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L-Leucine Increases Skeletal Muscle IGF-1 but Does Not Differentially Increase Akt/mTORC1 Signaling and Serum IGF-1 Compared to Ursolic Acid in Response to Resistance Exercise in Resistance-Trained Men

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Key words: cell signaling, muscle protein synthesis, branched-chain amino acid, resistance exercise

Objective: Ursolic acid administration following resistance exercise increases mammalian target of rapamycin complex 1 (mTORC1) activity and skeletal muscle IGF-1 concentration in murines in a manner similar to L-leucine yet remains unexamined in humans. This study examined serum and skeletal muscle insulin-like growth factor-1 (IGF-1) and Akt/mTORC1 signaling activity following ingestion of either ursolic acid or L-leucine immediately after resistance exercise.

Methods: Nine resistance-trained men performed 3 lower-body resistance exercise sessions involving 4 sets of 8–10 repetitions at 75%–80% one repetition maximum (1-RM) on the angled leg press and knee extension exercises. Immediately following each session, participants orally ingested 3 g cellulose placebo (PLC), L-leucine (LEU), or ursolic acid (UA). Blood samples were obtained pre-exercise and at 0.5, 2, and 6 hours postexercise. Muscle biopsies were obtained pre-exercise and at 2 and 6 hours postexercise.

Results: Plasma leucine increased in LEU at 2 hours postexercise compared to PLC ($p = 0.04$). Plasma ursolic acid increased in UA at 2 h and 6 hours postexercise compared to PLC and LEU ($p < 0.003$). No significant differences were observed for serum insulin ($p = 0.98$) and IGF-1 ($p = 0.99$) or skeletal muscle IGF-1 receptor (IGF-1R; $p = 0.84$), Akt ($p = 0.55$), mTOR ($p = 0.09$), and p70S6K ($p = 0.98$). Skeletal muscle IGF-1 was significantly increased in LEU at 2 hours postexercise ($p = 0.03$) and 6 hours postexercise ($p = 0.04$) compared to PLC and UA.

Conclusion: Three grams of L-leucine and ursolic acid had no effect on Akt/mTORC1 signaling or serum insulin or IGF-1; however, L-leucine increased skeletal muscle IGF-1 concentration in resistance-trained men.

INTRODUCTION

Skeletal muscle is the largest organ in the human body and integral in the regulation of locomotion, exercise performance, disease prevention, and metabolic homeostasis. Resistance exercise has been shown to elicit elevations in muscle protein synthesis (MPS), and the mammalian/mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway appears to be the primary regulator of the anabolic response [1]. Additionally, systemic growth factors including insulin-like growth factor-1 (IGF-1) regulate mTORC1 through interaction with its trans-membrane receptor (IGF-1R). Increased mTORC1 activity leads to the phosphorylation of its downstream targets including eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and 70-kDa S6 protein kinase (P70S6K), which promotes cap-dependent translation initiation [2].

Various nutritional interventions, amino acids in particular, appear to augment the anabolic response to resistance exercise [1,3]. The branched-chain amino acid L-leucine appears to be the primary nutritional regulator of MPS via direct activation of the nutrient-sensitive mTORC1 signaling pathway [4]. Though the exact mechanism remains to be elucidated, increased leucine concentrations in skeletal muscle apparently translocate mTORC1 to the surface of the lysosomal

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membrane, which contains the mTORC1 activator Ras homologue enriched in the brain (Rheb) [5].

Recent research has identified ursolic acid as a potential natural anabolic compound, stimulating hypertrophy while also attenuating atrophy [6]. Ursolic acid (3β-hydroxy-12-urs-12-en-28-oic acid) is a pentacyclic triterpenoid carboxylic acid found in herbs and fruits, such as Fuji apple peel, which may contain as much as 65 mg of ursolic acid [7,8]. Recently, the therapeutic effects of ursolic acid–containing plants, including anticancer, antiviral, anti-inflammatory, antioxidant, and hepatoprotective effects, have been confirmed in the scientific literature [8]. Mice fed an ursolic acid–enriched diet for 16 weeks exhibited increased skeletal muscle Akt activity. In the same study, C2C12 myotubes treated with ursolic acid were shown to enhance ligand-dependent activation of the insulin and IGF-1 receptors. Furthermore, mRNA expression of atrogin-1 and MuRF1 were repressed, whereas IGF-1 mRNA was induced in mice fed an ursolic acid–enriched diet for 5 weeks [6]. When ursolic acid was injected into rats following exercise, mTORC1 activity, as measured by phosphorylation of state of the proline-rich Akt substrate of 40 kDa ($PRAS40^{Thr246}$), was attenuated compared to exercise-only rats and skeletal muscle IGF-1 was increased as compared to exercise alone [9].

Recent research has been investigating L-leucine for its role in increasing MPS, both on its own and with coingestion of additional amino acids and carbohydrates [10–14]. Both L-leucine and ursolic acid are reported to induce signaling through mTORC1; however, the impact of ursolic acid appears to be upstream of mTORC1 [6,9]. To date, there are no data in humans to substantiate the molecular mechanism(s) through which ursolic acid may increase MPS and subsequent resistance exercise-mediated hypertrophy.

Since previous research has demonstrated ursolic acid to activate mTORC1 signaling and elicit an anabolic response [6,9], the exercise/sports nutrition industry is marketing ursolic acid as an impactful means of augmenting muscle mass when ingested in conjunction with resistance training. Moreover, ursolic acid is also being marketed as being superior to L-leucine in its anabolic capacity. Because there are no data currently available to substantiate these claims, the purpose of the current study was to compare the effects of orally administered ursolic acid and L-leucine immediately after resistance exercise on serum insulin and IGF-1, skeletal muscle IGF-1, and signaling proteins specific to the Akt/mTORC1 pathway (IGF-1R, Akt, mTOR, p70S6K).

METHODS AND PROCEDURES

Experimental Design

The study was a randomized, 3-condition crossover design in which participants received a separate nutritional supplement immediately following an identical bout of resistance exercise as outlined in Fig. 1. One session constituted the placebo session and the other 2 were the experimental sessions involving either ursolic acid or L-leucine. Muscle samples were collected pre-exercise and 2 and 6 hours postexercise. Blood samples were collected at identical time points with an additional sample collected 0.5 hours postexercise.

Participants

Nine apparently healthy, resistance-trained men (22.8 ± 4.4 years, 174.7 ± 6.7 cm, 83.5 ± 19.0 kg) volunteered to serve as study participants. Inclusion criteria required participants to resistance train at least thrice weekly for a minimum of one year prior to the onset of the study. Based on self-report from each participant, the average resistance training age for all 9 participants was 8.1 ± 2.9 years. Twelve participants were initially recruited for the study, completed informed consent forms, and participated in an initial familiarization session. However, only 9 completed the study, because 2 participants became ill due to circumstances unrelated to the study and one sustained an orthopedic injury unrelated to the

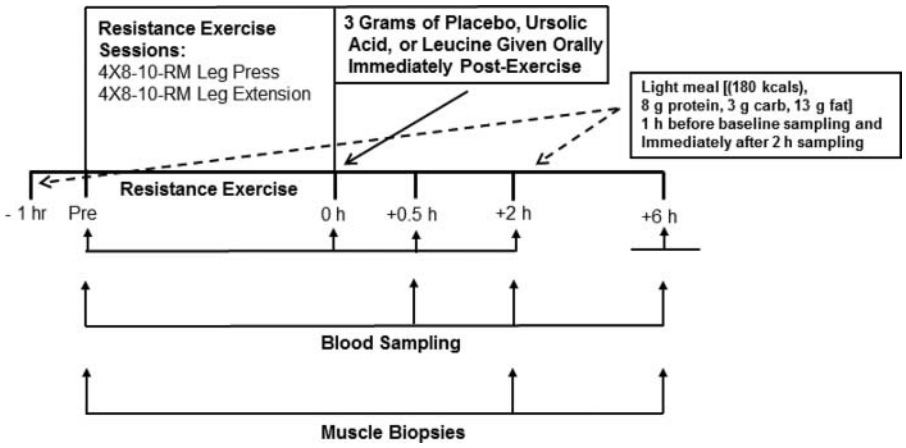

Fig. 1. Illustration of the experimental protocol for the testing sessions during the course of the study.

study. Only participants considered low risk for cardiovascular disease and with no contraindications to exercise as outlined by the American College of Sports Medicine and who had not consumed any nutritional supplements (excluding multivitamins) one month prior to the study were allowed to participate. Approval to conduct the study was granted by the Institutional Review Board for Human Subjects of Baylor University. Additionally, all experimental procedures involved in the study conformed to the ethical consideration of the Declaration of Helsinki.

Familiarization Session

After reporting to the Exercise and Nutritional Biochemistry Lab (EBNL), participants who met the entry criteria were familiarized to the study protocol via a verbal and written explanation outlining the study design and then signed university-approved informed consent documents. Participants completed a medical history questionnaire and underwent assessments for body composition and muscle strength assessments for the 2 lower-body exercises involved in the study.

Body Composition Testing

During the familiarization session, total body mass (kg) was determined on a standard dual-beam balance scale (Detecto, Bridgeview, IL). Total body water was determined with bioelectrical spectroscopy (ImpediMed Ltd., Pinkenba, QLD, Australia). Fat mass and fat-free mass were determined using dual-energy x-ray absorptiometry (Hologic Discovery Series W, Waltham, MA). Quality control calibration procedures were performed on a spine phantom prior to each participant. Previous studies in our lab have shown the accuracy of dual-energy x-ray absorptiometry for body composition to be ±3.7% as assessed by direct comparison with hydrodensitometry and scale weight.

Muscle Strength Assessments

In order to determine muscular strength, participants performed one-repetition maximum (1-RM) tests on angled leg press (Nebula, Versailles, OH) and knee extension (Cybex, Medway, MA) exercises during the familiarization session based on previous studies [15,16]. Participants completed a standardized warmup consisting of 5 to 10 repetitions at approximately 150% and 50% of their total body mass for the angled leg press and knee extension, respectively. The participant rested for 1 minute and then completed 3 to 5 repetitions at approximately 175% and 75% of his total body mass. The weight was then increased conservatively and the participant attempted to lift the weight for one repetition. If the lift was successful, the participant rested for 2 minutes before attempting the next weight increment. This procedure continued until the participant failed to complete the lift. The 1-RM was recorded as the maximum weight that the participant was able to lift for one repetition. Once the 1-RM for each leg was determined, participants were asked to perform and practice 2 sets with the proposed resistance for the exercise sessions without muscle sampling to familiarize them with the protocol and to ensure that they were able to complete the protocol before being formally admitted to the study.

Dietary Analysis

Participants were required to record their dietary intake for 4 days prior to each of the 3resistance exercise sessions and asked not to change their dietary habits during the course of the study. The dietary recalls were evaluated with the Food Processor dietary assessment software program (EHSA Research, Salem, OR) to determine the average daily macronutrient consumption of fat, carbohydrate, and protein in the diet for the duration of the study.

Resistance Exercise Session Protocol

Participants were instructed to refrain from resistance exercise during the course of the study and to avoid any form of exercise for 48 hours prior to each exercise session prior to report to the EBNL. Three resistance exercise sessions occurred in conjunction with the ingestion of each of 3 supplements and were performed 7–10 days apart. Participants reported to the lab for each exercise session after an 8-hour overnight fast. However, in an attempt to help prevent participants beginning each exercise session in a catabolic scenario, upon reporting to the lab participants were provided a light snack (Atkins Advantage caramel chocolate nut roll; 180 kcal, 13 g total fat, 8 g protein, 3 g carbohydrate) 1 hour before commencing the exercise sessions. To further help avoid a catabolic scenario and also deter hunger pangs until after the 6-hour sampling point, another Atkins Advantage caramel chocolate nut roll was provided immediately following the 2-hour sampling point. During each of the 3 resistance exercise sessions, participants performed 4 sets of 8–10 repetitions with 75%–80% of the 1-RM on the angled leg press and knee extension exercises [16]. In all cases, 2 minutes of rest separated sets and exercises. All exercise sessions were supervised by study personnel.

Nutrient Supplementation Protocol

In a randomized, double-blind fashion, one of 3 supplements was orally ingested in capsule form immediately after the completion of each exercise session. Within 12 gelatin capsules, all of the same size, shape, and color, the placebo supplement (PLC) consisted of 3 g of cellulose (Nutricology, Alameda, CA) and the 2 experimental supplements consisted of 3 g (0.036 g/kg equivalent) of ursolic acid (UA; Smart Powders, Graham, NC) and L-leucine (LEU; Source Naturals, Santa Cruz, CA). The 3 g dose of

ursolic acid was extrapolated from a 200 mg intraperitoneal injection dose in mice through normalization to body surface area, which equates to 0.016 g/kg [6,17]. The remaining treatment condition doses were matched to the 3 g ursolic acid dose. To assure the purity of each supplement, independent raw materials analyses were obtained. L-Leucine, it was certified to contain L-leucine at 99% purity, confirmed by high-performance liquid chromatography analysis (Threshold Enterprises, Ltd., Scotts Valley, CA). Ursolic acid was certified to ursolic acid extract at a purity of 98%, confirmed by liquid chromatography–mass spectrometry (Advanced Analytical Testing Service, Inc. Ontario, Canada).

Venous Blood Sampling and Muscle Biopsies

Venous blood was collected from the antecubital vein using a Vacutainer apparatus (Becton Dickson, Franklin Lakes, NJ) using standard phlebotomy procedures. Sample collection tubes were allowed to stand at room temperature for 10 minutes and then centrifuged. Serum and plasma were separated and transferred to sterile cryovials and stored at −80°C for later analysis. At each resistance exercise session, blood samples were obtained immediately before and 0.5, 2, and 6 hours postexercise.

Precutaneous muscle biopsies (15–20 mg) were obtained from the middle portion of the vastus lateralis muscle at the midpoint between the patella and the greater trochanter of the femur at a depth between 1 and 2 cm as previously described [15]. The initial leg biopsied was selected by a web-based random number generator (www.random.org). The biopsy area was shaved clean of leg hair, washed with antiseptic soap, and cleaned with rubbing alcohol. In addition, the biopsy site was further cleansed by swabbing the area with betadine. A small area of the clean skin approximately 2 cm in diameter was anesthetized with a 1.0 ml subcutaneous injection of lidocaine. Once anesthetized, a 16-gauge fine-needle aspiration biopsy instrument (Tru-Core I biopsy instrument, Medical Device Technologies, Gainesville, FL) was inserted into the skin at an approximate depth of 1 cm to extract the muscle sample. After removal, adipose tissue was trimmed from the muscle specimens and were immediately frozen in liquid nitrogen and stored at −80°C for later analysis. After the initial biopsy, the subsequent biopsy attempts were made to extract tissue from approximately the same location as the initial biopsy by using the pre-biopsy scar, depth markings on the needle, and a puncture site that was made approximately 0.5 cm to the former from medial to lateral. The opposite leg was used for the subsequent exercise session, and for the final exercise session the leg was again randomly determined as with the initial leg. At each resistance exercise session, muscle samples were obtained before and at 2 and 6 hours postexercise.

Plasma Leucine Analysis

Plasma leucine concentrations were determined spectrophotometrically from a branched chain amino acid assay kit (Sigma-Aldrich, St. Louis, MO). All samples were analyzed in duplicate in one run to avoid batch effects and the assay was performed at 450 nm wavelength with a microplate reader (iMark, Bio-Rad, Hercules, CA) against a leucine standard, and plasma leucine concentrations were determined using data reduction software (Microplate Manager, Bio-Rad). The overall intra-assay percentage coefficient of variation was 5.71%.

Plasma Ursolic Acid Analysis

Ursolic acid was extracted from plasma based on previous methodology [18]. Briefly, 100 μL aliquots of sample were spiked with 100 μL of the internal standard, glycyrrhetic acid (Sigma-Aldrich) at a working solution (50 ng/mL). One milliliter of ethyl acetate was added, and samples were vortex mixed for 2 minutes followed by centrifugation at 2000 × g for 10 minutes. Two hundred microliters of the upper organic layer was transferred and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 200 μL of acetonitrile, vortex mixed for 30 seconds, and centrifuged at 12,000 × g for 5 minutes. The supernatant was transferred to an injection vial for ultraperformance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) analysis.

Extracts were analyzed by UPLC-MS/MS analysis on an Acquity UPLC system coupled to a Xevo TQ-S tandem quadrupole mass spectrometer (Waters Corp., Milford, MA). Final extracts were injected (10 μL) into the UPLC system and separated on a 15 cm × 2.1 mm (5 μm) Eclipse Plus-C8 column (Agilent Technologies, Palo Alto, CA) at a constant flow rate of 500 μL/min. The mobile phase consisted of an 80:20 ratio of acetonitrile and water. The isocratically eluted analytes were introduced into the mass spectrometer using negative electrospray ionization. Electrospray source conditions were as follows: capillary voltage 2.5 kV; source block temperature 150°C; desolvation gas (N_2) temperature 600°C at a flow of 900 L/h; cone gas flow 150 L/h; optimized cone voltages 2 and 94 V for the ursolic acid (Sigma-Aldrich) and the internal standard, respectively. The argon collision gas pressure was maintained at 3.4×10^{-3} mbar in the collision cell. Mass spectrometric detection was performed using multiple reaction monitoring mode. The following multiple reaction monitoring transitions were monitored for both detection and quantitation purposes: m/z 455 > 455 for ursolic acid and m/z 469 > 409 for the internal standard at optimized collision energies of 46 and 44 eV, respectively.

Analyte concentrations in both controls and samples were determined using matrix-matched calibration as previously described [18]. Stock working solutions were prepared at 5, 10, 50, 100, and 500 ng/mL concentrations in acetonitrile. Calibration standards were prepared by

evaporating 100 μL of each working solution under a stream of nitrogen in a disposable Eppendorf tube (Sigma Aldrich, St. Louis, MO). One hundred microliters of blank human plasma was subsequently added to a microfuge tube, votex mixed for 30 seconds, and subjected to the extraction methodology described above. Continuous calibration verification (CCV) and matrix spike samples were prepared at the 100 and 50 ng/mL levels using 100 μL of either blank plasma or a dosed plasma sample. Ursolic acid concentrations were determined using an internal standard procedure. Response factor was calculated by dividing the peak area for ursolic acid by the peak area for the internal standard, and a calibration curve was prepared by plotting a linear regression ($R^2 > 0.999$) of the analyte response versus the analyte concentration. During UPLC-MS/MS analysis, calibration was monitored through use of blanks and CCV standards with an acceptability criterion of $\pm$15%. In a given run, one blank, one CCV sample, and one matrix spike/matrix spike duplicate pair were inserted between every 12 samples. Recovery of spiked amounts (criteria 80%–120%) as well as matrix spike/matrix spike duplicate pair relative percentage difference (criteria 15%) were evaluated for quality assurance purposes.

Serum Insulin and IGF-1 Analysis

Serum samples were analyzed using commercially available enzyme-linked immunoabsorbent assays (ELISA) kits for insulin and IGF-1 (Alpha Diagnostic Laboratories, San Antonio, TX) as previously described [19–22]. According to the manufacturer, the sensitivity for these assays is 0.5 μIU/ml and 0.01 ng/mL, respectively, for insulin and IGF-1. Standard curves were generated using control peptides. All samples were analyzed in duplicate in one run to avoid batch effects, and the assays were performed at 450 nm wavelength with a microplate reader (iMark). Insulin and IGF-1 concentrations were determined using data reduction software (Microplate Manager). The overall intra-assay percentage coefficient of variation was 6.92% and 7.34% for insulin and IGF-1, respectively.

Cell Extraction and Total Protein Content

Approximately 15 mg of each muscle sample was homogenized using a commercial cell extraction buffer (Biosource, Camarillo, CA) and a tissue homogenizer. The cell extraction buffer was supplemented with phenylmethanesulphonylflouride and a protease inhibitor cocktail (Sigma Chemical Company, St. Louis, MO) with broad specificity for the inhibition of serine, cysteine, and metalloproteases [16,21,23]. Total protein content was determined spectrophotometrically at a wavelength of 595 nm and using bovine serum albumin as the standard [16,21,23]. All samples were analyzed in duplicate in one run to avoid batch effects; protein concentrations were determined using data reduction software (Microplate Manager) and total protein content was expressed relative to muscle wet weight.

Skeletal Muscle IGF-1 and mTORC1 Signaling Pathway Intermediates

From muscle tissue samples obtained at the 3 resistance exercise sessions, IGF-1 was determined using a commercially available ELISA kit (Alpha Diagnostic Laboratories). The phosphorylated levels of IGF-1R^{Tyr1131}, Akt^{Thr308}, mTOR^{Ser2448}, and P70S6K^{Thr389} were determined by phosphoprotein ELISA kits (Cell Signaling Technology, Danvers, MA) as we have previously demonstrated [21,23]. All samples were analyzed at 450 nm in duplicate in one run to avoid batch effects and concentrations determined with data reduction software (Microplate Manager). The overall intra-assay percentage coefficients of variation were 6.34%, 8.58%, 7.04%, 9.36%, and 7.96%, respectively, for IGF-1, IGF-1R, Akt, mTOR, and P70S6K.

Statistical Analyses

Statistical analyses were performed by utilizing separate 3 $\times$ 4 (Condition $\times$ Time) factorial analyses of variance (ANOVA) with repeated measures for blood variables. For muscle variables, a 3 $\times$ 3 (Condition $\times$ Time) factorial ANOVA was employed. Further analyses of the main effects were performed by separate one-way ANOVAs. Significant between-condition differences were determined through Tukey's post hoc test. In addition, area under the curve (AUC) was determined for serum IGF-1 and plasma leucine and ursolic acid using a trapezoidal method and analyzed using one-way ANOVA and Tukey's post hoc tests. An *a priori* power calculation showed that 10 participants per condition was adequate to detect a significant difference between conditions in the marker of Akt in response to resistance exercise, given a type I error rate of 0.05 and a power of 0.80. The index of effect size utilized was partial eta squared (η^2), which estimates the proportion of variance in the dependent variable that can be explained by the independent variable. Partial eta squared effect sizes were as follows: weak = 0.17, medium = 0.24, strong = 0.51, very strong = 0.70 [24]. All statistical procedures were performed using SPSS 20.0 software (IBM, New York, NY) and an alpha level $\leq$ 0.05 was adopted throughout.

RESULTS

Anthropometrics, 1-RM, Resistance Exercise Volume, and Dietary Analysis

Physical, anthropometric, and 1-RM data describing the participants are presented in Table 1. Resistance exercise

Table 1. Physical, Anthropometric, and 1-RM Data for Participants at the Onset of the Study

Variable	Mean ± SD
Age (years)	22 ± 4.41
Height (cm)	174 ± 6.77
Body mass (kg)	83 ± 19.04
Lean mass (kg)	65 ± 11.79
Fat mass (kg)	16 ± 9.73
Leg press 1-RM (kg)	386 ± 113.15
Knee extension 1-RM (kg)	74 ± 14.37

1-RM = one repetition maximum.

volumes were 30,821 ± 9002, 30,765 ± 9007, and 30,963 ± 9501 kg for PLC, LEU, and UA, respectively. No significant differences were observed in resistance exercise volume between trials ($p > 0.05$).

The caloric and macronutrient content prior to each condition are provided in Table 2. No significant differences were observed for total kilocalories, fat, carbohydrate, or protein intake per day between the 3 trials ($p > 0.05$).

Plasma Leucine Concentration

For plasma leucine, a significant Time × Condition interaction was observed ($p = 0.028$; $\eta^2 = 0.65$). In addition, there wer significant main effects for time observed ($p = 0.035$; $\eta^2 = 0.67$) and condition ($p = 0.044$; $\eta^2 = 0.63$). Post hoc analyses revealed a significant difference in plasma leucine concentration in LEU compared to PLC and UA that occurred 2 hours postexercise (Fig. 2). The inset graph represents AUC for plasma leucine and also revealed a significant difference for the LEU supplement ($p = 0.025$).

Plasma Ursolic Acid Concentration

Plasma ursolic acid revealed a significant Time × Condition interaction ($p = 0.003$, $\eta^2 = 0.87$) and significant main effects for time ($p = 0.015$, $\eta^2 = 0.73$) and condition ($p = 0.001$, $\eta^2 = 0.974$). Post hoc analyses revealed that UA was significantly increased at 2 and 6 hours postexercise compared to PLC and LEU. In addition, AUC analysis showed a significant increase for UA compared to PLC and LEU for plasma ursolic acid ($p = 0.001$; $\eta^2 = 0.975$; Fig. 3).

Serum Insulin and IGF-1 Concentration

Serum insulin revealed no significant main effects for time ($p = 0.363$; $\eta^2 = 0.11$) or condition ($p = 0.620$; $\eta^2 = 0.01$) or the Time × Condition interaction ($p = 0.98$; $\eta^2 = 0.009$). In addition, AUC analysis showed no significant differences between PLC, LEU, and UA for serum insulin ($p = 0.85$; $\eta^2 = 0.01$; Fig. 4).

Serum IGF-1 revealed no significant main effects for time ($p = 0.89$; $\eta^2 = 0.006$) or condition ($p = 0.36$; $\eta^2 = 0.02$) or the Time × Condition interaction ($p = 0.99$; $\eta^2 = 0.003$). In addition, AUC analysis showed no significant differences between PLC, LEU, and UA for serum IGF-1 ($p = 0.75$; $\eta^2 = 0.02$; Fig. 5).

Skeletal Muscle IGF-1

Skeletal muscle IGF-1 levels resulted in a significant Time × Condition interaction ($p = 0.041$; $\eta^2 = 0.51$). In addition, significant main effects for time ($p = 0.05$; $\eta^2 = 0.27$) and condition ($p = 0.001$; $\eta^2 = 0.81$) were observed. Post hoc analyses revealed that LEU was significantly increased at 2 and 6 hours postexercise compared to PLC and UA (Fig. 6).

Skeletal Muscle Akt/mTORC1 Signaling Intermediates

Phosphorylated- IGF-1R^{Tyr1131} revealed no statistically significant main effects for time ($p = 0.86$, $\eta^2 = 0.004$) or condition ($p = 0.068$; $\eta^2 = 0.072$) or the Time × Condition interaction ($p = 0.83$; $\eta^2 = 0.02$; Fig. 7).

The levels of phosphorylated-Akt^{Thr308} revealed no statistically significant main effects for time ($p = 0.92$; $\eta^2 = 0.002$) or condition ($p = 0.83$; $\eta^2 = 0.005$) or the Time × Condition interaction ($p = 0.55$; $\eta^2 = 0.041$; Fig. 8).

Phosphorylated-mTOR^{2448} revealed no statistically significant main effects for time ($p = 0.52$; $\eta^2 = 0.018$) or condition ($p = 0.41$; $\eta^2 = 0.025$) or the Time × Condition interaction ($p = 0.18$; $\eta^2 = 0.083$; Fig. 9).

For phosphorylated-p70S6K^{Thr389}, there were no statistically significant main effects for time ($p = 0.94$; $\eta^2 = 0.001$)

Table 2. Four-Day Dietary Intakes of Participants Prior to Each of the 3 Testing Sessions[a]

Variable	PLC	LEU	UA	*p*-Value
Calories (kcals/day)	2030.6 ± 709.6	2175.9 ± 916.5	1879.0 ± 731.1	0.73
Fat (g/day)	88.2 ± 51.3	92.0 ± 52.4	71.4 ± 31.5	0.62
Carbohydrate (g/day)	229.9 ± 104.1	214.0 ± 82	201.1 ± 124.0	0.84
Protein (g/day)	106.9 ± 33.3	117.6 ± 80.3	112.3 ± 53.4	0.93

PLC = placebo, LEU = leucine, UA = ursolic acid.

[a]Data are presented as mean ± SD. There were no significant differences for any of the dietary variables at the 3 testing sessions ($p > 0.05$).

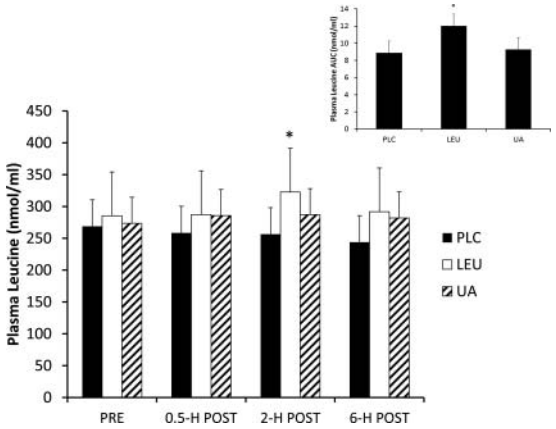

Fig. 2. Plasma leucine concentrations in response to PLC, LEU, and UA supplementation and resistance exercise. *A significant increase was observed at 2 hours postexercise for LEU compared to PLC and UA ($p = 0.044$). The inset graph represents the AUC for plasma leucine and also revealed a significant difference for the LEU supplement ($p = 0.02$). Data are presented as means ± SD.

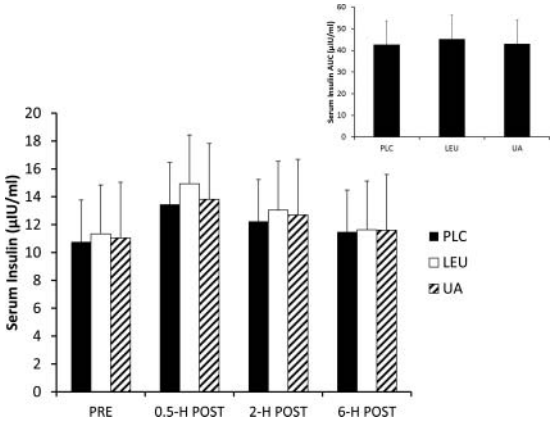

Fig. 4. Serum insulin concentrations in response to PLC, LEU, and UA supplementation and resistance exercise. Three were no significant increases at any time point and between exercise sessions ($p > 0.05$). The inset graph represents the AUC for serum insulin and also revealed no significant differences ($p > 0.05$). Data are presented as means ± SD.

or condition ($p = 0.56$; $\eta^2 = 0.016$) or the Time × Condition interaction ($p = 0.98$; $\eta^2 = 0.006$; Fig. 10).

DISCUSSION

The present study investigated the effect of ursolic acid and L-leucine on signaling proteins within the Akt/mTORC1 pathway following a single bout of resistance exercise. A secondary objective was to determine whether ursolic acid and L-leucine had any differential effects on serum and muscle IGF-1 concentrations. At the 3 g dose provided, we observed no supplement-induced increases in the phosphorylation state of IGF-1R, Akt, mTOR, and p70S6K for either supplement. Additionally, supplementation did not induce increases in serum IGF-1; however, skeletal muscle IGF-1 concentrations were significantly increased at 2 and 6 hours postexercise in response to the LEU condition.

Plasma leucine concentrations were greater at 2 hours postexercise in the LEU condition compared to the PLC and UA conditions. It has been shown that an oral 3.42 g dose of leucine caused a peak in plasma leucine concentrations at 30

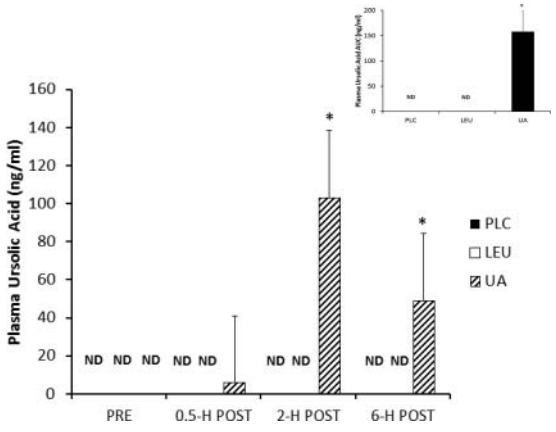

Fig. 3. Plasma ursolic acid concentrations in response to PLC, LEU, and UA supplementation and resistance exercise. *A significant increase was observed at 2 and 6 hours postexercise for UA compared to PLC and LEU ($p = 0.003$). ND = not detectable. For example, no ursolic acid was detectable at pre-exercise prior to ingesting all 3 supplements, and at 0.5, 2, and 6 hours postexercise, ursolic acid was not detectable after ingesting the PLC and LEU supplements. The inset graph represents the AUC for plasma ursolic acid and also revealed a significant difference for the UA supplement ($p = 0.001$). Data are presented as means ± SD.

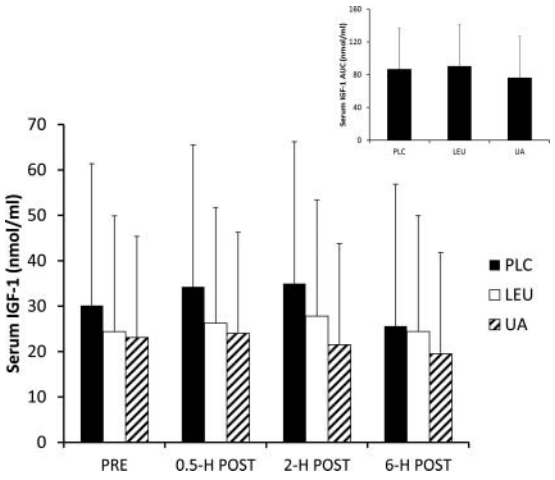

Fig. 5. Serum IGF-1 concentrations in response to PLC, LEU, and UA supplementation and resistance exercise. There were no significant increases at any time point and between exercise sessions ($p > 0.05$). The inset graph represents the AUC for serum IGF-1 and also revealed no significant differences ($p > 0.05$). Data are presented as means ± SD.

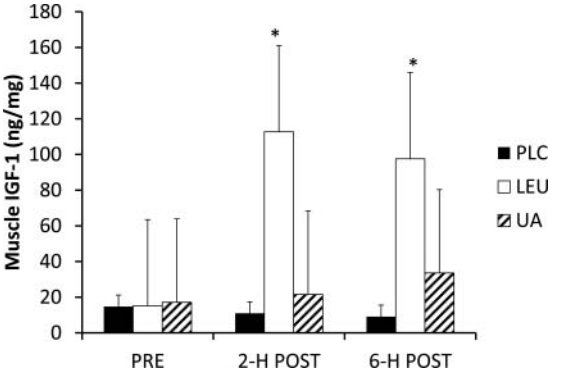

Fig. 6. Skeletal muscle IGF-1 peptide concentrations in response to PLC, LEU, and UA supplementation and resistance exercise. *A significant increase was observed at 2 and 6 hours postexercise for LEU compared to PLC and UA ($p = 0.041$). Data are presented as means ± SD.

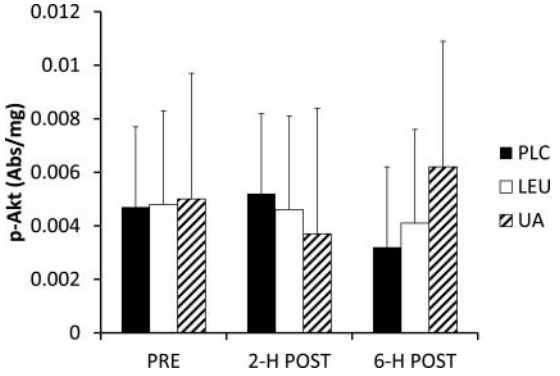

Fig. 8. Skeletal muscle concentrations of phosphorylated Akt in response to PLC, LEU, and UA supplementation and resistance exercise. There were no significant increases at any time point and between exercise sessions ($p > 0.05$). Data are presented as means ± SD.

minutes postingestion. Plasma leucine was still significantly elevated at 60 minutes postingestion and, though not statistically significant, was still elevated above baseline at 120 minutes postingestion [14]. Previous work has demonstrated a dose–response effect for leucine relative to its bioavailability in humans; increasing the dose from 1.85 to 3.50 g in healthy participants resulted in higher arterial leucine concentrations [13].

Ursolic acid is a lipophilic compound, thereby limiting the bioavailability in the body [25]. Ursolic acid is poorly absorbed in the gastrointestinal tract and may undergo hepatic first-pass metabolism [26,27]. In humans, pharmacokinetic data have shown that ursolic acid intravenously delivered by nano-liposomes at a dose of 98 mg/m^{-2} produced a peak concentration of 3404 ng/ml at 4 hours following intravenous infusion [18]. In the present study, participants orally ingested 3 g of ursolic acid at an average relative dose of 35.93 mg/kg^{-1}. Though we did not have a 4-hour sampling point as in Xia et al.'s study [18], based on our sampling points we observed the average peak concentration of ursolic acid to be 103 ng/ml, which occurred 2 hours postingestion. In addition, the concentration of ursolic acid, albeit lower, was still significantly elevated at 6 hours postingestion. Similarly, a previous study in which participants orally ingested 32 mg of ursolic acid reported a peak plasma ursolic acid concentration of 27.85 μg/L^{-1} that occurred 1.67 hours following ursolic acid ingestion [27]. Our data indicate that ursolic acid is bioavailable over the course of a 6-hour period yet is unable to augment serum and muscle IGF-1 concentrations and the activity of signaling proteins in the Akt/mTORC1 pathway.

IGF-1 is a known humoral myogenic activator through binding and subsequent activation of the IGF-1R [28]. In the current study, there was no overall indication that serum IGF-1 was affected by resistance exercise in any of the 3 conditions. This result is in line with previous work from our lab using the same resistance exercise protocol, which also revealed no

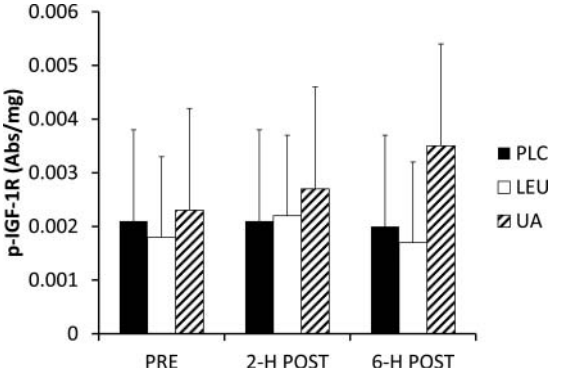

Fig. 7. Skeletal muscle concentrations of phosphorylated IGF-1R in response to PLC, LEU, and UA supplementation and resistance exercise. There were no significant increases at any time point and between exercise sessions ($p > 0.05$). Data are presented as means ± SD.

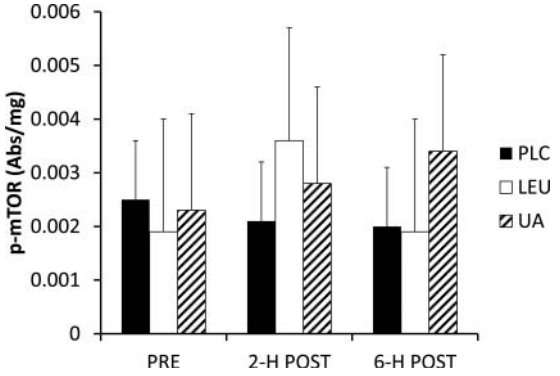

Fig. 9. Skeletal muscle concentrations of phosphorylated mTOR in response to PLC, LEU, and UA supplementation and resistance exercise. There were no significant increases at any time point and between exercise sessions ($p > 0.05$). Data are presented as means ± SD.

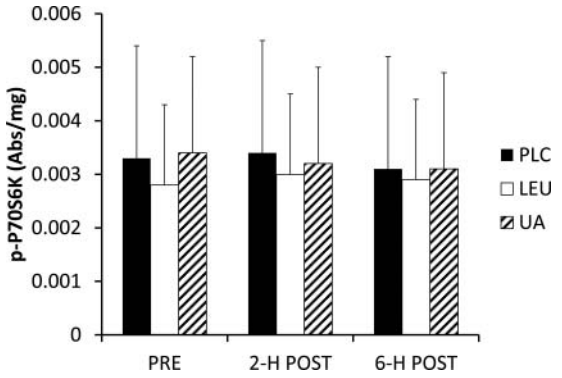

Fig. 10. Skeletal muscle concentrations of phosphorylated P70S6K in response to PLC, LEU, and UA supplementation and resistance exercise. There were no significant increases at any time point and between exercise sessions ($p > 0.05$). Data are presented as means ± SD.

exercise-induced effects on the hepatically derived release of IGF-1 [19,20]. Others have also demonstrated the unresponsiveness of serum IGF-1 to a single bout of resistance exercise [28]. Our present study is also in agreement with Ogasawara et al. [9], who, using a similar study design, did not observe an increase in serum IGF-1 concentrations in rats injected with UA or in combination with exercise.

Leucine has been shown to robustly increase MPS through mTORC1 signaling in human skeletal muscle by promoting the formation and subsequent translocation to the lysosome surface [5,13,14]. Our present study is noteworthy because we demonstrated that 3 g of leucine did not induce significant increases in the phosphorylation status of the Akt/mTORC1 pathway signaling proteins at any time point measured. A number of human studies that attribute a robust postprandial mTORC1 response to leucine often include the coingestion of amino acids or carbohydrates but not leucine per se [10–13,23]. However, in response to the ingestion of 3 g of leucine plus 9.5 g of protein, 35 g of carbohydrates, and 5.69 g of fat, significant increases in the phosphorylation of $mTOR^{Ser2448}$ were observed at 90 minutes after resistance exercise only, with no increases in any of the other signaling intermediates. Furthermore, when leucine was increased to 5 g and protein reduced to 7.5 g, the phosphorylation of $mTOR^{Ser2448}$ remained increased up to 4.5 h following resistance exercise [11]. However, a study using only 3.42 g of leucine observed a significant increase in $p70S6K^{Thr389}$ at 50 and 100 minutes following ingestion [14]. In addition, a recent study observed a significant increase in phosphorylation of $mTOR^{Ser2448}$ at 60 and 90 minutes after resistance exercise with intermittent ingestion of a total of 3.78 g of leucine [29]. Although statistically significant differences were not observed, our results demonstrate a 1.90-fold increase the phosphorylation of $mTOR^{Ser2448}$ during the LEU condition from preexercise to 2 hours postexercise compared to 0.84 and 1.22 for the PLC and UA conditions, respectively. Data support the notion that a prolonged anabolic response does not occur without additional essential amino acids and carbohydrate availability [10–13,19,22,29]. Therefore, it is plausible that by collecting muscle samples at 120 minutes following resistance exercise we may not have allowed for an appropriate time frame in which to observe a statistically significant leucine-induced increase in the phosphorylation state of $mTOR^{Ser2448}$.

Previous myotube studies have shown the hypertrophic effects of ursolic acid due to prolongation of hormone-mediated IGF-1R activity and subsequent phosphorylation of the mTOR signaling pathway [6]. A single bout of resistance exercise in conjunction with intraperitoneal injections of ursolic acid in rats had no effect on the phosphorylation of Akt^{Ser473} yet increased Akt^{Thr308}. In addition, it appears to have prevented the attenuation of $PRAS40^{Thr246}$ and $p70S6K^{Thr389}$ phosphorylation while also increasing skeletal muscle IGF-1 when compared to resistance exercise only [9]. Mice fed an ursolic acid–enriched diet for 5 weeks had improved body composition and strength and at week 16 showed improved Akt activity [6,30]. However, the results of the present study do not agree with these previous observations, because ursolic acid did not increase the activity of $IGF\text{-}1R^{Tyr1131}$, Akt^{Thr308}, $mTOR^{2448}$, or $p70S6K^{Thr389}$.

In the present study, skeletal muscle IGF-1 was unaltered in response to ursolic acid and resistance exercise. However, a significant increase with the LEU condition at 2 and 6 hours postexercise was observed. The results indicate that leucine-induced elevations in skeletal muscle IGF-1; however, Akt/mTORC1 signaling remained unaltered. Our results are in agreement with Bolster et al. [31], who did not observe elevated mTORC1 signaling in rats but did see changes downstream of mTORC1 as a result of leucine. In the present study, we provide evidence for leucine-induced increases in skeletal muscle IGF-1 concentration but not for ursolic acid, as previously observed [6].

Acute administration of ursolic acid has been shown to cause a robust increase in myotubes [6] and attenuate the decrease in rats [9] of mTORC1 signaling. In order to investigate the effects of oral ursolic acid administration, we obtained muscle biopsies from human vastus lateralis muscle. Despite a nonsignificant increase in mTOR, we observed ursolic acid to induce a fold change of 1.48 from baseline to 6 hours postexercise in the phosphorylation of $mTOR^{Ser2448}$. Human skeletal muscle and myotubes differ in their handling of intracellular calcium and force production [32], and myotubes can circumvent the effects of non-muscle tissue [6]. The effects of non-muscle tissue are important considerations because ursolic acid has been suggested to induce a number of physiological effects, and based on the receptors with which ursolic acid can interact, indicates that the effects could be a result of weak global and/or nonselective ligand–receptor binding [8,25,33].

For instance, evidence suggests that ursolic acid mediates 5' adenosine monophosphate-activated protein kinase activation induced by a decrease in the cellular adenosine monophosphate/adenosine triphosphate ratio as a result of uncoupling protein (UCP) upregulation [34]. However, administration of ursolic acid through intraperitoneal injections, as in murine models [6,9,30], could have attenuated the hepatic first-pass metabolic effect of oral ursolic acid administration [26,27]. As a result, it is also possible that skeletal muscle needs to be "loaded" through chronic usrolic acid ingestion, regardless of species [6,8,30,34].

Though it may be perceived to be a limitation that we provided a light snack 1 hour prior to the exercise sessions and immediately following the 2-hour sampling point, it should be emphasized that the snacks had no preferential effects on any of the Akt/mTOR signaling intermediates assessed in any condition. The 8 g of protein and 3 g of carbohydrate did not significantly elevate serum insulin or plasma leucine levels in any condition. We have previously shown that oral ingestion of 10 g of whey protein (1.5 g leucine) or 10 g of carbohydrate (maltodextrose) immediately prior to a single bout of resistance exercise had no significant effects on serum insulin or mTOR and p70S6K activity [22]. As a result, we are confident that the provision of snacks in the present study had no confounding effects of the outcome measures assessed.

It is plausible that our resistance exercise protocol did not induce a strong enough stimulus to provoke activation in mTORC1 signaling in resistance-trained participants. However, we used the same protocol in a previous investigation with untrained participants and observed resistance exercise-induced changes in mTOR and p70S6K [23]. Therefore, it is important to bear in mind that a limitation of our study may be a difference in resistance training status compared to other studies. Increased training status has been demonstrated to attenuate postexercise anabolic responses including MPS rates and intracellular anabolic signaling [35–37]. Therefore, it is conceivable that the 3 g dose of leucine was not enough to increase the activity of the Akt/mTOR signaling intermediates we observed in resistance-trained individuals. There are a number of studies using low-protein/low-leucine doses in response to resistance exercise; however, those studies employed untrained participants. For example, it has been shown that 5 and 10 g doses of whey protein resulted in increased MPS; however, there were no increases in the activity of mTOR pathway intermediates assessed [38]. Another study demonstrated that 3.5 g of leucine compared to 1.8 g within 10 g essential amino acid solutions resulted in increased MPS 1 hour following resistance exercise but not at 2 and 3 hours, though mTOR activity was elevated at all 3 time points; however, there were no significant increases in Akt activity [13]. Based on the lack of congruence between these studies and the present study, it is conceivable that resistance-trained individuals have an elevated leucine threshold relative to its effectiveness of Akt/mTORC1 activity and MPS. However, more studies must be conducted before we can know whether or not this presumption is valid. In addition, caution should be taken when comparing studies that measure dynamic changes MPS and those that measure static proxy markers of phosphoproteins.

Based on disagreement with previous studies, the present study is impactful in demonstrating that, at the time points measured, in resistance-trained individuals (1) ursolic acid appears to be ineffective, even at a dose of 3 g, at upregulating Akt/mTOR pathway activity and (2) a 3 g dose of leucine may be insufficient at increasing Akt/mTOR but increases skeletal muscle IGF-1. Furthermore, we have demonstrated that 3 g of L-leucine or ursolic acid did not augment Akt/mTORC1 signaling activity, suggesting that ursolic acid does not differentially effect mTORC1 signaling when compared to L-leucine. Based on the results presented herein, we conclude that 3 g of L-leucine and ursolic acid had no effect on serum IGF-1 or Akt/mTORC1 signaling; however, L-leucine increased skeletal muscle IGF-1 concentration in resistance-trained men.

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Author Contributions

D.D.C. served as the study coordinator and was involved in participant recruitment, testing, data collection, and laboratory analyses and assisted in article preparation. N.A.S., M.B.S., S. K.M.B., and T.L.A. were involved in data collection and testing. A.J.R. was involved in conducting the liquid chromatography–mass spectrometry for plasma ursolic acid. D.S.W. was the principal investigator and was responsible for developing the experimental design. He was also involved in training and mentoring graduate students for laboratory analyses, testing, data collection, laboratory analyses and provided primary oversight during the course of the study as well as supervised article preparation.

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