Published in final edited form as:

Nutrition. 2015 ; 31(0): 1018–1024. doi:10.1016/j.nut.2015.02.011.

The aromatic amino acid tryptophan stimulates skeletal muscle IGF1/p70s6k/mTor signaling in vivo and the expression of myogenic genes in vitro

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Abstract

Objectives—Nutrition plays a key role in the maintenance of muscle and bone mass, and dietary protein deficiency has in particular been associated with catabolism of both muscle and bone tissue. One mechanism thought to link protein deficiency with loss of muscle mass is deficiency in specific amino acids that play a role in muscle metabolism. We tested the hypothesis that the essential amino acid tryptophan, and its metabolite kynurenine, might directly impact muscle metabolism in the setting of protein deficiency.

Methods—Adult mice (12 mo) were fed a normal diet (18% protein), as well as diets with low protein (8%) supplemented with increasing concentrations (50, 100, and 200 μ M) of kynurenine (Kyn; or with tryptophan (Trp; 1.5 mM). Myoprogenitor cells were also treated with Trp and Kyn in vitro to determine their effects on cell proliferation and expression of myogenic differentiation markers.

Results—Results indicate that all mice on the low protein diets weighed less than the group fed normal protein (18%). Lean mass measured by DXA was lowest in mice on the high kynurenine diet, whereas percent lean mass was highest in mice receiving tryptophan supplementation and percent body fat was lowest in mice receiving tryptophan. ELISA assays showed significant increases in skeletal muscle IGF-1, leptin, and the myostatin antagonist follistatin with tryptophan supplementation. mRNA microarray and gene pathway analysis performed on muscle samples demonstrate that mTor/eif4/p70s6k pathway molecules are significantly up-regulated in muscles from mice on Kyn and Trp supplementation. In vitro, neither amino acid affected proliferation of myoprogenitors, but tryptophan increased the expression of the myogenic markers MyoD, myogenin, and myosin heavy chain.

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Conclusion—Together, these findings suggest that dietary amino acids can directly impact molecular signaling in skeletal muscle, further indicating that dietary manipulation with specific amino acids could potentially attenuate muscle loss with dietary protein deficiency.

Keywords

aging; sarcopenia; C2C12 cells; pathway analysis; muscle atrophy

Introduction

Aging is associated with significant changes in musculoskeletal health, including bone loss and loss of muscle mass and strength, which together increase the risk for falls and bone fractures [1, 2]. The factors underlying loss of muscle and bone mass with age are likely to include a number of different pathways and mechanisms such as reduced protein synthesis, cellular senescence, and tissue catabolism secondary to increased inflammation [3]. Nutrient-related factors are also acknowledged to be important for maintaining muscle and bone mass with increasing age [4]. Caloric restriction, for example, has been shown in various animal models to be the most effective countermeasure for slowing the aging process. We have, however, recently demonstrated that caloric restriction can have a negative impact on muscle and bone mass [5]. Aging itself is associated with a marked decrease in caloric intake in older human subjects, and data from the Health and Nutrition and Health Examination Survey (HANES) indicate that as much as sixteen percent of the US population over the age of sixty-five consumes less than 1,000 calories. The prevalence of malnutrition among institutionalized older subjects increases to between twenty-three to sixty percent of that population [6, 7], and it is this institutionalized population that is at the most significant risk for fracture [8].

The importance of overall nutrient intake [5] as well as gut [9] adipocyte [10] and skeletal muscle [11]- derived hormone signals to bone mass was previously documented by our group and termed the “entero-osseous axis” [9] to describe the relationship between nutrient-stimulated gut hormone release and bone formation. Here we focus on the role of nutrition and skeletal muscle, as epidemiological data support an association between protein intake and muscle mass and between low protein intake and muscle wasting [12–15]. Likewise, specific amino acids have also been linked directly to muscle mass, and dietary protein deficiency as well as amino acid deficiency have been linked directly with age-related sarcopenia [16–17]. The amino acid tryptophan has been previously shown to decline with age in serum of elderly men [18], and its metabolite kynurenine has been observed to accumulate in the peripheral tissues of rats with advanced age [19]. Tryptophan is an essential amino acid and a precursor of serotonin. Serotonin in turn regulates the secretion of pituitary growth hormone (GH), which can induce the production of liver-derived insulin-like growth factor-I (IGF-I). IGF-I signaling is known to play a key role in the regulation of muscle mass [20], and so we sought to determine whether dietary supplementation with tryptophan could rescue the reduction of muscle mass that occurs in adult animals on a low protein diet. We also sought to determine whether kynurenine might induce age-associated changes in skeletal muscle, such as decreased expression of myo-anabolic factors, in adult mice on a protein-deficient diet.

Materials & Methods

In Vivo Animal Study

All animal procedures were approved by the Institutional Animal Care & Use Committee (IACUC) at Georgia Regents University. Twelve-month-old, male C57BL/6 mice were obtained from the aged rodent colony at the National Institute on Aging and randomly assigned to five groups of 10 mice each. Each group was fed one of five specific diets (Table 1) for a period of eight weeks: 1) 18% protein; 2) 8% protein + tryptophan (1.5 mM); 3) 8% protein + kynurenine (50 μ M); 4) 8% protein + kynurenine (100 μ M); and 5) 8% protein + kynurenine (200 μ M). Relative doses are based on our in vitro experiments showing that 100 μ M kynurenine stimulates cellular responses in vitro. Kynurenine has a molecular weight 208 g/mol, and we sought to produce estimated kynurenine concentrations for final blood concentrations of 50, 100 or 200 μ M. Given an approximate mouse weight of 30 gm and volume of distribution at 60% (water; 18 ml), then for a 100 μ M kynurenine in vivo concentration the daily dose of kynurenine should be ~0.374 mg. If a mouse ingests 5 gm of food per day, then adding .075 mg/g of food or 750 mg kynurenine/10 kg of food is expected to yield a 100 μ M concentration in vivo.

The animals were anesthetized using isoflurane for dual-energy x-ray absorptiometry (DXA) scanning at the end of the eight week treatment period. Body composition results were obtained from total body imaging in less than 5 minutes. Whole-body bone fat mass, % fat mass, lean mass, and % lean mass were measured by DXA with a PIXImus instrument (Lunar, Madison, WI). The animals were weighed and then euthanized according to AVMA-approved procedures using CO₂ followed by thoracotomy. The left tibialis anterior was dissected free after euthanasia, weighed, and snap frozen in liquid nitrogen. The primarily fast-twitch extensor digitorum longus (EDL) muscle was dissected free and transected at mid-belly. Half of the EDL was snap frozen for microarray and the other half was used for cryostat sectioning. The right EDL was dissected free for ELISA assays.

ELISA assays and Immunohistochemistry

Protein was isolated from the right extensor digitorum longus muscle of each mouse for ELISA analysis according to the protocol previously described by our lab [21]. IGF-1 (Cat # MG100), Follistatin (Cat # DFN00), and Leptin (Cat # MOB00) ELISA kits were purchased from R & D Systems. Assays were performed according to manufacturer's protocol and samples were assayed without dilution. Myostatin (Cat # K1012) ELISA kits were purchased from Alpco diagnostic and performed according to manufacturer's protocol as we have described previously [21]. Frozen sections of the EDL muscle were stained using primary antibodies to follistatin (goat anti-human polyclonal, R&D Systems clone AF-669) and IGF-1 (rabbit anti-human polyclonal, Santa Cruz Biotechnology clone H-70) with donkey anti-goat Alexa Fluor 488 (abcam ab150129) and goat anti-rabbit Alexa Fluor 546 (Life Technologies A11010) secondary antibodies following procedures we have described previously [22].

Microarray analysis

Microarray analysis was performed at the Georgia Regents University Cancer Center Integrated Genomics Shared Resources Core facility. Briefly, extensor digitorum longus (EDL) muscles were homogenized in Trizol reagent (Cat #: 15596-026, Invitrogen Life Technologies, Carlsbad, CA, USA) for microarray. Total RNA was extracted using a standard chloroform protocol. For miRNA isolation, miRNeasy Micro kit (Cat #: 217084, Qiagen, Valencia, CA, USA) was utilized. mRNA was converted into double stranded cDNA using a T7-oligo (dT) promoter primer sequence and purified for use as a template for in vitro transcription. Transcription reactions were performed with T7 polymerase and biotinylated nucleotide analog/ribonucleotide mix for cDNA amplification. The biotinylated cRNA was prepared in the hybridization mix with oligonucleotide B2 and four control bacterial and phage cDNA. Labeled cRNA was hybridized to the Mouse 430 2.0 GeneChip array (Affymetrix) containing 45,000 probesets corresponding to 21,814 unique genes according to the manufacturer's instructions. Ingenuity Pathway Analysis (IPA) was used to decipher the possible biological relevance of gene expression changes established. (Ingenuity Systems, <http://www.ingenuity.com> website; Redwood City, CA, USA). Gene sets established by analysis of mRNA expression (significant expression changes), were subjected to Interactive Pathways Analysis (IPA) and significant pathways ($p < 0.05$) were compared to each other. Analysis settings for IPA used the reference set of Ingenuity Knowledge Base (Genes Only) with both direct and indirect relationships included. The top canonical pathways from both up- and down-regulated genes were assessed under each diet condition.

In vitro assays

C2C12 cells were plated in a 48 well plate at 5,000 cells/cm² and allowed to attach in proliferation medium for 48 hours. The proliferation medium used here for C2C12 was DMEM (high glucose) supplemented with 10% FBS and penicillin-streptomycin. Proliferation medium was removed, cells washed with PBS, and KRB buffer supplemented with 0, 25 uM, 50 uM, 100 uM, 200 uM, or 400 uM tryptophan (Sigma-Aldrich, CAS #73-22-3) or kynurenine [23]. Proliferation was measured after 24 hours of treatment using the bromodeoxyuridine (BrdU) incorporation assay (Cell Proliferation ELISA, BrdU; colorimetric) (cat# 2752) (Millipore, USA,) to determine the effect of the oxidized nutrients on C2C12 proliferation. Briefly, cells were seeded (5,000 cells/cm²) in a 96-well plate for 24 hours. BrdU and treatment were added to cells for 24 hours in a low serum medium. After removing the culture medium, the cells were fixed and the DNA was denatured in one step by adding FixDenat. The anti-BrdU-POD was added to be incorporated in newly synthesized, cellular DNA. The immune complexes were detected by the subsequent substrate reaction. The reaction product was quantified by measuring the absorbance at 450nm using a scanning multi-well spectrophotometer (plate reader). In a separate set of experiments, cells were treated as described above with 0, 25, or 100 uM of tryptophan or kynurenine, and mRNA isolated after 24 hrs for real-time PCR. For the treatment of C2C12 cells with various amino acids, cells were washed twice with PBS and incubated under following condition: 4:1 ratio of KRB buffer and DMEM (high glucose) supplemented with 2% horse serum. Composition of KRB buffer is as follows: 120 mM NaCl, 3.5 mM KCl, 1.2

mM NaH₂PO₄, 1.2 mM MgSO₄·7H₂O, 25.9 mM NaHCO₃, 1.25 mM CaCl₂·2H₂O, and glucose 0.1%.

Real-time PCR

qRT-PCR analysis of C2C12 cells was performed as described previously [21, 22]. Briefly, cells were lysed in TRIzol® reagent (Invitrogen) for RNA isolation and subsequent cDNA synthesis (iScript™ kit; Bio-Rad, Hercules, CA, USA). One hundred nanograms of cDNA were amplified in duplicates in each 40-cycle reaction using an iCycler™ (Bio-Rad) with annealing temperature set at 60°C, ABsolute™ QPCR SYBR® Green Fluorescein Mix (ABgene, Thermo Fisher Scientific), and custom-designed qRT-PCR primers for myogenic genes that we have used previously (21, 22; Thermo Fisher Scientific). A melt curve was used to assess the purity of amplification products. GAPDH was used as the control gene. Delta Ct values for each treatment group were calculated as $Ct = Ct_{\text{Target gene}} - Ct_{\text{Control gene}}$. The magnitude of the difference between the groups was estimated using deltadelta Ct values for each target gene and these were calculated as $Ct = Ct_{\text{TRT}} - Ct_{\text{vehicle}}$ and fold change was calculated as 2^{-Ct} . We also ran a real-time PCR muscle-specific (myogenesis and atrophy-related genes; Qiagen, USA) array to compare gene expression in muscle samples from mice on the 18% protein diets versus those on the 8% protein + tryptophan to validate changes in myogenic gene expression detected in vitro.

Statistical Analysis

Data are represented by mean±SD. One-way analysis of variance (ANOVA) with five groups was used to analyze the data with a Dunnett's post-hoc comparison-to-control test used for significant results. The analysis was performed on the ranks of the ELISA data (follistatin, IGF-1, myostatin, and leptin) to reduce the influence of outlying observations. T-tests were used to assess the effect of the various treatments on the control gene (GAPDH) for data obtained from the RT-PCR experiments. The difference in Ct expression between the treatments (K25, K100, T25, and T100) and vehicle were assessed using a one-way ANOVA. SAS® 9.3 (SAS Institute, Inc., Cary, NC) was used to analyze the data and significance was determined at alpha=0.05.

Results

Mice fed a normal (18%) protein diet weighed significantly more than mice in the groups on the other diets, and while all the groups on lower protein (8%) weighed less than those fed normal protein they did not differ significantly from one another in body weight (Fig. 1A). Tibialis anterior muscle mass did not differ significantly among any of the groups (Fig. 1B); however, once the muscle weights were normalized for body mass the two groups on the higher kynurenine diets showed greater relative muscle mass than the other groups (Fig. 1C). Percent body fat decreased significantly in all groups on low protein, except for the group on the highest kynurenine supplement (Fig. 1D). This same high kynurenine group also showed a significant decrease in lean mass (Fig. 1E). Percent lean mass was significantly greater in the tryptophan group and middle dose kynurenine group compared to the 18% protein group (Fig. 1F).

ELISA assays showed that tryptophan supplementation increased relative protein (normalized for total protein) levels of follistatin, IGF-1, and leptin, though none of the groups differed significantly in relative myostatin protein levels (Fig. 2). Mice on the lowest dose of kynurenine also showed an increase in muscle-derived IGF-1 relative to mice on normal protein diet (Fig. 2B), and immunohistochemistry showed abundant localization of follistatin and IGF-1 in muscle sections from mice on Kyn or Trp supplementation (Fig. 2C). Integrated pathway analyses performed on gene expression array data from muscles of mice on normal protein versus mice fed low protein plus either kynurenine or tryptophan produced similar results. Specifically, both the kynurenine and tryptophan groups showed increased expression of genes involved in eif2, eif4/p70s6k, and mTor signaling relative to normal protein diet (Fig. 3). Dose-response studies demonstrated no effect of either kynurenine or tryptophan on myoblast proliferation (Fig. 4A). Gene expression data demonstrate that while kynurenine downregulated the myogenic factor myoD, tryptophan increased expression of the three myogenic genes myogenin, myoD, and myosin heavy chain (Fig. 4B).

Myogenic PCR array demonstrated the tryptophan supplementation increased the relative expression of several genes compared to mice on the 18% protein diet (Fig. 4C). Specifically, the Trp-fed mice showed increased expression of MyoD, as well as Akt1 and Akt2, two factors essential for myogenic differentiation and myotube maturation. In addition, mice with Trp supplementation showed elevated expression of MuSK, a muscle-specific kinase critical for neuromuscular junction formation, and increased expression of Atp2A1, and ATPase involved in excitation-contraction in fast-twitch skeletal muscle fibers.

Discussion

Dietary protein is well-known to have important effects on the maintenance of muscle mass throughout growth, development, and aging; however, the role of specific amino acids as signaling molecules in muscle has received less attention. Previous work suggests that tryptophan and its metabolite kynurenine may have direct effects on cell signaling via the extracellular calcium sensing receptor, which we have recently demonstrated in bone cells [24, 25]. It has previously been shown that tryptophan can significantly impact muscle mass through its metabolite serotonin, and animals deficient in tryptophan are known to show low levels of growth hormone (GH) and significant muscle atrophy, a downstream effect of reduced GH-IGF1 signaling [26]. Our data are consistent with a role for dietary tryptophan in stimulating IGF1 signaling within the setting of dietary protein deficiency, in that tryptophan supplementation increased muscle-derived IGF-1 as well as the expression of genes in the eif4/p70s6k/mTor pathway, known to be activated by IGF1. Eif4 is recognized as playing an important role of muscle protein synthesis [27], and dietary supplementation with tryptophan has been shown to stimulate muscle protein synthesis in swine [28]. Our in vitro data showed that tryptophan induced the expression of myogenic factors in C2C12 myoblasts, suggesting that this amino acid can impact muscle cells directly, as well as indirectly through the serotonin-GH-IGF1 axis. The microarray pathway data also revealed that in both groups with low protein in the diet, the eif2 pathway is significantly up-regulated. This pathway inhibits protein synthesis [29], and appears to be strongly activated in the presence of dietary protein deficiency.

The data presented in this study also highlight some important differences in the response of skeletal muscle to either kynurenine or tryptophan supplementation. While tryptophan was shown to increase follistatin, leptin, and IGF-1 in muscle, low dose kynurenine actually decreased muscle-derived leptin and high dose kynurenine resulted in loss of muscle mass and preservation of body fat. In contrast, relative lean mass was highest in mice with tryptophan supplementation. Follistatin is an important regulator of myofiber hypertrophy as it is a potent antagonist of both activin and myostatin, two factors known to be involved in the suppression of muscle growth and development [30]. Likewise, the leptin receptor is highly expressed in skeletal muscle and is upregulated with mechanical loading [31], and leptin deficiency is associated with muscle atrophy and impaired proliferation and differentiation of myoprogenitors [23]. In addition, kynurenine increased the expression of genes involved in eif4/p70s6k/mTor signaling approximately 1.5 fold, whereas tryptophan increased the expression of these genes closer to 3-fold (Fig. 3). Together, the in vivo data suggest that tryptophan supplementation can impact a number of peptides in skeletal muscle that play important role(s) in the regulation of myofiber size and muscle mass. The in vivo data are further supported by in vitro experiments showing that, in contrast to kynurenine, tryptophan supplementation consistently increased the expression of myogenic markers myogenin, myoD, and myosin heavy chain whereas kynurenine did not consistently stimulate the expression of myogenin and myoD.

Although dietary protein deficiency is associated with impaired muscle growth and development, there has been some question as to its role in age-associated loss of muscle mass [32], which occurs at a rate of 5–8% per decade after age 30 [33]. Findings from the Health ABC study, for example, suggested that lower protein intake stimulated muscle loss [34]; however, in this study total protein intake was still within the normal recommended daily allowance and so the subjects were not considered to be protein deficient [32]. The relationship is confounded further by the fact that the response of older adults to lower levels of amino acids is blunted compared to that of younger individuals [32], although some of this age-related insensitivity to amino acids can be overcome with leucine supplementation [35]. Muscle protein synthesis is also generally attenuated in older individuals compared to the young [3], which may further complicate the relationship between nutrient-related stimuli and changes in either muscle mass or strength. Excessive consumption of tryptophan supplements has in some cases been linked to eosinophilia-myalgia syndrome, which causes inflammation and fibrosis in the fascia surrounding muscle [36]. This is a relatively rare condition, and when it occurs it most often observed in patients taking tryptophan supplements for insomnia. Our study provides new insights into the potential for different amino acids to impact signaling in skeletal muscle of mature animals, and suggests in turn that these molecules have the potential to affect multiple downstream pathways regulating muscle protein synthesis and myogenesis, particularly in the setting of dietary protein deficiency.

Acknowledgments

Funding for this research was provided by the National Institute on Aging (P01 AG036675). We are grateful for assistance provided by Penny Roon and Donna Kumiski in the GRU Histology Core facility.

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Highlights

- Dietary protein deficiency is associated with loss of muscle mass.
- Tryptophan increased % lean mass and decreased % body fat in adult mice on a low protein diet.
- Tryptophan supplementation also increased muscle-derived IGF-1, follistatin, and leptin.
- In vitro, tryptophan stimulated myosin heavy chain and myogenin expression.

Figure 1.

A. Body mass, B. Tibialis anterior (TA) mass, C. TA mass relative to body mass, D. % Body fat, E. Lean mass, and F. % lean mice for mice fed normal protein (18%) or low protein (8%) supplemented with different concentrations of kynurenine (Kyn) or tryptophan (Trp). Bar graph indicates group means and error bars represent one standard deviation. *p < 0.05 compared to 18% protein.

Figure 2.

ELISA data (A, B, D, E) and immunostained muscle sections (C) for muscle-derived follistatin (Fstn), IGF-1, myostatin (GDF-8), and leptin. ELISA data and stained sections are for the extensor digitorum longus muscle, and ELISA data are normalized for total protein in mice fed normal protein (18%) or low protein (8%) supplemented with different concentrations of kynurenine (Kyn) or tryptophan (Trp). Bar graph indicates group means and error bars represent one standard deviation. * $p < .05$ compared to 18% protein.

Figure 3. Integrated pathway analysis for mRNA microarrays performed on muscle samples from mice fed 8% protein plus kynurenine (A, B) or tryptophan (C, D) compared with muscles from mice on normal (18%) protein. Data shown are for upregulated genes (1.5-fold +; A, C) and all genes (B, D).

Figure 4.

A. Relative proliferation of C2C12 myoblasts treated with different doses of kynurenine (Kyn) or tryptophan (Trp). B. Expression of myogenic markers myogenin (myog), myoD, and myosin heavy chain (MHC) in C2C12 myoblasts treated with different doses (25, uM, 100M) of kynurenine (K) or tryptophan (T). C. Real-time PCR data for whole-muscle samples in mice on 18% protein diet compared to mice fed 8% protein + tryptophan. Bar graph indicates species means and error bars represent standard errors. * $p < .05$, ** $p < .01$.

Table 1

Dietary formulations for the different groups included for study.

Diet Composition (g/kg) *	18% Prot	8% Prot + 50 uM Kyn	8% Prot + 100 uM Kyn	8% Prot + 200 uM Kyn	8% Prot + 1.5 mM Trp
Casein	207.0	92.0	92.0	92.0	92.0
Sucrose	430.0	520.0	520.0	520.0	520.0
Corn Starch	200.0	200.0	200.0	200.0	200.0
Mineral Mix	13.3	13.3	13.3	13.3	13.3
Vitamin Mix	10.0	10.0	10.0	10.0	10.0
Kyn or Trp Added	0.0	0.375	0.075	0.15	1.1

* Diets have a constant ratio of carbohydrate to fat (-9.3:1) on a weight basis.