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Effects of long-term exposures to low iron and branched-chain amino acid containing diets on aging skeletal muscle of Fisher 344 Brown Norway rats

Yuho Kim,

Department of Exercise Science, Syracuse University, 820 Comstock Ave, Room 201 WB, Syracuse, NY 13244, USA

Muscle Energetics Laboratory, National Heart, Lung, and Blood Institute, 10 Center Drive Room B1D400, Bethesda, MD 20892, USA

Sok Sambo Men,

Department of Nutrition Science, Syracuse University, 426 Ostrom Avenue, Syracuse, NY 13244, USA

Chen Liang,

Department of Exercise Science, Syracuse University, 820 Comstock Ave, Room 201 WB, Syracuse, NY 13244, USA

Candace N. Receno,

Department of Exercise Science, Syracuse University, 820 Comstock Ave, Room 201 WB, NY 13244, USA

Tom D. Brutsaert,

Department of Exercise Science, Syracuse University, 820 Comstock Ave, Room 201 WB, Syracuse, NY 13244, USA

Donna L. Korol,

Department of Biology, Syracuse University, 107 College Place, Syracuse, NY 13244, USA

Kevin S. Heffernan, and

Department of Exercise Science, Syracuse University, 820 Comstock Ave, Room 201 WB, Syracuse, NY 13244, USA

Keith C. DeRuisseau

Department of Exercise Science, Syracuse University, 820 Comstock Ave, Room 201 WB, Syracuse, NY 13244, USA

Abstract

Aging skeletal muscle displays an altered iron status that may promote oxidative stress and sarcopenia. A low iron (LI) containing diet could reduce muscle iron status and attenuate age-

Corresponding author: Keith C. DeRuisseau (email: kederuis@syr.edu); Department of Exercise Science, Syracuse University, 820 Comstock Ave, Room 201 WB, Syracuse, NY 13244, USA.

Conflict of interest statement

The authors declare that they have no conflict of interest.

related muscle atrophy. Supplemental branched-chain amino acids (BCAA) may also alleviate sarcopenia by promoting muscle protein synthesis and iron status improvement. This study examined individual and combined effects of LI and BCAA diets on anabolic signaling and iron status in skeletal muscle of aging rats. Twenty-nine-month-old male F344BN rats consumed the following control-base diets: Control + regular iron (35 mg iron/kg) (CR; n = 11); Control + LI (~6 mg iron/kg) (CL; n = 11); 2×BCAA + regular iron (BR; n = 10); and 2×BCAA + LI (BL; n = 12) for 12 weeks. Although LI and/or 2×BCAA did not affect plantaris muscle mass, 2×BCAA groups showed lower muscle iron content than did CR and CL groups ($P < 0.05$). p70S6 kinase phosphorylation was greater in 2×BCAA and LI animals compared to CR animals ($P < 0.05$). Interactions between IRON and BCAA were observed for proteins indicative of mitochondrial biogenesis (PGC1- α) and oxidative capacity (COX-2 and citrate synthase) ($P < 0.05$) wherein the combined diet (BL) negated potential benefits of individual diets. Antioxidant capacity, superoxide dismutase (SOD) activity, and oxidative injury (3-nitrotyrosine (3-NT), protein carbonyls, and 4-hydroxynonenal (4-HNE)) were similar between groups. In conclusion, 12 weeks of LI and 2×BCAA diets showed significant impacts on increasing anabolic signaling as well as ameliorating iron status, however these interventions did not affect muscle mass.

Keywords

aging; sarcopenia; iron status; BCAA; protein synthesis

Introduction

Advancing age is often accompanied by sarcopenia, which is characterized by a measurable decrease in muscle size and function (Rosenberg 1997). A growing body of evidence suggests that an increase in skeletal muscle iron status could play a pivotal role in the development of sarcopenia (Altun et al. 2007; Hofer et al. 2008; Jung et al. 2008; Seo et al. 2008; Xu et al. 2008; DeRuisseau et al. 2013; Xu et al. 2013). Although iron is an essential element necessary for oxygen transport, energy production, and other enzymatic functions, uncontrolled iron status can be toxic because of its high reactivity with hydrogen peroxide (H_2O_2). Iron-catalyzed formation of hydroxyl radicals ($\cdot OH$) can damage biomolecules such as DNA/RNA (Xu et al. 2008), proteins (Xu et al. 2012), and lipids (Reardon and Allen 2009). It was revealed that the increased muscle iron status with age might lead to elevated oxidative stress (Altun et al. 2007; Hofer et al. 2008; Jung et al. 2008; Xu et al. 2008). Thus, attenuation of muscle iron status changes during aging could alleviate the degree of muscle mass loss and dysfunction by mitigating oxidative stress.

Administration of a low iron (LI) containing diet could be an effective means to decrease systemic iron status and downregulate the skeletal muscle iron level of aging animals. Previous studies showed the effectiveness of LI-containing diets in attenuating iron accumulation in liver (Otogawa et al. 2008) and heart (Naito et al. 2011) disease models that feature the pathological condition of iron overload. Additionally, recent work showed that a long-term (i.e., 12 weeks) LI diet reduced iron accumulation in skeletal muscle of adult rats (Arruda et al. 2013). Thus, prolonged administration of a LI-containing diet could be an effective strategy to reduce skeletal muscle iron levels and attenuate sarcopenia.

Sarcopenia is also affected by the balance between muscle protein synthesis and breakdown. In aging skeletal muscle the rate of protein synthesis may be reduced (Dickinson et al. 2013), thereby accelerating the advance of sarcopenia. The administration of branched-chain amino acids (BCAA; Leucine, Isoleucine, and Valine) can upregulate protein synthesis in aging skeletal muscle of humans (Volpi et al. 1998; Volpi et al. 2003; Chevalier et al. 2011) and rodents (Vary et al. 1999; Crozier et al. 2005). Specifically, BCAA activate mTOR that, in turn, stimulates two separate down-stream targets (P70 ribosomal protein S6 kinase 1 (p70S6K1) and 4E binding protein 1 (4EBP1)) that are responsible for initiation of protein translation (Anthony et al. 2000) (For reviews, see (Anthony et al. 2001; Kimball and Jefferson 2006b; Kimball and Jefferson 2006a)). Furthermore, a link between mTOR and tissue iron status was suggested since mTOR was shown to alter transferrin receptor (TfR1) stability (Bayeva et al. 2012), a key marker of intracellular iron status. BCAA appear to decrease iron status and TfR1 levels in iron overload conditions, specifically liver diseases (Iwasa et al. 2013; Korenaga et al. 2015). Thus, while BCAA may be an effective means to stimulate protein synthesis they may also provide benefit to aging muscle by reducing iron levels.

Iron accumulation may lead to mitochondrial dysfunction particularly in skeletal muscles (Chabi et al. 2008; Seo et al. 2008), which in turn may disrupt muscle physiology. For instance, proteins important for mitochondrial function and biogenesis such as cytochrome oxidase (COX) and PGC-1 α are decreased in skeletal muscles of very old rats (Chabi et al. 2008), suggesting an increased iron status may play a role in mitochondrial dysfunction. Moreover, long-term administration of BCAA may not only boost mitochondrial biogenesis (e.g., PGC-1 α) but also ameliorate oxidative stress in skeletal muscles of middle-aged animals (D'Antona et al. 2010). Thus, we postulated that LI and BCAA diet would positively influence mitochondrial biogenesis in aging skeletal muscle.

The overall purpose of this study was to determine the effect of LI and/or BCAA on: (1) iron status; (2) anabolic signaling; (3) markers of mitochondrial biogenesis; and (4) oxidative stress and antioxidative ability in skeletal muscle of old rats. Since plantaris displays pronounced atrophy and increased iron concentration with advancing age (DeRuisseau et al. 2013), we hypothesized that long-term administration of LI and/or 2 $\times$ BCAA would augment plantaris muscle mass and result in: (1) lower muscle iron status (i.e., lower non-heme iron concentration and total iron content; greater expression of TfR1); (2) greater anabolic signaling (i.e., increased phosphorylated mTOR and p70S6K1); (3) greater mitochondrial biogenesis (i.e., increased expression of PGC-1 α), and mitochondrial oxidative capacity (i.e., increased citrate synthase (CS) & cytochrome c oxidase subunit 2 (COX-2)); and (4) lower oxidative stress (i.e., decreased protein carbonyl, 3-nitrotyrosine (3-NT) and 4-hydroxynonenal (4-HNE)), and enhanced antioxidative ability (i.e., increased superoxide dismutase (SOD) activity and antioxidant capacity). Furthermore, we tested the hypothesis that co-administration of LI and 2 $\times$ BCAA diets would have an additive effect on the outcomes compared with the individual dietary treatments. Hence, this study investigated individual, as well as combined effects of LI and 2 $\times$ BCAA on the specified outcomes.

Materials and methods

Animals

Male Fisher 344 × Brown Norway (F344BN) F1 rats (n = 44) were obtained from the National Institute on Aging at 29 months of age since significant decreases in muscle mass have been noted to occur around this age (Hagen et al. 2004; Hepple et al. 2004). Upon arrival to the animal facility at Syracuse University, all rats were acclimated for a minimum of three days and were randomly divided into four groups (n = 10–12/group). Rats were first grouped into either control (1×BCAA) or 2×BCAA diet groups, and then further divided into subgroups by iron level in the diet (regular iron containing [RI] diet or low iron [LI] containing diet). Thus, the following four groups of animals were studied: (1) Control + RI (CR; n = 11), (2) Control + LI (CL; n = 11), (3) 2×BCAA + RI (BR; n = 10), and (4) 2×BCAA + LI (BL; n = 12). The AIN-93M diet served as the base for all diets. All rats were singly housed in Optirat cages (Animal Care Systems, CO) and had access to their respective diets and water *ad libitum*. Reverse osmosis water was provided to all rats in an effort to minimize environmental iron exposure. Diets and water were replaced weekly. The animal room conditions were automatically controlled: 12:12 light/dark cycle, 40 to 60% humidity and 20–25°C. All procedures were approved by the Syracuse University Institutional Animal Care and Use Committee and followed guidelines by the American Physiological Society for the use of animals in research.

Dietary treatments

Once experimental groups were established, rats in each group were fed their respective diets for 12 weeks. This study administered four different diets: (1) CR (TD.94048; AIN-93M + 1×BCAA + RI), (2) CL (TD.140351; AIN-93M + 1×BCAA + LI), (3) BR (TD.140352; AIN-93M + 2×BCAA diet + RI), and (4) BL (TD.140353; AIN-93M + 2×BCAA + LI). As shown in Table 1, all diets were based on the AIN-93M purified diet and purchased from Teklad lab animal diets (Harlan Laboratories, Madison, WI). The 2×BCAA diets (i.e., BR and BL diets) were designed to contain twice the amount of BCAA (Leucine: Isoleucine: Valine = 1.6: 1.0: 1.2) compared to the control diets (i.e., CR and CL diets). The LI diets minimized background iron (6 mg iron/kg; ~6 ppm) by the use of microcrystalline cellulose and an iron deficient mineral mix. Since adult rats require ~35 mg iron/kg diet (i.e., 35 ppm) (Reeves et al. 1993; Arruda et al. 2013) the regular iron diets contained 36–46 mg iron/kg. Total body mass and food consumption were measured weekly. Diets were maintained in a refrigerator at 4°C for short-term storage or frozen for longer-term storage. All diets were used within six months from the day of arrival at the facility.

Tissue collection and muscle mass measurement

Following 12 weeks of dietary administration, rats were anesthetized with an intraperitoneal injection of Fatal Plus (100–120 mg/kg) (Vortech Pharmaceuticals Ltd., Dearborn, MI). Once in a surgical plane of anesthesia the (right and left) lower limb skeletal muscles (plantaris, soleus, gastrocnemius, TA, and EDL) were quickly collected. The muscle wet mass was measured after removal of excess connective tissue and fat. The muscles were quickly frozen and stored at –80°C until biochemical analyses were conducted. The total

diaphragm (costal and crural regions) and a section of the liver were also collected and stored at -80°C .

Non-heme iron measurement

Non-heme iron levels were determined using previously described protocols (Rebouche et al. 2004; Jung et al. 2008; DeRuisseau et al. 2013). Plantaris muscle and liver (30–50 mg) were homogenized in (18.6 M Ω) water (1:10 w:v) on ice using a glass Potter Elvehjelm style homogenizer. The homogenates were gently mixed with a protein precipitation solution (1N HCl and 10% trichloroacetic acid) in 1:1 volume. After incubating for 1 hr at 95°C , the samples were centrifuged at $8,200 \times g$ for 10 min and the supernatants were gently mixed with reaction solution (0.508 mM ferrozine, 1.5 M sodium acetate, and 0.1% thioglycolic acid). Sample blanks were prepared by incubating samples with 1.5 M sodium acetate and 0.1% thioglycolic acid. The sample reaction and blank mixtures were incubated at room temperature for 30 min and reaction absorbance values were obtained by using a microplate reader (PowerWave HT; BioTek, Winooski, VT) at 562 nm. Non-heme iron concentration was calculated by comparing absorbance values to a standard curve that was generated using an iron atomic absorption standard solution (#305952; Sigma Aldrich, St. Louis, MO). Total non-heme iron content (nmol/muscle mass) was calculated by multiplying the non-heme iron concentration (nmol/gww) with an average of left and right plantaris muscle mass values.

Western blotting

Western blotting was performed to examine protein expression levels related to mTOR signaling (mTOR and p70S6K1), mitochondrial biogenesis (PGC-1 α) and oxidative capacity (CS and COX-2), iron uptake (TfR1), and oxidative stress (3-NT and 4-HNE). Using a glass Potter Elvehjelm style homogenizer, ~50 mg of plantaris was homogenized in RIPA Lysis buffer (1:10 w:v; sc-34162A; Santa Cruz Biotechnology, Dallas, TX). After centrifugation at $10,000 \times g$ for 10 min at $5-6^{\circ}\text{C}$, the protein concentration of the soluble fraction was assessed using the RC/DC protein assay (#500-0122; Bio-Rad, Hercules, CA).

Samples were prepared by mixing with 4 $\times$ Laemmli sample buffer (#161-0747; Bio-Rad) including β -mercaptoethanol and boiling at 100°C for 5 min. Proteins (30–40 μg) were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (0.2 μm ; #161-0112; Bio-Rad) using a mini-trans blot system (Bio-Rad) at 275 mA for 2 hrs. The membranes were blocked in 5% non-fat milk or bovine serum albumin (BSA) in Tris-buffered saline containing Tween-20 (TBS-T) for 1 hr at room temperature and were subsequently incubated with each primary antibody overnight at 4°C .

The following primary antibodies were used: anti-phospho-mTOR (Ser2448; #2971), anti-mTOR (#2972), anti-phospho-p70S6K1 (Thr389; #9205), and anti-p70S6K1 (#9202) from Cell Signaling Technology (Boston, MA); anti-PGC-1 α (H-300; sc-13067) and anti-COX-2 (sc-23984) from Santa Cruz; anti-citrate synthase (GTX110624) from GeneTex (Irvine, CA); anti-TfR1 (ox-26; MCA-155R) from AbD Serotec (Raleigh, NC); anti-nitrotyrosine (#AB5411) from Millipore (Temecula, CA); anti-4-HNE (#HNE11-S) from Alpha

Diagnostic International (San Antonio, TX). After overnight incubation with gentle agitation at 4°C the membranes were washed with TBS-T and incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hr at room temperature: goat anti-rabbit IgG-HRP (#31460) from Thermo Fisher Scientific (Rockford, IL); goat anti-mouse IgG-HRP (sc-2005) or donkey anti-goat IgG-HRP (sc-2020) from Santa Cruz Biotechnology (Dallas, TX).

After incubation with secondary antibodies the membranes were washed and treated with enhanced chemiluminescence (ECL) substrate (#170-5061, Bio-Rad) to develop the HRP bound to the secondary complex. Bio-Rad ChemiDoc XRS+ System with Image Lab Software (version 4.1) was used to digitize blots. Using the captured images, densitometry analysis was performed to assess background adjusted volumes of selected bands. All protein band intensities were normalized to an internal control protein (e.g., GAPDH or β -Actin) and expressed as arbitrary units (A.U.). In addition, phosphorylation of mTOR and p70S6K1 was assessed by normalizing to its total protein level.

Antioxidant capacity

Skeletal muscle antioxidant capacity was assessed using a commercially available kit (Cayman Chemical, Ann Arbor, MI). Muscle tissues were homogenized in buffer (5 mM potassium phosphate, pH = 7.4, 0.9% sodium chloride) and centrifuged at $10,000 \times g$ at 4°C for 15 min. Then, 10 μ L of sample was added to 10 μ L metmyoglobin and 150 μ L chromogen. Reactions were initiated with an addition of 40 μ L hydrogen peroxide working solution. After a 5 min incubation, samples were read at 750 nm using a spectrometer (PowerWave HT; BioTek, Winooski, VT). Results are expressed as μ mol/mg protein.

Total SOD activity

Total SOD activity was measured using a commercially available assay kit (Sigma-Aldrich, St. Louis, MO). Briefly, plantaris muscle was homogenized in buffer (50 mM Tris-HCl, pH = 7.5, 5 mM EDTA and 1 mM DTT) and centrifuged at $10,000 \times g$ for 15 min at 4°C. Then, 20 μ L of supernatant was added to 200 μ L of WST working solution, and 20 μ L of either enzyme working solution or dilution buffer was added and mixed thoroughly. Samples were incubated at 37°C for 20 min and were read at 450 nm using a spectrometer (PowerWave HT; BioTek, Winooski, VT). The percent inhibition value was normalized to the sample protein concentration to obtain SOD activity units.

Protein carbonyls

Protein carbonyls were measured using the OxyBlot assay kit (Millipore, Billerica, MA). Briefly, muscle tissues (~10 mg) were homogenized in ice-cold buffer (5 mM Tris-HCl, pH = 7.4; 5 mM dithiothreitol) and centrifuged at $1,500 \times g$ for 10 min. Proteins were derivatized to 2,4-dinitrophenylhydrazine (DNP). These DNP-derivatized protein samples were separated using polyacrylamide gel electrophoresis via 12% gradient polyacrylamide gels containing 0.1% SDS. After electrophoresis, the proteins were transferred to nitrocellulose membranes. The membranes were then soaked in PBS buffer containing 5% skim milk and 0.05% Tween, and subsequently incubated with a primary antibody specific for the DNP moiety of the proteins. This step was followed by incubation with a HRP-

conjugated antibody directly against the primary antibody. The membranes were then treated with ECL substrate (#170-5061, Bio-Rad) to develop the HRP bound to the antibodies complex. The blots were quantified using Bio-Rad ChemiDoc XRS+ System with Image Lab Software (version 4.1).

Statistical analysis

Descriptive statistics are reported as means $\pm$ standard errors of the mean (SE). Changes in body mass and food intake were assessed by two-way repeated measures analysis of variance (ANOVA) using Time $\times$ Experimental Group. For other analyses, two-way ANOVA was applied to examine significance of main effects of iron treatments (RI vs. LI), main effects of BCAA treatment (control vs. 2 $\times$ BCAA), and their interaction (IRON $\times$ BCAA). Homogeneity of variance was determined by Levene's test, and if it was violated ($P < 0.05$), either log ($\log_{10}X$ used for PGC-1 α , COX-2, and protein carbonyl) or inverse transformation ($1/X$ used for non-heme iron concentration and 3-NT) was applied to statistical tests to meet the assumption. Although figures depict non-transformed data, when applicable, these were checked with figures containing transformed data to ensure whether the patterns between them were consistent. Upon detection of significant interactions ($P < 0.05$), Tukey's post hoc tests were performed to identify means that were significantly different. Extreme outlier values (indicated on SPSS generated interquartile boxplots) were removed from analyses. Prism 6 (GraphPad Software, CA) and IBM SPSS statistics 21.0 were used and statistical significance was set at $P < 0.05$.

Results

Body mass and muscle mass characteristics

Body mass was measured weekly throughout the 12-week dietary administration period. No significant difference in body mass was observed between groups (Fig. 1A). All groups increased body mass over the 12-week period but no significant group differences were observed in the percent change of body mass. Fig. 1B displays food consumption across groups reported as an average intake (grams/day). Although all rats had a similar amount of food consumption over the dietary experimental procedure, CR rats consumed significantly more food than did BL rats during the last week of the experiment (week 12; $P < 0.001$). When we examined data for total food intake we observed that one rat in the mixed diet group (BL; BCAA + Low-iron diet) dramatically decreased its food intake during the last two weeks, which likely skewed the result. However, this rat's body weight was not significantly altered as compared to its initial weight (i.e., decreased by $\sim 5\%$), so we did not exclude it from food intake data. Considering that the cumulative food consumption was similar between groups, it was likely that all animal groups had a similar caloric intake. When considering iron intake (i.e., calculation of daily iron intake by multiplying total amount of food intake with iron level in each diet), rats on LI and BL consumed 0.11 mg iron/day, while animals on the regular iron diets (CR and BR) consumed 0.91 mg iron/day on average. Meanwhile, rats in the 2 $\times$ BCAA diet groups consumed approximately 0.27 (Ile), 0.42 (Leu), and 0.32 (Val) g/day. As shown in Table 2, there was no significant difference in muscle wet mass between groups for plantaris, soleus, EDL, gastrocnemius, TA, and diaphragm.

Non-heme iron status in skeletal muscle and liver

Based on previous reports that LI (Otogawa et al. 2008; Naito et al. 2011; Arruda et al. 2013) and BCAA (Iwasa et al. 2013; Korenaga et al. 2015) diets decrease tissue iron status in various iron overload conditions, we examined whether LI and/or 2×BCAA diets would alter the muscle iron status. The main effect of 2×BCAA diets on non-heme iron concentration level of the aged plantaris approached statistical significance ($P = 0.059$; Fig. 2A). However, when expressed per muscle, 2×BCAA diets showed a significant main effect on the plantaris total non-heme iron content ($F(1, 30) = 4.305$, $P = 0.046$; Fig. 2B). That is, non-heme iron status was likely to be lower in 2×BCAA diet groups (i.e., BR and BL) compared to control diet groups (i.e., CR and CL).

The expression level of the key iron-regulating component TfR1 was similar across all treatment groups (Fig. 2C). As shown in Fig. 2D, rats provided with LI diets showed a lower liver non-heme iron concentration ($F(1, 28) = 5.304$, $P = 0.029$), which was not affected by 2×BCAA administration.

Anabolic signaling

No significant main effect of, or interaction between IRON and BCAA on mTOR phosphorylation was observed (Fig. 3A). However, as presented in Fig. 3B, two-way ANOVA revealed a significant interaction between IRON and BCAA for phospho-p70S6K1 ($F(1, 30) = 4.575$, $P = 0.044$) despite no main effects of LI or 2×BCAA. Post hoc analysis indicated that phospho-p70S6K1 levels of LI and 2×BCAA dietary intervention groups (i.e., CL, BR, and BL) was greater than the CR group ($P < 0.05$, CR vs. CL; $P < 0.01$, CR vs. BR and BL, respectively). Furthermore, total mTOR and p70S6K1 protein expression levels were not different between groups relative to internal protein (i.e., GAPDH) levels.

Mitochondrial biogenesis and oxidative phosphorylation

PGC-1 α protein expression levels demonstrated a noteworthy response pattern. Specifically, a significant crossover interaction was observed between IRON and BCAA ($F(1, 27) = 6.189$, $P = 0.019$; Fig. 4A) such that LI diets produced opposite effects depending upon dietary BCAA levels. A subtle, but greater level of plantaris PGC1 α was observed in rats consuming CL diet (i.e., 1×BCAA), but decreased among rats consuming 2×BCAA. Furthermore, post hoc analysis revealed that BL animals had significantly lower PGC-1 α protein expression compared to both CL and BR groups ($P = 0.007$, CL vs. BL; $P = 0.033$, BR vs. BL). Compared to CR animals, however, neither LI nor 2×BCAA dietary administration altered PGC-1 α expression level.

As shown with mitochondrial biogenesis (i.e., PGC-1 α), similar protein expression patterns were reflected in the results of mitochondrial oxidative capacity (COX-2 and CS; Fig. 4B and C). Again, significant crossover interactions between IRON and BCAA were observed for both COX-2 and CS protein expression ($P < 0.05$). Similar to PGC-1 α , COX-2 and CS protein expression levels were slightly greater in LI only fed animals (i.e., CL) than control animals (i.e., CR), but this effect was not observed when LI was combined with 2×BCAA. In addition, 2×BCAA only diet group (i.e., BR) also showed slightly greater CS protein levels in the aging muscle as compared to CR animals. Post hoc analysis revealed no

significant differences between any groups for COX-2 or CS expression. However, BL animals showed lower COX-2 levels as compared to the CL group, which approached statistical significance ($P = 0.051$). Hence, similar to the result for mitochondrial biogenesis, long-term co-administration of LI and 2×BCAA diminished the response of COX-2 and CS compared to effects imparted by LI and 2×BCAA administered individually.

Oxidative stress

Effects of LI and 2×BCAA diets on markers of antioxidant ability (antioxidant capacity and Total SOD) and oxidative injury (protein carbonyl, 3-NT, and 4-HNE) are shown in Fig. 5 and Fig. 6. Antioxidant capacity (Fig. 5A) and Total SOD activity (Fig. 5B) were not different between groups. In addition, protein carbonyl (Fig. 6A) and 3-NT levels (Fig. 6C) in the plantaris were shown to be similar between groups, and we also observed no significant difference between groups for 4-HNE (Fig. 6E).

Discussion

This study showed significant effects of LI and/or 2×BCAA on upregulating anabolic signaling in aging skeletal muscle of rats. In particular, aged animals fed 2×BCAA exhibited a lower iron status in skeletal muscle. Similar to previous findings (D'Antona et al. 2010; Arruda et al. 2013), LI and 2×BCAA diets did not alter body mass of old rats, and muscle mass values were not changed by the dietary treatments.

We investigated whether LI and 2×BCAA diets could decrease muscle iron status of aged rats. Notably, the LI diet did not affect the muscle iron level despite its ability to lower the liver iron concentration. Since the liver is crucial for body iron storage and regulation of systemic iron status, it is possible that it may be more responsive to LI diet administration than skeletal muscle. Although prolonged administration of a LI diet (10 mg iron/kg) significantly lowered the skeletal muscle iron concentration of adult rats (Arruda et al. 2013), the dietary efficacy was not observed among aged animals in the current study. It is feasible that the liver iron status was not lowered to the point that compromised tissue iron delivery. In such instance, the muscle would likely receive an adequate supply of iron from the circulation. However, without hematological measures of iron status we can only infer that iron delivery was adequate in our rats that received LI diets. In growing rats that had an initial adequate iron status, levels of various iron status indicators (e.g., transferrin saturation, serum iron, muscle cytochrome c) showed greater depletion over a 54-day period of iron deficiency when rats consumed 2 mg iron/kg vs. 6 mg iron/kg (Dallman et al. 1982). Thus, what seems like a small difference in iron intake resulted in measurable differences in the iron status. Changes in iron status also occurred prior to the depletion of liver iron stores. Irrespective of why the muscle non-heme iron level was unchanged among rats receiving LI diets the muscle iron status likely did not play a role in the increased p70S6 kinase anabolic signaling.

In contrast to the response of LI diet-treated animals, administration of 2×BCAA diets (i.e., alone or in combination with LI) led to a decrease in the muscle iron content. This is an interesting result in light of the finding that neither liver iron status nor muscle TfR1 protein expression was altered by 2×BCAA diets. The 2×BCAA-induced reduction in muscle iron

content may not have been sufficient to significantly alter the iron concentration, which could explain why the muscle TfR1 expression and oxidative stress were unaffected. However, it remains unclear as to why the 2×BCAA diets altered the iron status of the muscle, but not the liver. We conjecture that an upregulation in the protein synthesis pathway (i.e., p70S6K1) caused an increased need for iron in the BCAA metabolite-accelerated TCA cycle, or iron from the non-heme pool was diverted for heme synthesis.

It is well established that supplementation with BCAA can boost skeletal muscle anabolic signaling (D'Antona et al. 2010), even amongst elderly subjects (Guillet et al. 2004; Cuthbertson et al. 2005; Casperson et al. 2012; Zeanandin et al. 2012). Thus, the increased anabolic signaling observed in the 2×BCAA dietary groups was anticipated. Notably, this study revealed an uncoupled expression pattern between up-stream mTOR and down-stream p70S6K1. Specifically, phospho-p70S6K1 levels were increased despite unaltered expression of phospho-mTOR. A similar expression pattern was also identified in other studies. For example, while mTOR phosphorylation by BCAA was diminished by the aging process, p70S6K1 phosphorylation levels appeared to be comparable to the young controls (Guillet et al. 2004; Cuthbertson et al. 2005). Also, p70S6K1 phosphorylation can be mediated by an mTOR-independent mechanism such as c-Jun-N-terminal kinase (JNK), which leads to a conformational change in an inhibitory domain (Thr421 and Ser424) of p70S6K1 (Martin et al. 2014). Thus, simultaneous detection of elevated phospho-mTOR and phospho-p70S6K1 in aged muscle may not be imperative for an increased muscle protein synthesis to occur. Future studies are warranted to determine whether LI and/or BCAA-associated increase in anabolic signaling via these alternative pathways (e.g. JNK) would affect muscle protein synthetic rates, as well as muscle mass.

The rise in muscle iron concentration with age is associated with mitochondrial dysfunction, which is believed to be a causal factor in the development of sarcopenia. Contrary to our hypothesis, the LI and 2×BCAA dietary treatments showed no main effects on the protein markers of mitochondrial oxidative capacity in the aged rats. Administration of BCAA was shown previously to increase mitochondrial biogenesis in middle-aged mice (D'Antona et al. 2010) and C2C12 cells (Sun and Zemel 2009; Liang et al. 2014). Thus, it is unclear as to why the 2×BCAA diet did not increase PGC-1 α and markers of mitochondrial oxidative capacity (i.e., COX-2 and CS). It is possible that mitochondrial adaptation of skeletal muscle in response to the dietary treatments may be diminished by aging (Ljubicic et al. 2009). Otherwise, 4E-BP1, which is also a down-stream component of the mTOR signaling pathway, may be more related with mitochondrial biogenesis/function rather than p70S6K1 (Morita et al. 2013).

Since mTOR signaling may influence iron homeostasis and vice versa (Galvez et al. 2007; Ndong et al. 2009; Bayeva et al. 2012), we speculated that the combination of 2×BCAA with prolonged administration of LI diet would further elevate mTOR signaling in muscle via reductions in the muscle iron status. Since the LI diet was not successful in lowering the muscle iron status, it is not surprising that the combined diet did not produce additive effects. Interestingly, however, significant crossover-interactions between LI and 2×BCAA diets on markers of mitochondrial biogenesis and oxidative capacity were observed. The interactions suggest a potential beneficial effect of individual dietary treatments on

mitochondrial markers that may be attenuated when LI and 2×BCAA dietary interventions are combined. Nonetheless, additional work would be required to further investigate the potential effects of LI and 2×BCAA on the interference phenomenon.

The various measures we employed to assess oxidative stress showed no detectable difference between the groups. The lack of significant change in the muscle iron concentration may explain why none of the dietary interventions elicited an effect on the oxidative stress measures. Interestingly, while co-administration of LI and 2×BCAA led to significant decrease in mitochondrial biogenesis and oxidative capacity, the combined diet (BL) did not appear to exert toxic effects. Hence, it is feasible that the BL-inducible mitochondrial dysregulation is not linked with oxidative stress.

A few study limitations should be noted. First, because mature rats (i.e., 29 months old) maintained on a long-term (12 weeks) LI diet (10 mg iron/kg) did not display significant changes in hemoglobin (Arruda et al. 2013), we excluded the possibility that the LI diets (~6 mg iron/kg) would result in significant changes to hematological parameters and therefore they were not assessed in this study. Second, the non-heme iron measurement did not provide insight into possible dietary effects on iron distribution changes (e.g., cytosol vs. mitochondria) within the muscle. Third, it is unknown whether mTOR downstream substrates other than p70S6K1 (i.e., 4E-BP1) were altered by LI and 2×BCAA diet interventions.

In conclusion, within the confines of our experimental design, LI and/or 2×BCAA did not increase skeletal muscle mass. However, LI and 2×BCAA diets led to an increase in p70S6K1 phosphorylation in aging muscle of rats, and BCAA enriched diets (2×BCAA) lowered muscle iron status. Moreover, we observed a potential for individual LI or 2×BCAA dietary treatments to upregulate markers of mitochondrial biogenesis and oxidative capacity in muscle from aged rats. However, using these findings from muscle as the basis for recommendations for elderly individuals to consume less iron would be premature given the potential for development of anemia. While factors including chronic low-grade inflammation, poor diet, and medications such as aspirin can contribute to the development of anemia, the restriction of dietary iron in some individuals could exacerbate the problem (Fairweather-Tait et al. 2014). For future work, changes to the dietary administration strategy (e.g., longer period of administration or different diet composition), particularly under inflammatory conditions, could be utilized to further explore possible benefits of LI and BCAA diets to attenuate sarcopenia.

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A **B** **Fig. 1.**

Body mass and food consumption. (A) There was no significant difference in body mass between groups over 12 weeks of dietary intervention. (B) No significant difference was detected in food consumption between groups, except during the last week (i.e., week 12);

** $P < 0.001$, CR vs. BL. $n = 8$ per group.

Fig. 2.

Iron status was evaluated by non-heme iron concentration and content in plantaris muscle and liver. (A) Non-heme iron concentration revealed no significant difference between groups. (B) 2xBCAA (main effect) showed a lower total non-heme iron content in plantaris muscle compared to control groups, $*P < 0.05$, 2xBCAA vs. Control. (C) There was no significant difference in TfR1 protein levels between groups. A representative image of TfR1 normalized to β -Actin was shown. (D) A significant main effect of LI diet on non-heme iron concentration was observed in the liver, $*P < 0.05$, RI vs. LI. $n = 7-9$ per group.

Fig. 3. Anabolic signaling was assessed by mTOR and p70S6K1 pathway. (A) Phosphorylation-mTOR at Ser2448 was normalized to mTOR in plantaris muscle and there was no significant difference between groups. (B) Phosphorylation-p70S6K1 at Thr389 was normalized to p70S6K1 in plantaris muscle. There was a significant interaction between IRON and BCAA on p70S6K1 ($\dagger P < 0.05$) and post hoc analysis identified differences between CL/BR/BL vs. CR; $*P < 0.05$, CL vs. CR; $**P < 0.01$, BR vs. CR; $**P < 0.01$, BL vs. CR. (C) Representative images of Western blot for phosphorylation of both mTOR and p70S6K1. $n = 8-9$ per group.

Fig. 4. Mitochondrial biogenesis and capacity as assessed by PGC-1 α , COX-2, and CS protein expression. (A) A significant interaction between IRON and BCAA was identified for PGC-1 α protein expression level ($\dagger P < 0.05$). Post hoc analysis detected significant differences between CL/BR vs. BL; $** P < 0.01$, CL vs. BL; $* P < 0.05$, BR vs. BL. (B) There was a significant interaction between IRON and BCAA for COX-2 ($\dagger P < 0.05$). (C) A significant interaction was observed between IRON and BCAA on CS ($\dagger P < 0.05$). (D) Representative images of Western blot for PGC1- α , CS, and COX-2 are shown. $n = 8-9$ per group.

Fig. 5. Muscle antioxidative ability was assessed by antioxidant capacity and Total SOD activity. (A) No significant difference between groups ($P > 0.05$) for antioxidant capacity was observed. (B) Total SOD activity was similar between groups ($P > 0.05$). $n = 7-8$ per group. A.U. = Activity units.

Fig. 6.

Muscle oxidative injury was determined by protein carbonyl, 3-nitrotyrosine (NT) and 4-HNE protein levels. (A, C, and E) No significant effect of LI and/or 2×BCAA diets was observed in protein carbonyl, 3-NT, and 4-HNE levels in the aging skeletal muscle of rats, respectively. (B, D and F) Images of protein carbonyl, 3-NT and 4-HNE were obtained from the whole cell lysates of plantaris muscles, respectively. Arrows indicate prominent bands

that were used for evaluating protein carbonyl, 3-NT and 4-HNE, respectively. n = 8–9 per group.

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Table 1

Diet composition (g/Kg).

	Control Diets		2×BCAA Diets	
	Control (Regular iron) (TD.94048)	Iron-deficient (TD.140351)	2×BCAA (Regular iron) (TD.140352)	Iron-deficient 2×BCAA (TD.140353)
Casein	140.0	140.0	140.0	140.0
Iron (mg/kg)	36 – 46	0 – 6	36 – 42	0 – 6
L-Cysteine	1.8	1.8	1.5	1.5
L-Isoleucine	7.0	7.0	15.0	15.0
L-Leucine	11.2	11.2	23.2	23.2
L-Valine	8.4	8.4	17.4	17.4
Corn Starch	436.542	436.542	436.542	436.542
Maltodextrin	155.0	155.0	155.0	155.0
Sucrose	100.0	100.0	100.0	100.0
Soybean Oil	40.0	40.0	40.0	40.0
Cellulose	50.0	50.0 (microcrystalline)	50.0	50.0 (microcrystalline)
Mineral Mix (AIN-93M-MX)	35.0	35.0 (Fe Deficient)	35.0	35.0 (Fe Deficient)
Vitamin Mix (AIN-93M-VX)	10.0	10.0	10.0	10.0
Choline Bitartrate	2.5	2.5	2.5	2.5
TBHQ, Antioxidant	0.008	0.008	0.008	0.008
Food Color	–	0.15 (Red)	0.15 (Green)	0.15 (Yellow)

Table 2

Muscle mass (grams).

	Control		2×BCAA	
	RI	LI	RI	LI
Plantaris	0.63 ± 0.03	0.66 ± 0.02	0.63 ± 0.02	0.63 ± 0.02
Soleus	0.27 ± 0.01	0.28 ± 0.01	0.26 ± 0.01	0.29 ± 0.02
Extensor digitorum longus	0.27 ± 0.01	0.28 ± 0.01	0.27 ± 0.01	0.28 ± 0.01
Gastrocnemius	2.91 ± 0.13	3.07 ± 0.09	3.01 ± 0.09	2.98 ± 0.12
Tibialis anterior	1.12 ± 0.04	1.18 ± 0.02	1.10 ± 0.06	1.10 ± 0.05
Diaphragm	2.25 ± 0.11	2.19 ± 0.21	1.97 ± 0.19	1.98 ± 0.10

Values are mean ± SE. RI, Regular iron diet; LI, Low iron diet.