ingestion of 10 g of EAA within human skeletal muscle. Importantly, new members of the miRNA muscle family, miR-499, -208b, and -23a, were upregulated, whereas the expression levels of the growth-related genes myostatin and MEF2C decreased and MyoD1 and FSTL1 increased in human skeletal muscle following EAA ingestion. These data provide novel information that EAA can acutely regulate gene and miRNA expression in human skeletal muscle, both of which may contribute to the anabolic effect of EAA ingestion.

The primary finding of this study was that miR-499, -208b, and miR-23a were significantly elevated following EAA ingestion in human skeletal muscle. Very little information is available regarding the function of these muscle miRNA. Van Rooij and Olson (7,16,17) suggested that miR-499 and -208b play a role in the slow phenotype of skeletal muscle by targeting repressors of the β myosin heavy chain gene. Supporting this, McCarthy et al. (18) identified that Sox6 and Purβ (inhibitors of the slow myosin gene) were upregulated whereas miR-499 and -208b were downregulated following hindlimb suspension in rats. Data on miR-23a suggest that an upregulation of this miRNA was necessary for the hypertrophy effects of isoproterenol and aldosterone in cardiac cells (24). Furthermore, Wada et al. (28) indicated that myoblasts overexpressed with miR-23a resisted the glucocorticoid-inducted muscle atrophy by downregulation of its target, atrogin-1. Finally, Safdar et al. (34) reported that miR-23a expression was acutely downregulated following 90 min of treadmill running in mice. Taken together, these studies suggest the muscle miRNA have various roles in skeletal muscle such as controlling muscle mass, muscle fiber phenotype, and adaptations associated with aerobic exercise.

TABLE 1 Plasma concentrations of amino acids, insulin, glucose, and FFA in young adults before and after EAA ingestion[1]

		Min							
	Basal	15	30	45	60	90	120	150	180
Leucine, *μmol/L*	164.9 ± 6.1	728.6 ± 151.9*	1135.0 ± 81.8*	1209.7 ± 99.2*	952.8 ± 106.3*	634.5 ± 84.9*	487.6 ± 56.5*	327.3 ± 26.1	287.9 ± 21.5
Isoleucine, *μmol/L*	50.4 ± 4.5	147.5 ± 23.9*	219.4 ± 11.5*	224.7 ± 13.7*	171.4 ± 13.1*	109.7 ± 10.8*	79.6 ± 6.2	54.5 ± 2.4	46.7 ± 3.1
Valine, *μmol/L*	213.2 ± 14.8	333.4 ± 29.7*	453.4 ± 19.1*	500.0 ± 9.0*	452.9 ± 20.0*	360.6 ± 21.4*	311.2 ± 14.4*	255.4 ± 7.6	234.7 ± 7.9
Insulin, *pmol/L*	32.9 ± 5.3	72.7 ± 8.2*	91.8 ± 11.5*	72.9 ± 11.7*	55.6 ± 7.8*	35.7 ± 6.5	31.2 ± 6.3	35.0 ± 5.9	27.4 ± 6.2
Glucose, *mmol/L*	4.9 ± 0.1	4.8 ± 0.1	4.8 ± 0.1	4.8 ± 0.1	4.8 ± 0.1*	4.8 ± 0.1*	4.8 ± 0.1*	4.7 ± 0.1*	4.7 ± 0.1*
FFA, *mmol/L*	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.1*	0.5 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	1.0 ± 0.1*

[1] Values are the mean ± SE, $n = 7$. *Different from basal, $P < 0.05$.

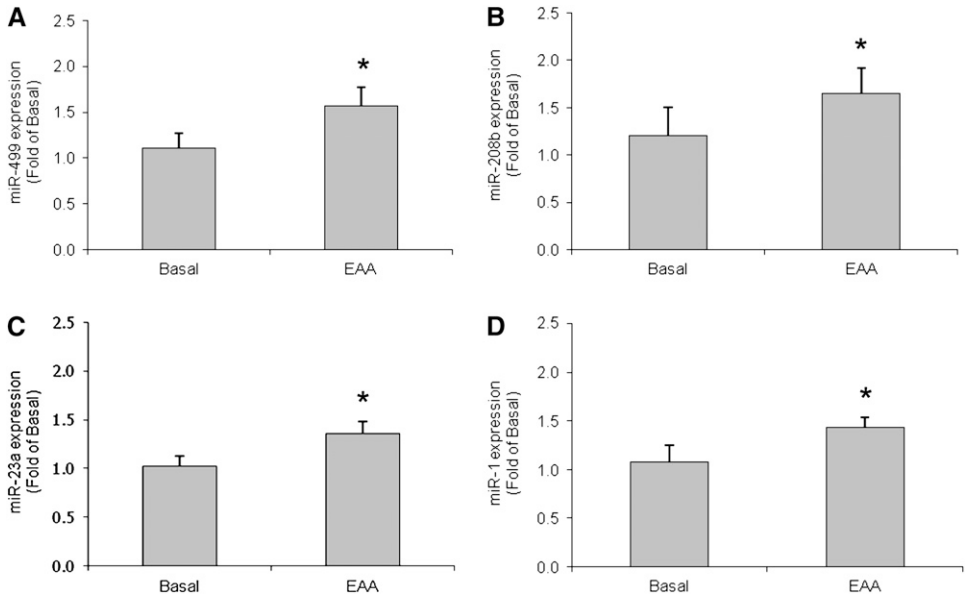

FIGURE 1 Expression of miR-499 (*A*), -208b (*B*), -23a (*C*), and -1 (*D*) in skeletal muscle of young adults before and 3 h after EAA ingestion. Values are the mean ± SE, $n = 7$. *Different from basal, $P < 0.05$.

The miRNA expressed in muscle (miR-1, -133a, and -206) target and repress many genes that are responsible for muscle cell growth and development. Of the 3 miRNA listed above, we found an increased expression of miR-1 following EAA ingestion whereas its primary transcript, pri-miR-1–2, was unchanged. McCarthy and Esser (13) also noted a disagreement in expression pattern of pri-miR-1–2 and the mature transcript, miR-1, following compensatory hypertrophy in mouse plantaris muscle. Thus, the synthesis of a mature miRNA transcript appears to be much more complicated than an up- or down-regulation of the primary transcript. An increase in miR-1 is opposite to what we have previously observed following the anabolic combination of EAA and resistance exercise (14). We suggest that miR-1 may be "putting the brakes on" muscle growth, because the demand for enhanced muscle remodeling is not needed with consuming 10 g of EAA alone in healthy, young adults or perhaps miR-1 is targeting a mRNA transcript that negatively regulates cell size.

To expand the understanding of these miRNA, we conducted a search on a miRNA database to determine whether these miRNA targeted any of the measured muscle growth-related genes in this study. We found that miR-499 and -208b are predicted to target the human myostatin gene. Myostatin, a transforming growth factor-β family member, is known most notably for its role as an inhibitor of muscle growth (41). Interestingly, we show that myostatin mRNA expression decreased following an anabolic stimulus of EAA in humans. It appears that myostatin may be a target of at least miR-208, since Callis et al. (23) reported that overexpression of miR-208a in mouse heart caused cardiac hypertrophy by suppressing genes such as myostatin. A highly predicted target of miR-23a in humans is MEF2C. The general function of the MEF2 family appears to be promotion of a slower muscle phenotype (42) and an association with the expression of metabolic genes (43,44). Therefore, in addition to their role as a regulator of fiber phenotype, an interesting possibility is that perhaps miR-499 and -208b work in concert to target the myostatin transcript while miR-23a may downregulate gene targets such as MEF2C, because EAA ingestion is primarily anabolic and exerts its effect by increasing muscle protein synthesis.

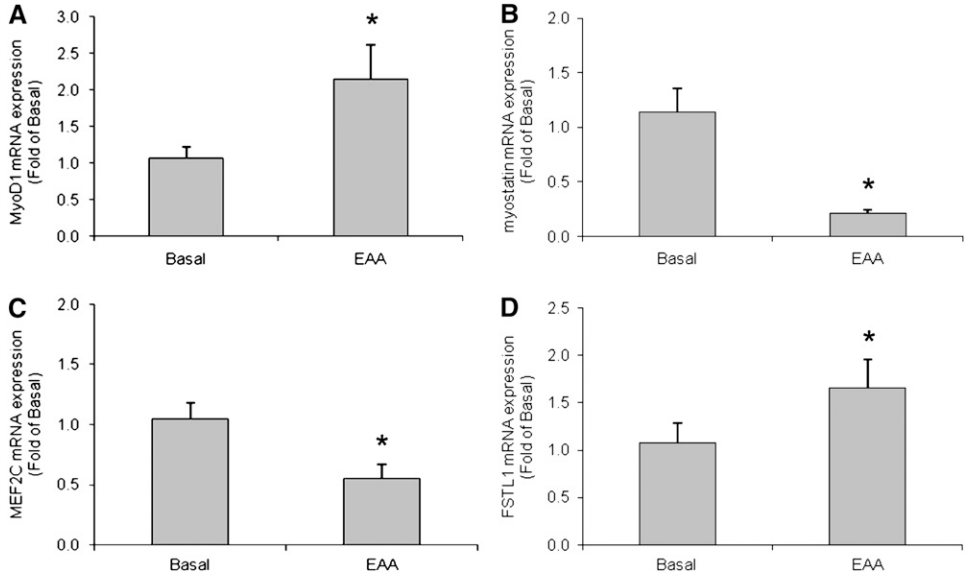

FIGURE 2 Growth-related gene expression of MyoD1 (*A*), myostatin (*B*), MEF2 (*C*), and FSTL1 (*D*) in skeletal muscle of young adults before and 3 h after EAA ingestion. Values are the mean ± SE, $n = 7$. *Different from basal, $P < 0.05$.

Making inferences between miRNA and their targets is a limitation and needs verification. For instance, we report that FSTL1 mRNA expression, a positive muscle growth regulator and target of miR-206 (31), was significantly elevated following EAA ingestion. Counter to our hypothesis, pri-miR-206 was significantly increased and miR-206 was unchanged following EAA ingestion, a similar expression pattern found by McCarthy and Esser (13) but following compensatory hypertrophy. The disassociation between miR-206 and FSTL1 was also identified by Allen et al. (45) who found that 7 d of spaceflight resulted in decreased expression of miR-206 and FSTL1 in rodent gastrocnemius muscle. miR-206 expression levels also vary dependent on the muscle type (13). Therefore, relationships between targets and miRNA may become confounded when working with mixed muscle types such as the vastus lateralis or gastrocnemius. The same argument can be applied to the increase in miR-1 and lack of change of its target HDAC4 (46) following EAA ingestion. Furthermore, miRNA regulation of gene targets may not only occur by means of degradation (8). Finally, some of the gene targets (i.e. MEF2, SRF) (46) may also be regulators of miRNA expression through a novel feedback mechanism (7,47). It is readily apparent that a major challenge of miRNA research is the identification of gene targets.

Data published recently has indicated that skeletal muscle growth-related genes were acutely regulated following a mixed meal (55% carbohydrates, 30% fats, and 15% protein) in older men and women (6). We also showed that growth-related genes (MyoD1, myostatin, MEF2C, FSTL1) are altered but that several miRNA were changed from baseline in response to a nutrient solution containing EAA only in young participants. However, one limitation of our study is that we are unable to definitively show that the changes in muscle growth-related genes and miRNA are due solely to EAA, because energy and insulin may also be playing a role. For example, insulin has been noted as a regulator of gene and miRNA expression (48) and we report modest increases in insulin concentrations during the first hour following EAA ingestion and a small increase in the blood FFA concentration at the end of the experiment. On the other hand, amino acid concentrations increased to a very large extent and were sustained (Table 1) for a longer time than insulin, which provides additional support for EAA being the primary regulator of the changes in gene and miRNA expression reported in our study. Additionally, it is not known whether miRNA expression patterns in human skeletal muscle can be potentially altered by circadian rhythms and therefore future experiments are needed to determine what role, if any, circadian changes may have on miRNA expression.

In conclusion, we show that EAA rapidly induce the expression of miRNA and several growth regulating genes expressed in human skeletal muscle. Further work is needed to elucidate the precise role of miRNA in the regulation of adult muscle mass following protein anabolic (or catabolic) stimuli and to determine what specific mRNA are targeted by these novel post-transcriptional regulators.

Acknowledgments

B.R. and M.D. developed the research proposal; E.V. developed research design; M.D. and B.R. wrote the manuscript; M.D. analyzed the data; E.G., C.F., M.D., and S.D. collected data and reviewed manuscript. All authors read and approved the final draft of the manuscript.

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