ABSTRACT

A Comparison of the Effects of Ursolic Acid and L-Leucine Supplementation on Markers of Muscle Protein Synthesis via Akt-mTOR Signaling Response to Resistance Exercise

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Resistance exercise (RE) stimulates skeletal muscle protein synthesis (MPS) during post-exercise recovery due to up-regulation of the mammalian target of rapamycin (mTOR) signaling pathway L-leucine supplementation is also known to stimulate MPS by activating mTOR signaling. However, recent research has discovered a natural compound called ursolic acid which also appears to stimulate MPS by activating the mTOR signaling pathway, and has been presumed to occur due to IGF-1 receptor (IGF-1R) up-regulation. Ursolic acid is a natural pentacyclic triterpenoid carboxylic acid that is widely found in apple skin and other fruits such as cranberries. The main purpose of this study was to compare the effects of a single dose of ursolic acid or L-leucine supplementation given immediately after resistance exercise on IGF-1 (a serum regulator of MPS) and the subsequent effects of IGF-1 on phosphorylating/activating its receptor ($IGF\text{-}1R^{Tyr1131}$). Furthermore, the purpose was to also determine the effects on signaling intermediates of MPS contained within the Akt/mTOR pathway (phosphorylated levels of Akt^{Thr308}, $mTOR^{Ser2448}$, $p70S6K^{Thr389}$). In a randomized, cross-over design, nine

apparently healthy, resistance-trained [regular, consistent resistance training (i.e. thrice weekly) for at least 1 year prior to the onset of the study], men between the ages of 18-30 performed three separate testing sessions of lower-body resistance exercise involving 4 sets of 8-10 repetitions at 75-80% 1-RM on the angled leg press and knee extension exercises. Immediately after each resistance exercise session, participants orally ingested 3 grams (0.043 g/kg equivalent) of cellulose placebo (PLC), L-leucine (LEU), or ursolic acid (UA). A venous blood sample was obtained before, and 0.5, 2, and 6 hr post-exercise, whereas a vastus lateralis muscle biopsy was obtained before and 2 and 6 hr post-exercise. Each testing session was separated by 7 days to allow full recovery between sessions. Statistical analyses were performed utilizing separate two-way ANOVA for each criterion variable employing a probability level of ≤ 0.05. Using ELISA, no significant differences were observed among the three supplements for serum IGF-1 ($p > 0.05$). Also using ELISA, for skeletal muscle phosphoproteins, no significant differences existed among the three supplements for phosphorylated IGF-1R, Akt, and p70S6K ($p > 0.05$). However, the LEU supplement significantly increased phosphorylated mTOR compared to UA and PLC ($p = 0.001$). At the 3 g dose provided, ursolic acid was unable to increase IGF-1R signaling and, unlike L-leucine, ursolic acid had no positive effect on mTOR signaling activity. Therefore, ursolic acid appears to have no effect on mTOR activity when ingested immediately following resistance exercise.

A Comparison of the Effects of Ursolic Acid and L-Leucine Supplementation on Markers of Muscle Protein Synthesis via Akt-mTOR Signaling in Response to Resistance Exercise

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1-RM – 1-repetition maximum

GTP – guanosine triphosphate

IGF-1 – insulin like growth factor-1

LEU – leucine

MPS – muscle protein synthesis

mRNA – messenger ribonucleic acid

mTOR – mammalian target of rapamycin

mTORC1 – mammalian target of rapamycin complex 1

MuRF1 – muscle RING-finger protein-1

Nf- κB – nuclear factor - κB

PI3K - phosphoinositide 3-kinase

RE – resistance exercise

p70S6K – S6 kinase 1

PLC - placebo

IGF-1 R – insulin like growth factor 1 receptor

Ser – serine

4E-BP – 4E binding protein

Thr – threonine

AA – amino acid

GH – growth hormone

TSC1 – tuberous sclerosis 1complex

Akt – protein kinase B

TSC1/2 – tuberous sclerosis 1/2 complex

DNA – deoxyribonucleic acid

UA – ursolic acid

EAA's – essential amino acids

eEF2 – eukaryotic elongation factor 2

eEFK2 – eukaryotic elongation factor-2 kinase

eIF – eukaryotic initiation factor

ERK – extracellular signal-regulated kinases

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CHAPTER ONE

Introduction

RE stimulates skeletal MPS during post-exercise recovery (Dreyer et al., 2007). For example, the mammalian target of rapamycin (mTOR) signaling pathway is activated during and after a single bout of RE, which was associated with a significant increase in MPS (Dreyer et al., 2006). Essential amino acids (EAAs) in nutrient supplements serve as substrates for new protein synthesis, stimulate insulin release, and largely independent of insulin, directly activate the nutrient-sensitive mTOR signaling pathway (Dennis et al., 2011). However, of the EAAs, leucine independently stimulates MPS by activating components of the mTOR signaling pathway (Crozier et al., 2005).

Recent research has discovered a natural compound which also appears to stimulate MPS by activating the mTOR signaling pathway (Kunkel et al., 2011; Ogasawara et al., 2013), just as with the amino acid, leucine. This compound, ursolic acid, is a natural pentacyclic triterpenoid carboxylic acid that is widely found in the waxy coats of apples and other fruits such as cranberries. It has been shown to play an important role in many biological functions such as; antioxidant, anti-inflammatory, trypanocidal, anti-heumatic, antiviral, and anti-tumoral properties (Ikeda et al., 2008). Microarray analysis identified ursolic acid as an inhibitor of muscle atrophy in humans and rodents (Kunkel et al., 2011). A study that fed rats a high fat diet (HFD) lacking or containing 0.14% ursolic acid demonstrated that significant muscle hypertrophy in rodents compared to a control HFD only group (Kunkel et al., 2012). A more recent

study has shown that ursolic acid administered through intraperitoneal injection immediately following electrically stimulated isometric exercise in rats was effective in increasing the activity of mTORC1, a key signaling regulator in MPS (Ogasawara et al., 2013).

Activation of mTOR can occur through two separate signaling pathways. Specifically through insulin induced activation of the PI3K/Akt pathway, or a currently undefined mechanism involving the unique class 3 PI3K receptor, hVps34; which has been demonstrated to be necessary for p70S6K activation (Byfield, Murray, & Backer, 2005). The stimulation of the mTOR pathway via IGF-1, which inhibits protein degradation via the ubiquitin pathway, has been examined in various models. Studies performed both on cell culture and rodents have suggested that the transcriptional regulation of atrogin-1 and MuRF1 is controlled by an IGF-1/Akt dependent signaling pathway (Sandri et al., 2004 and Stitt et al., 2004). One of the most highly up-regulated genes in skeletal muscle after ursolic acid supplementation is IGF-1, which is known to be transcriptionally induced in hypertrophic muscle; increased IGF-1 expression represses atrogin-1, DDIT4L, and MuRF1 mRNAs (Adams & Haddard, 1996; Kunkel et al., 2011; Sacheck et al, 2004). Ursolic acid rapidly stimulates IGF-1 receptor (IGF-1R) and insulin receptor activity. However, this only occurred if IGF-1 or insulin, respectively, was present; suggesting that ursolic acid first enhances the capacity of pre-existing IGF-1 and insulin to activate skeletal muscle their respective receptors (Kunkel et al., 2011).

Similar to ursolic acid, the degree to which leucine stimulates mTORC1 appears to be reliant upon concomitant increases in insulin concentrations. Leucine elicits a much

stronger impact on mTORC1 signaling than other AAs, as leucine withdrawal alone is as effective as complete AA starvation at suppressing mTORC1 signaling (Hara et al., 1998). Increased MPS following leucine ingestion is associated with enhanced translation initiation via activation of mTORC1, downstream targets p70S6K, and 4E-BP1 (Anthony et al., 2000; Cuthbertson et al., 2005; and Fujita et al., 2007). Leucine has been shown to cause increased Akt phosphorylation at Ser^{473}, and is a potent regulator of p70S6K signaling (Yarasheski et al., 2003). Due to the supposed similarity in the mechanisms of action of ursolic acid and leucine on the mTOR signaling pathway, investigation into their effects can provide a valuable tool when comparing these two nutritional supplements.

Purpose of the Study

The main purpose of this study is to compare the effects of a single 3 gram dose of ursolic acid and leucine administered orally immediately after RE on serum regulators of MPS (ursolic acid, leucine, and IGF-1) and to also determine the effects of various markers of skeletal MPS on the Akt/mTOR pathway (IGF-1 R, Akt, mTOR, p70S6k, total protein content).

Hypotheses

H_1: At baseline, no significant differences will exist between groups for serum leucine & IGF-1.

H_2: At baseline, no significant differences will exist between groups for skeletal muscle IGF-1 R, Akt, mTOR, & p70S6k

H_3: No significant difference will be observed for volume load between testing sessions.

H_4: No significant difference will exist for calorie macronutrient intake between testing sessions.

H_5: After RE, serum ursolic acid and leucine will not significantly increase in any of the three groups, & when also compared to baseline.

H_6: After RE, serum IGF-1 will not significantly increase in any of the three groups, but will significantly increase when compared to baseline.

H_7: After RE, skeletal muscle IGF-1 R, Akt, mTOR, & p70S6k will not significantly increase in any of the three groups, but will significantly increase when compared to baseline.

Delimitations

1. Ten apparently healthy males between the ages of 18 to 30 who are recreationally resistance-trained [persons who resistance train for general health and body composition not perform, with consistency, the volume of resistance training normally required in order to compete in professional strength or bodybuilding competitions or competitive athletic events].

2. Participants will be recruited from Baylor University and within the surrounding Waco, TX area by flyers and online advertisements.

3. Participants will be excluded from the study if they have consumed any dietary supplement (except a multivitamin) or pharmaceutical used as a potential ergogenic aid for three months prior to the study.

4. All participants will be considered low risk for cardiovascular disease, with no contraindication to exercise as outlined by the American College of Sports Medicine (ACSM).

5. All participants will be tested at the Baylor Laboratory for Exercise Science and Technology (BLEST) and Exercise Nutritional Biochemical Laboratory (EBNL) in accordance with Helsinki Code after signed university approved informed consent documents.

Limitations

1. The results of the study will only be applicable to the larger population of recreationally, resistance-trained men between 18 and 30 years of age.

2. Inferences are limited to the time points at which samples are collected.

3. Each participant's difference in inherent circadian rhythm due to sleep schedule and daily stresses that may affect criterion variables.

Assumptions

1. All laboratory equipment will be functioning properly to produce valid and reliable measurements. Proper calibration and the use of trained research staff will minimize any potential for errors.

2. Participants will put forth maximal effort during the maximal strength testing session, and all of the following RE sessions.

3. All participants will follow the guidelines provided for completion of the study.

4. All participants will maintain their normal dietary habits throughout the study.

5. All participants will have refrained from exercise for 48 before each of the testing sessions.

6. All participants will have adequate sleep (approximately 8 hours) before each of the testing sessions.

7. Participants will accurately answer all relevant questions regarding medical history and resistance training experience.

Definitions

1-repetition maximum – (1-RM) the maximum amount of weight able to be lifted for one repetition (Brooks et al., 2005).

Concentric contraction – a shortening contraction (Brooks et al., 2005)

Eccentric contraction – a lengthening contraction (Brooks et al., 2005)

Gene – a segment of DNA that can be transcribed into RNA (Boron & Boulpaep, 2009).

Gene expression – process of transforming genetic information into RNA and then into proteins (Boron & Boulpaep, 2009).

Hypertrophy – enlargement of muscle fibers via addition of newly constructed myofibrils (Tiidus, 2008).

Insulin-like growth factor-1 – (IGF-1) a hormone similar in molecular structure to insulin. It plays an important role in childhood growth and continues to have anabolic effects in adults (Adams & Haddad, 1996).

Messenger ribonucleic acid – (mRNA) RNA molecules that convey genetic information from DNA to the ribosome, where they specify the amino acid sequence of the protein products of gene expression (Boron & Boulpaep, 2009).

Transcription – the first step of gene expression, in which a particular segment of DNA is copied into RNA (Boron & Boulpaep, 2009)

Transcription coactivator – a protein that increases gene expression by binding to a transcription factor (Boron & Boulpaep, 2009).

Transcription factor – (TF) proteins that regulate gene transcription by binding to specific DNA sequences (Boron & Boulpaep, 2009).

Translation – the process in which proteins are created from RNA templates (Boron & Boulpaep, 2009).

Volume load – amount of work performed during a RE session approximated by multiplying the load by the number of repetitions (Tran et al., 2006)

CHAPTER TWO

Literature Review

Introduction

Ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) is a natural pentacyclic triterpenoid carboxylic acid that is widely found in the waxy coats of apples and other fruits such as cranberries. It has been shown to play an important role in many biological functions such as; antioxidant, anti-inflammatory, trypanocidal, anti-heumatic, antiviral, and anti-tumoral properties (Ikeda et al., 2008). Kunkel et al. (2011) identified ursolic acid through microarray analysis as an inhibitor of skeletal muscle atrophy. Skeletal muscle hypertrophy has been observed following 7-weeks of supplementing a HFD with 0.14% ursolic acid (Kunkel et al., 2012). This gives premise to ursolic acid as a possible therapy for sarcopenia and other muscle wasting conditions. Ursolic acid could provide a safe supplementation strategy to those who are trying to increase muscle mass, or maintain muscle mass during fasting periods.

Recent research data suggests ingestion of ursolic acid stimulates MPS by activating the mammalian target of rapamycin (mTOR) signaling pathway, just as with the amino acid, L-leucine (Anthony et al., 2001; Kunkel et al., 2012; Ogasawara et al., 2013). Despite observing no significant increases in plasma IGF-1 due to ursolic acid Kunkel et al., (2011) noted ursolic acid increased all IGF-1 exons levels, which suggest ursolic acid-mediated IGF-1 induction may be to localized skeletal muscle. The mTOR signaling pathway is activated during and after a single bout of RE, which was associated

with a significant increase in MPS. Essential amino acids (EAAs) in nutrient supplements serve as substrates for new protein synthesis, stimulate insulin release, and largely independent of insulin, directly activate the nutrient-sensitive mTOR signaling pathway (Dreyer et al., 2007). This provides a valuable way to examine the effects of ursolic acid on the mTOR signaling pathway.

The review of literature is broken into the following sections: (1) Introduction, (2) PI3K/Akt/mTOR Signaling Pathway, (3) Ursolic acid and Resistance Training, (4) Ursolic Acid and Skeletal Muscle Gene Expression, (5) Effects of Leucine Supplementation, and (6) Conclusion.

PI3K/Akt-mTOR Signaling Pathway

The PI3K/Akt/mTOR pathway, activated by IGF-1, plays an important role in muscle growth, and gene regulation (Zhang et al., 2013). Akt, activated by PI3K, is a serine/threonine kinase involved in the regulation of cellular metabolism and has been shown to induce rapid skeletal muscle hypertrophy (Farnfield et al., 2009). mTOR is a serine/threonine kinase partially downstream of Akt and responsible for the complex integration of anabolic stimuli mediating cell growth. Activation of mTOR can occur through insulin induced activation of the PI3K/AKT pathway or a currently undefined mechanism involving the unique class 3 PI3K, hVps34; which has been demonstrated to be necessary for p70S6K activation (Byfield, Murray, & Backer, 2005).

Acting as a nutrient, redox, and energy sensor while controlling protein synthesis, mTORC1, is composed of mTOR, MLST8, PRAS40, and DEPTOR (Dunlop and Tee, 2009; Kim et al., 2002). Mitogenic signaling upstream of mTORC1 is complex, many signaling pathways converge at the TSC1/2 level. In response to growth factors or insulin

stimulation, signaling cascades are triggered, resulting in TSC2 phosphorylation, and the subsequent dissociation of the TSC1/2 complex (Tee, Anjum, and Blenis, 2003). Loss of TSC1/2 allows GTP loading of Rheb, the Rheb-GTP acts as a potent activator of mTORC1 signal transduction (Tee et al., 2003) and is thought to enhance recognition of mTORC1 substrates 4E-BP1 and p70S6K to mTOR for their optimal phosphorylation (Sato et al., 2009). Enhanced cellular capacity to drive translation occurs through mTORC1 by increasing ribosomal biogenesis and, consequently, ribosome numbers. Due to the energy costly nature of protein synthesis, during limited nutrient and/or energy supply cells will inhibit mTORC1. Muscle mass is enhanced by mTORC1, through repressed autophagy, and governing MPS via the phosphorylation of downstream substrate targets p70S6K, and 4E-BP1 (Dodd and Tee, 2012).

Phosphorylation of 4E-BPs by mTORC1 allows for cap-dependent translation to occur efficiently (Richter and Sonenberg, 2005). Directly activated by mTORC1, p70S6K promotes protein synthesis through the phosphorylation of multiple downstream substrates associated with ribosomes, which interact directly with the 40s ribosomal subunit. Downstream of mTOR, activation of p70S6K is strongly linked to muscle hypertrophy, with phosphorylation of Thr^{389} closely associated with complete activation of the kinase (Pearson et al., 1995).

Translation initiation factors such as eIF3 and eIF4B, elongation factors such as eEFK2, and mRNA processing factors including CBP-80 are regulated by p70S6K (Dodd and Tee, 2012; Peterson and Sabatini, 2005; Tee and Blenis, 2005; Wang et al., 2001). It has also been hypothesized that p70S6K regulates ribosomal biogenesis which would

indirectly function to enhance overall rates of protein synthesis (Mahoney, Dempsey, and Blenis, 2009).

Ursolic Acid and Resistance Exercise

Ursolic acid has been identified as a potent stimulator of muscle protein anabolism through the PI3K/Akt/mTOR pathway (Kunkel et al., 2011). Mice that ingested a HFD containing 0.14% ursolic acid increased Akt phosphorylation at Ser^{473} (Kunkel et al., 2012). HFD's supplemented with 0.14% or 0.27% ursolic acid increased plasma IGF-1 concentrations as compared to a HFD only in mice (Kunkel et al., 2011). By increasing IGF-1 the PI3K/Akt pathway is induced, and thus gene regulation is increased. Ursolic acid has also reduced both obesity, glucose intolerance, and fatty liver disease in mice that were fed high-fat diets without decreasing food intake (Kunkel et al., 2012). Kunkel et al., 2011 demonstrated that ursolic acid enhances IGF-1-induced phosphorylation of IGF-1 R, Akt, and p70S6K in C2C12 myotubes. Sprague-Dawley rats were intraperitoneal injected with ursolic acid immediately following electrically stimulated isometric RE had significantly increased IGF-1 skeletal muscle concentrations at both 1-Hr and 6-Hr following RE compared to (Ogasawara et al., 2013). Taken together, the available data suggest that all effects of ursolic acid were consistent with increased skeletal muscle IGF-1/Akt/mTOR signaling activity.

It is hypothesized that ursolic acid rapidly stimulates IGF-1R and insulin receptor activity. However, ursolic acid alone did not significantly increase phosphorylation of the IGF-1R or insulin receptor; insulin or IGF-1, respectively, must be present for ursolic acid's effects (Kunkel et al., 2011). This would suggest that ursolic acid either inhibits receptor dephosphorylation, facilitates hormone-mediated receptor autophosphorylation,

or act as a ligand with insulin and IGF-1R's. Ursolic acid may first enhance the capacity of pre-existing IGF-1 and insulin to activate their respective skeletal muscle receptors. In turn this activates Akt, p70S6K, and ERK leading to altered skeletal muscle gene expression in a manner than reduces atrophy and promotes hypertrophy. However, currently it remains ambiguous as to the exact mechanism with which ursolic acid exhibits its effects.

It appears that a synergistic effect between ursolic acid and RE is necessary to see a significant increase in IGF-1 skeletal muscle concentration. Muscle contractions, such as that occurring in RE, are known to accelerate muscle anabolism, as repeated application of such stimuli leads to the gradual accumulation of muscle proteins (Philp, Hamilton, and Barr, 2011; Yarasheski, 2003). Work by Ogasawara et al. (2013) supports the notion that RE is necessary to see significantly increased skeletal muscle hypertrophy due to ursolic acid. They found ursolic acid significantly increased exercise-induced muscle IGF-1 concentrations at 1-Hr and 6-Hr following RE. RE alone increased muscle IGF-1 concentrations 1 and 6 hours after RE compared to control groups (without ursolic acid or RE), however, ursolic acid alone did not cause any changes in muscle IGF-1 concentrations compared to control groups. High intensity muscle contractions are known to increase the phosphorylation of mTORC1 downstream targets: p70S6K and rpS6, for more than 18 hours after muscle contraction (Ogasawara et al., 2013).

Part of ursolic acid's molecular effects could be attributed to inhibition of mRNA's associated with skeletal muscle atrophy (Kunkel et al., 2011). In addition to decreased muscle atrophy, mice fed a HFD containing 0.14% ursolic acid showed significantly decreased body weight, epididymal fat weight, and retroperitoneal fat

weight (Kunkel et al., 2012). A key regulator of translation initiation, mTORC1, has been shown to play a role in MPS and muscle hypertrophy (Goodman et al., 2011). Mice fed a HFD with 0.27% ursolic acid significantly increased expression of IGF-1 mRNA, and phosphorylated Akt in the skeletal muscle of mice (Kunkel et al., 2011). The increase in IGF-1 gene expression following a hypertrophic stimulus such as RE or mechanical loading is a late event. In muscles that hypertrophy secondary to chronic ursolic acid treatment, phosphorylated Akt levels were significantly increased (Kunkel et al., 2011).This suggest ursolic acid may acutely activate mTORC1 through increased IGF-1/Akt signaling.

Increased phosphorylated Akt concentrations in skeletal muscle helps potentially account for many or ursolic acid's effects, including reduced atrophy-associated mRNA expression and subsequent reduced muscle atrophy, increased muscle hypertrophy, reduced total body weight, white fat glucose intolerance, hepatic steatosis, and reduced adiposity (Kunkel et al., 2011; Rao et al., 2011; Jayaprakasan et al., 2006; Lai et al., 2004). Ursolic acid increased grip strength and skeletal muscle weight in mice fed a HFD with 0.14% ursolic acid for 6 weeks compared to a control HFD only group. In the same study mice who received the 6 week 0.14% ursolic acid diet demonstrated significantly increased fast and slow skeletal muscle fibers size increased without altering the ratio of the fibers, and exhibited significantly increased hypertrophy of the quadriceps and triceps (Kunkel et al., 2012). Mice that ingested a high-fat diet with 0.27% ursolic acid for 17-weeks were able to run significantly farther than control mice, and had significantly lower resting heart rates as compared to a control high-fat diet (Kunkel et al., 2012).

While Ursolic acid's effects on skeletal muscle are potentially sufficient to explain its effects, it can be speculated that increased brown fat could play an important role. Inter-capsular brown fat provides protection against obesity, due to a high rate of energy expenditure, was significantly increased in mice that were fed a HFD supplemented with 0.14% ursolic acid (Kunkel et al., 2012). Ursolic acid could increase skeletal muscle and brown fat, leading to increased energy expenditure, and thus a resistance to obesity, glucose intolerance, and fatty liver disease.

Ursolic Acid and Skeletal Muscle Gene Expression

The current literature available in regards to ursolic acid and its effect on skeletal muscle mRNA expression is limited to work done by Kunkel et al. (2011 & 2012), whom utilized mice models. Fasting mRNAs simulate many of the same effects that occur in muscle atrophy. Ursolic acid has been shown to reduce fasting levels of atrogin-1 and muscle RING-finger protein-1 (MuRF1) mRNAs (Kunkel et al., 2011). Atrogin-1 and MuRF1 are transcriptionally up-regulated by atrophy-inducing stress, and are required for muscle atrophy (Sacheck et al., 2007). Therefore, use of atrogin-1 and MuRF1 as markers for skeletal muscle atrophy in humans is appropriate.

One of the most highly up-regulated genes in skeletal muscle after 5-weeks of 0.27% ursolic acid HFD was IGF-1 (Kunkel et al., 2011), which is known to be transcriptionally induced in hypertrophic muscle (Adams & Haddad, 1996). Increased IGF-1expression has been demonstrated to represses atrogin-1, DDIT4L, and MuRF1 mRNAs (Sacheck et al., 2004), and ursolic acid reduces atrogin-1 and MuRF mRNAs. Repression of atrogin-1 and MuRF could be attributed to ursolic acid enhancing IGF-1-mediated inhibition of FoxO transcription factors. Data from Kunkel et al. (2011)

supports altered skeletal muscle gene expression in a manner known to reduce atrophy and promote hypertrophy due to 5-weeks of a HFD containing 0.27% ursolic acid. Specifically, changes in downstream mRNA expression include induction of IGF-1 (feed-forward mechanism that likely contributes to ursolic acid-mediated hypertrophy), repression of antrogin-1, and MuRF1, along with induction or repression of many other genes whose contributions to muscle atrophy or hypertrophy remain to be determined (Kunkel et al., 2011).

Ursolic acid ingestion leads to other mRNA expression changes that are desirable as well; causing an increase in the expression of uncoupling protein1 (UCP1), a brown fat marker (Kunkel et al., 2012). Ursolic acid reduced the steady-state level of Srebpf1 mRNA, which encodes SREBP-1c, a transcription factor that promotes lipogenesis and fatty liver disease. Accordingly ursolic acid reduced the expression of three key SREBP-1 target gen acetyl-CoA carboylase 1, fatty acid synthase, and stearoyl Co-A desaturase (Kunkel et al., 2012). As previously mentioned, ursolic acid improves insulin sensitivity and glucose intolerance. This occurs due to nuclear factor-κB (NF-κB)-mediated inflammatory signaling pathway being blocked, suppression of protein tyrosine phosphatase 1B (PTP1B) expression, and enhancement of insulin receptor phosphorylation (Lu et al., 2011). Although Kunkel et al. (2012) found no significant changes in muscle mass in PTP1B knockout mice as compared to control mice. PTP1B suppression could occur by ursolic acid blocking endoplasmic reticulum (ER) stress and inhibiting the IκB kinase/NF-κB-mediated inflammatory signaling pathway in the hippocampus of mice, which activates the PI3K/Akt-mTOR pathway (Le et al., 2011).

Effects of Leucine Supplementation

AA's are building blocks of protein composed of nitrogen, carbon, oxygen, and hydrogen. Exogenous AA supplementation in conjunction with resistance training increases the MPS response initiated by mechanical loading, through further enhancement of mTORC1 activation (Pasiakos, 2012). AA supplements can cause a more robust anabolic response which generally exceeds those elicited by exercise performed in the absence of nutrition (Drummond et al., 2009). Elevated AA concentrations lead to more efficient signaling of mTORC1, and subsequent MPS initiated by mechanical stress (Philp et al., 2011). Investigations consistently demonstrate enhanced mTORC1, MPS, and hypertrophic response to mechanical stimuli when RE is combined with AA supplementation (Drummond et al., 2009).

Combining the metabolic effects of RE with AA supplementation results in a more pronounced and sustained anabolic response than either stimulus elicited alone, of particular interest to the present study is the amino acid, leucine. Yarasheski et al. (2012) demonstrated a relatively small amount of leucine (0.75 grams) is able to achieve maximal MPS when other EAA's are provided as well. Studies using differing amounts of essential amino acids and leucine, demonstrate with higher amounts of EAA, and thus leucine improves MPS and overall net protein balance compared to a lower doses (Kassanos et la., 2005; and Paddon-Jones et la., 2004). Leucine supplementation forces muscle fibers to favor the anabolic phenotype, and is effective at maintaining muscle mass. Studies have demonstrated leucine reduces muscle atrophy by suppressing proteolysis, along with suppressing expression of key components of the ubiquitin/proteasome pathway (Busquets et al., 2000; and Combaret et al., 2005; Dodd

and Tee, 2012). Therefore, leucine has been investigated as a pharmaconutrient with the potential to promote increases in MPS and lean tissue mass, while decreasing proteolysis (Yarasheski et al., 2012).

The previously mentioned effects of leucine occur through up regulation of the mTORC1 signaling pathway (Dodd and Tee, 2012). Increased MPS following leucine ingestion is associated with enhanced translation initiation via activation of mTORC1; which then activates downstream targets p70S6K, and 4E-BP1 (Anthony et al., 2000; Cuthbertson et al., 2005; and Fujita et al., 2007). Leucine elicits a much stronger impact on mTORC1 signaling than other AAs, as Hara et al. (2008) demonstrated leucine withdrawal alone is as effective as complete AA starvation in suppressing mTORC1 signaling. One study demonstrated that leucine binds directly to the substrate recognition domain of UBR2 (leucine binding protein and negative regulator of mTORC1), preventing degradation and consequently promoting mTORC1 signaling (Kume et al., 2010).

Akt can directly activate mTOR through phosphorylation, or indirectly by phosphorylating (and inhibiting) TCS2 (Nave et al., 1999; Inoki et al., 2002; and Manning et al., 2002). Leucine supplementation has been demonstrated to cause increases in the phosphorylation status of Akt at Ser^{473}, and a potent regulator of p70S6K signaling (Yarasheski et al., 2012). This action has led to AA's being described as "priming" molecules, whose phosphorylation of mTOR at Ser^{2448} is a prerequisite for further phosphorylation of Akt (Anthony et al., 2001). This is supported by Yarasheski et al. (2012) who demonstrated an increase in phosphorylation status of Akt at Ser^{473} 1 hour following RE, significant increases in mTOR phosphorylation at Ser^{2448}, and

leucine/whey protein ingestion increased p70S6K phosphorylation at Thr^{389}. Further downstream; p70S6K signals to eukaryotic elongation factor 2 (eEF2) to enhance translation elongation (Brown and Proud, 2002).

Similar to ursolic acid, the degree to which leucine stimulates mTORC1 activity appears to be reliant upon concomitant increases in insulin concentrations. Work by Crozier et al., (2005) supports this demonstrating that early stimulation of MPS was not reliant on insulin, however, maximal signaling of the mTOR was dependent on circulating insulin concentrations when rats were orally administered leucine. Drummond et al., (2010) demonstrated EAA ingestion increased leucine delivery to the muscle, intramuscular leucine concentrations, MPS, and mTORC1 activation. Due to the similarity in the hypothesized molecular mechanisms of action of ursolic acid and leucine on the mTOR signaling pathway, investigation into their effects can provide a valuable tool when comparing these two nutritional supplements.

Conclusion

Currently there is a lack of literature investigating ursolic acid supplementation in human skeletal muscle in regard to; both the effects it may have on training adaptations, and the molecular mechanism through which it carries out these effects. Initial studies have demonstrated significantly increase muscle hypertrophy, conservation of muscle protein by preventing proteolysis, and a host of other properties such as: antioxidant, anti-inflammatory, trypanocidal, anti-heumatic, antiviral, and anti-tumoral (Kunkel et al., 2011). It has been shown that ingestion of ursolic acid through long duration diet, and intraperitoneal injection leads to an increase expression of IGF-1 mRNA and therefore provides evidence for the mechanism of action being through the PI3K/Akt/mTOR

signaling pathway, similar to leucine (Kunkel et al., 2011; Kunkel et al., 2012; Ogasawara et al, 2013). Data already supports leucine supplementation working through the mTOR signaling pathway and promoting mTORC1 signaling (Hara et al., 1998). Leucine leads to an increased phosphorylation state of Akt, which then in turn acts upon mTOR (Yarasheski et al., 2012). Additionally, concentrations of circulating insulin affect the degree to which ursolic acid and leucine stimulates the mTOR signaling pathway (Crozier et al., 2005; Kunkel et al., 2011). The resulting data of a mixed methods design study comparing the effects of the two nutritional supplements will provide evidence as to whether or not the effects of ursolic acid are carried out through the mTOR signaling pathway.

CHAPTER THREE

Methods

Experimental Approach

In a randomized, cross-over design, participants visited the laboratory on 7 separate occasions in the following manner: visit 1 = entry/familiarization session, visit 2 = testing/ RE session 1, visit 3 = 6 hour follow-up for session 1, visit 4 = testing/ RE session 2, visit 5 = 6 hour follow-up for session 2, visit 6 = testing/ RE session 3, visit 7 = 6 hour follow-up session for session 3. Relative to the testing sessions (visits 2, 4, 6) participants performed a RE session involving the angled leg press, and knee extension exercises on three occasions separated by two weeks. One session constituted the control/placebo session and the other two were the experimental sessions involving either ursolic acid or leucine (Figure 1). The approach is based on the premise that since RE is known to increase MPS (Dryer et al., 2006), the proposed experimental model allowed for the determination of the ability of ursolic acid and/or leucine to augment MPS when ingested immediately

Participants

Nine apparently healthy, resistance-trained [regular resistance training (i.e. thrice weekly) for at least 1 year prior to the onset of the study], men between the ages of 18-30 volunteered to serve as participants of this study. Enrollment was open to men of all ethnicities. Only participants considered low risk for cardiovascular disease and no contraindications to exercise as outlined by the American College of Sports Medicine,

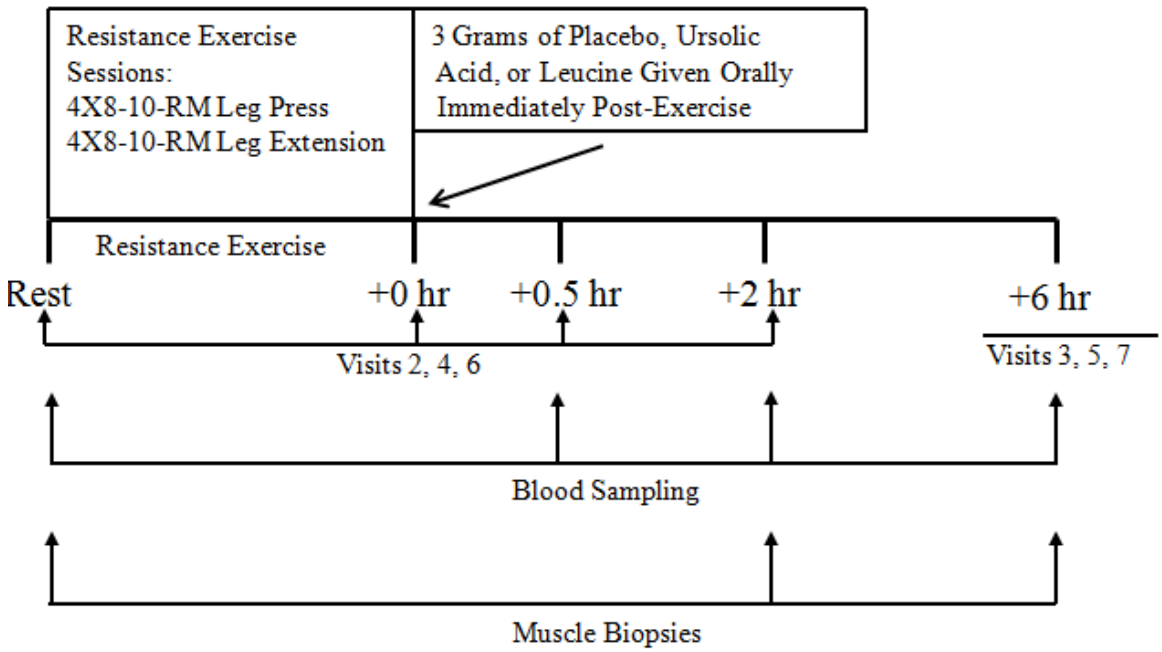

Figure 1. An illustration of the experimental protocol for the testing sessions during visits 2-7.

(ACSM), and who did not consumed any nutritional supplements (excluding multi-vitamins) one month prior to the study were allowed to participate. All eligible participants signed university-approved informed consent documents and approval was granted by the Institutional Review Board for Human Subjects. Additionally, all experimental procedures involved in the study conformed to the ethical consideration of the Helsinki Code.

Study Site

All supervised testing sessions and supplement assignment were conducted in the Resistance Training and Assessment Laboratory (RTAL) at Baylor University. All sample analyses were completed in the Exercise and Biochemical Nutrition Laboratory (EBNL) at Baylor University.

Independent and Dependent Variables

The independent variable was the RE and supplementation protocol (placebo, ursolic acid, and leucine). Dependent variables in blood included: plasma ursolic acid, serum leucine, and serum IGF-1. In skeletal muscle, the variables included: IGF-1 R, Akt, mTOR, p70S6k, and total protein content.

Entry and Familiarization Session (Visit 1)

Participants who expressed interest in participating in the study were interviewed through email to determine whether they qualified to participate in the current study. Participants who appeared to meet the eligibility criteria were invited to attend an entry/familiarization session. After the reported to the lab, participants completed a medical history questionnaire and underwent a general physical examination to determine whether they meet eligibility criteria. Participants who met the entry criteria were familiarized to the study protocol via a verbal and written explanation outlining the study design and underweant assessments for body composition and muscle strength assessments for the two lower-body exercises involved in the study. At the conclusion of the familiarization session, participants were given an appointment in which to attend their first testing session. In addition, each participant was instructed to refrain from exercise for 48 hours prior to each testing session, ate an Atkins© Advantage™ caramel chocolate nut roll 1-hr prior to reporting for each testing session, and another 1-hr prior to the 6-hr post-RE time point. They recorded their dietary intake for four days (including the bars the day of testing) prior to each of the three testing sessions involved in the study.

Muscle Biopsies (Visits 2-7)

Precutaneous muscle biopsies (~30 mg) were obtained from the middle portion of the vastus lateralis muscle of a leg picked randomly, at the midpoint between the patella and the greater trochanter of the femur at a depth between 1 and 2 cm. 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. 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. Two muscle samples were obtained at each of the three RE sessions and one 6 hours after each session, for a total of nine muscle samples being obtained during the course of the study. At each testing session, muscle samples were obtained: immediately prior to the commencing the testing session, 2 hours after RE, and 6 hours after RE. For subsequent RE session, the opposite leg of the previous RE session, was used for sample collection.

Blood Sampling (Visits 2-7)

Venous blood samples were obtained into 10 ml vacutainer tubes from a 20 gauge intravenous catheter inserted into the antecubital vein. Blood samples were allowed to stand at room temperature for 10 min and then centrifuged. The serum and plasma were removed and frozen at -80°C for later analysis. At each testing session, blood samples were obtained: immediately prior to the commencing the testing session, 0.5 hour after exercise, 2 hours after exercise, and 6 hours after the exercise session for a total of 12 blood samples obtained during the course of the study.

Body Composition Testing (Visit 1)

At the entry and familiarization session, total body mass (kg) was determined on a standard dual beam balance scale (Detecto). Total body water (total, intracellular, and extracellular) was determined with bioelectrical impedance [(BIA) Xitron 4200, San Diego, CA]. Percent body fat, fat mass, and fat-free mass, was determined using a calibrated Hologic 4500W dual-energy x-ray absorptiometry (DEXA). The DEXA segmented regions of the body (right arm, left arm, trunk, right leg, and left leg) into three compartments.

Dietary Analysis (Visits 2, 4, 6)

Participants were required to record their dietary intake for 4 days prior to each of the three RE sessions. The participants' diets were standardized and participants were 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 to determine the average daily macronutrient consumption of fat, carbohydrate, and protein in the diet for the duration of the study.

Muscle Strength Assessments (Visit 1)

In order to determine muscular strength, participants performed one-repetition maximum (1-RM) tests on angled leg press, and knee extension exercises during the familiarization session. Participants warmed up by completing 5 to 10 repetitions at approximately 50% of the estimated 1-RM. The participant rested for 1 minute, and then completed 3 to 5 repetitions at approximately 70% of the estimated 1-RM. The weight was then increased conservatively, and the participant attempted to lift the weight for one

repetition. If the lift is 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.

Assessment of Heart Rate & Blood Pressure (Visits 1-7)

At visits 1-7, heart rate and blood pressure were assessed. At the entry and familiarization session, these variables were obtained as part of the health history assessment. At visits 2, 4, and 6 heart rate and blood pressure were obtained at each of the 4 time points where blood samples are obtained. Heart rate and blood pressure was also obtained at visits 3, 5, and 7. Heart rate was determined by palpation of the radial artery using standard procedures. Blood pressure was assessed in the sitting position after resting for 5-min using a mercurial sphygmomanometer using standard procedures.

Resistance Protocol (Visits 2, 4, 6)

During each of the three RE 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. If muscle fatigue/failure occurred during a set, a spotter provided assistance until the participant completed the remaining repetitions and resistance was reduced for subsequent sets. In all cases, 2 minutes of rest separated sets and exercises. All RE sessions were supervised by study personnel.

Nutrient Supplementation Protocol (Visits 2, 4, 6)

In a randomized, doubled-blind fashion one of three nutrient supplements was orally ingested in capsule form immediately after the completion of each RE session.

Within twelve gelatin capsules, all of the same size, shape, and color, the control/placebo supplement consisted of 3 grams of cellulose (Nutricology, Alameda, CA) and the two experimental supplements consisted of 3 grams of leucine and 3 grams of ursolic acid. Since the purity of many over-the-counter nutrient supplements may be questionable, both L-leucine and ursolic acid will be 99% pure, confirmed by analytical GC/MS analyses by the manufacturer (Sigma Chemical Company, St. Louis, MO).

Serum IGF-1 Assessments

From the 12 blood samples obtained at the three RE sessions, IGF-1 levels were determined using commercially available enzyme-linked immunoabsorbent assays (ELISA) kits (Alpha Diagnostic Laboratories, San Antonio, TX). All samples were run in duplicate and the assays were performed at 450 nm wavelength with a microplate reader (iMark, Bio-Rad, Hercules, CA). Hormone concentrations were determined using data reduction software (Microplate Manager, Bio-Rad, Hercules, CA).

Serum L-Leucine and Plasma Ursolic Acid Assessments

Plasma levels of ursolic acid were determined by HPLC in the Laboratory of Ecological and Adaptational Physiology (LEAP) at Baylor University. L-leucine serum concentrations were determined from BCAA ELISA kits. Data from Caballero, Gleason, and Wurtman, (1991) demonstrated in 68 young males (age $\leq$ 40) in a fasted state total BCAA content of plasma when added individually for each (valine, isoleucine, and leucine) was 667 µmol/L. Leucine on its own accounted for 218 µmol/L, thus 32.7% of total serum BCAA is present as L-leucine.

Assessment of Skeletal Muscle Akt/mTOR Signaling Pathway Intermediates

From the nine muscle tissue samples obtained at the three RE sessions, the phosphorylated levels of IGF-1 R, Akt, mTOR, and p70S6 kinase were determined by phosphoprotein ELISA kits (Active Motif, Carlsbad, CA). All samples were run in duplicate and the assays were performed at 450 nm wavelength with a microplate reader (iMark, Bio-Rad, Hercules, CA). Protein concentrations were determined using data reduction software (Microplate Manager, Bio-Rad, Hercules, CA), and the final concentration was expressed relative to total muscle protein content.

Assessment of Skeletal Muscle Myofibrillar Protein Content

The content of myofibrillar protein from each muscle sample was determined spectrophometrically at a wavelength of 595 nm using bovine serum albumin as the standard. The final concentration was expressed relative to muscle wet-weight.

Statistical Analyses

Statistical analyses were performed by utilizing separate 3 x 4 [Session (placebo, ursolic acid, leucine) x Test (pre, post, 2-hour post, 6-hour post)] factorial analyses of variance (ANOVA) with repeated measures for blood variables. For muscle variables, a 3 x 3 [Session (placebo, ursolic acid, leucine) x Test (pre, 2-hour post, 6-hour post)] factorial ANOVA was employed. Further analyses of the main effects were performed by separate one-way ANOVAs. Significant between-group differences were determined through the Tukey's Post Hoc Test. An *a-priori* power calculation showed that 10 participants per group was adequate to detect a significant difference between groups in the marker of Akt in response to RE, 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 ($\eta 2$), which estimates the proportion of variance in the dependent variable that can be explained by the independent variable. Partial Eta squared effect sizes were determined to be: weak = 0.17, medium = 0.24, strong = 0.51, very strong = 0.70. All statistical procedures were performed using SPSS 19.0 software (Chicago, IL) and a probability level of < 0.05 was adopted throughout the study. All statistical procedures were performed using SPSS 20.0 software and a probability level of < 0.05 was adopted throughout.

CHAPTER FOUR

Results

Anthropometric and 1-RM Data

Thirteen participants completed baseline testing, of which nine finished the study; two dropped out due to time constraints, one for personal reasons, and one asked to be removed from the study. Physical, anthropometric, and 1-RM data describing the participants are expressed in Table 1. No significant differences occurred for volume load between session as all participants were able to lift the same load and perform the same amount of repetitions and sets for each session. Therefore we failed to reject our hypothesis that no significant differences observed for volume load between testing sessions.

Dietary Intake

All nine participants recorded their food intake beginning the 4 days prior to each exercise testing session throughout the 6-hr post-RE time point; therefore, statistical analyses were completed on food intakes. Nutritional content of the protein bars ingested before each time point were included in the analyses. As can been seen in Table 2, one-way *ANOVA* tests revealed no significant differences for total kilocalories per day, and the grams from fat, carbohydrate, or protein per day between resistance training sessions ($p > .05$). Therefore, we failed to reject out hypothesis that no significant differences will exist for calorie macronutrient intake between testing sessions.

Table 1
Anthropometric and 1-Rm data

Variable	Mean ± SD
Age (years)	22.8 ± 4.4
Height (cm)	174.7 ± 6.7
Bodyweight (kg)	83.5 ± 19.0
Lean mass (kg)	65.3 ± 11.7
Fat mass (kg)	16.2 ± 9.7
Leg Press 1-RM (kg)	386.6 ± 113.1
Leg Press Relative Strength (1-RM/ BW)	4.6 ± 1.1
Knee Extension 1-RM (kg)	74.1 ± 14.3
Knee Extension Relative Strength (1-RM/ BW)	0.9 ± 0.1

Note: SD = standard deviation; cm = centimeters; kg = kilograms; % = percent; BW = bodyweight; 1-RM = one-repetition maximum.

Table 2
Dietary Intake of Participants

Variable	PLC	LEU	UA	*p-v*alue
Kilocalories (kcals/day)	2030.58 ± 709.6	2175.9 ± 916.5	1879 ± 731.1	0.732
Fat (g/day)	88.2 ± 51.3	92.0 ± 52.4	71.4 ± 31.5	0.626
Carbohydrate (g/day)	229.9 ± 104.1	214.0 ± 82.0	201.1 ± 124.0	0.844
Protein (g/day)	106.9 ± 33.3	117.6 ± 80.3	112.3 ± 53.4	0.928

Note: All data are presented as mean ± SD. g = grams; kcals = kilocalories.

Serum Leucine Concentration

Serum leucine expressed as mean ± SD is presented in Figure 2, and Table 3. No statistically significant interaction between Time and Supplement [F (2, 36) = .477, p =

0.306, partial η^2 = .070] occurred. The main effect for Supplement demonstrated a statistically significant difference in serum leucine concentration [*F* (2, 27) = 69.698, *p* =.000, partial η^2 =.592]. Post-hoc analyses revealed a significant difference in serum leucine for UA compared to PLC (*p* = .000) and LEU (*p* = .000); however, this resulted simply from a higher baseline level of serum leucine during the UA condition, not due to an increase over time. Due to this we rejected our hypothesis that no differences will exist between supplement groups at baseline for serum leucine. The main effect for Time revealed no statistically significant differences for LEU [F (3, 36) = 2.638, p = 0.054, partial η2 = .076]. Therefore, we failed to rejected our hypothesis that serum leucine will not significantly increase following RE when compared to baseline.

Serum IGF-1 Concentration

Serum IGF-1 expressed as mean ± SD is presented in Figure 3, and table 3. Main effects revealed no statistically significant differences for Time [*F* (3, 27) = 150.283, *p* = .897, partial η^2 = .006], Supplement [*F* (2, 36) = 772.755, *p* = .363, partial η^2 = .021], and the Time x Supplement interaction [*F* (6, 36) = 232.718, *p* = .999, partial η^2 =.003]. We failed to reject our hypothesis that at baseline no significant difference will exist between supplement groups for serum IGF-1. We reject our hypothesis that serum IGF-1 will significantly increase after RE when compared to baseline.

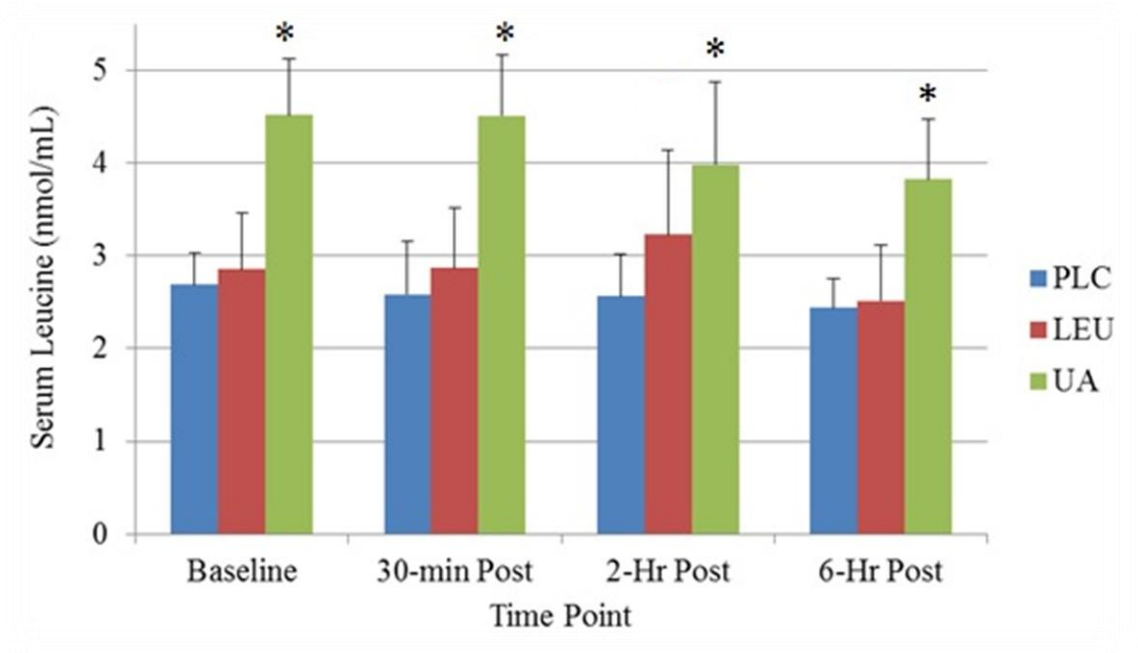

Figure 2. Serum leucine concentration (nmol/mL) mean ± SD for each time point and supplement. Hr = hours; Min= minutes; * = significantly different from PLC and LEU for all time points.

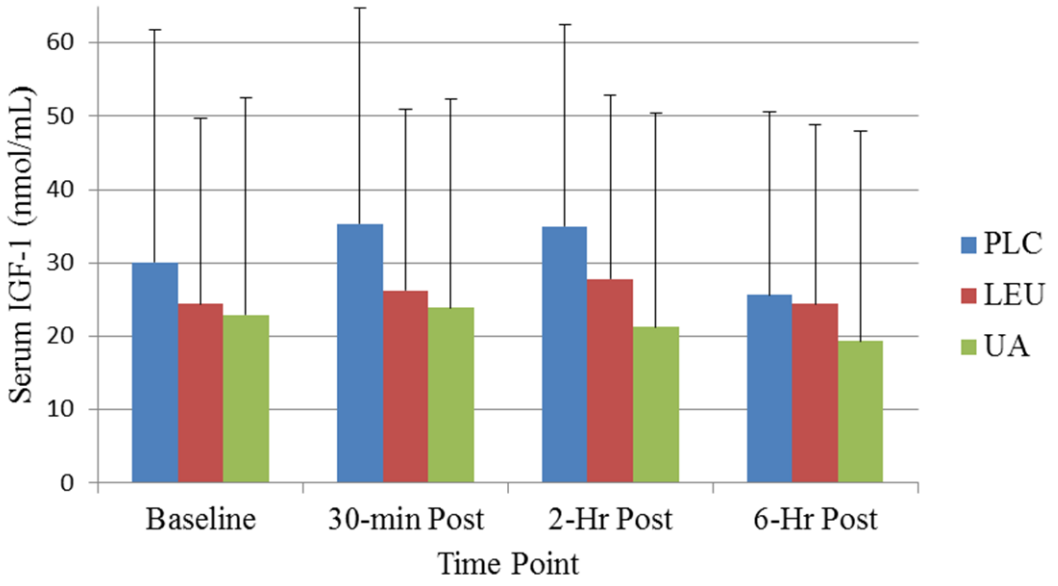

Figure 3. Serum IGF-1 concentration (nmol/mL) mean ± SD for each time point and supplement. Hr = hours.

Table 3
Serum Concentrations for Timepoint & Supplement

Variable	Time Point	PLC	LEU	UA	T ($p < .05$)	S ($p < .05$)	T x S ($p < .05$)
Serum Leucine (nmol/ml)	Baseline	2.688 ± 0.339	2.854 ± 0.611	4.515 ± 0.605	0.054	0.000*	0.306
	30-Min Post	2.586 ± 0.573	2.872 ± 0.641	4.507 ± 0.654			
	2-Hr Post	2.564 ± 0.455	3.227 ± 0.917	3.986 ± 0.886			
	6-Hr Post	2.439 ± 0.321	2.517 ± 0.592	3.824 ± 0.643			
Serum IGF-1 (nmol/ml)	Baseline	30.121 ± 31.632	24.384 ± 25.309	22.920 ± 29.535	0.897	0.363	0.999
	30-Min Post	35.375 ± 29.400	26.254 ± 24.631	23.883 ± 28.466			
	2-Hr Post	34.967 ± 27.542	27.815 ± 25.105	21.238 ± 29.110			
	6-Hr Post	25.602 ± 24.983	24.420 ± 24.469	19.344 ± 28.637			

Note: All data are presented as mean ± SD. * = statistically significant effect; Min = minutes; Hr = hours; Post = time after resistance training session; PLC = placebo; LEU = leucine; UA = ursolic acid; T = time effect; S = Supplement effect; T x S =Time x Supplement effect; p- = phosphorylated.

Table 4

Skeletal Muscle Phosphoprotein Data for Timepoint & Supplement

Variable	Time Point	PLC	LEU	UA	T ($p < .05$)	S ($p < .05$)	T x S ($p < .05$)
	Baseline	.0030 ± .0014	.0022 ± .0005	.0031 ± .0028			
p- IGF-1R	2-Hr Post	.0022 ± .0006	.0022 ± .0019	.0019 ± .0009	0.179	0.456	0.725
	6-Hr Post	.0023 ± .0007	.0016 ± .0006	.0024 ± .0018			
	Baseline	.0049 ± .0023	.0049 ± .0036	.0052 ± .0046			
p-Akt	2-Hr Post	.0054 ± .0039	.0047 ± .0049	.0042 ± .0036	0.924	0.837	0.555
	6-Hr Post	.0033 ± .0010	.0041 ± .0025	.0046 ± .0030			
	Baseline	.0030 ± .0017	.0013 ± .0004	.0040 ± .0044			
p-mTOR	2-Hr Post	.0021 ± .0008	.0018 ± .0014	.0028 ± .0014	0.631	0.001*	0.600
	6-Hr Post	.0020 ± .0008	.0014 ± .0003	.0035 ± .0030			
	Baseline	.0050 ± .0022	.0028± .0008	.0047 ± .0052			
p-p70S6K	2-Hr Post	.0035 ± .0013	.0030 ± .0019	.0032 ± .0015	0.272	0.201	0.698
	6-Hr Post	.0031± .0009	.0027 ± .0007	.0040 ± .0037			

Note: All data are presented as mean ± SD. * = statistically significant effect; Hr = hours; Post = time after resistance training session; PLC = placebo; LEU = leucine; UA = ursolic acid; T = time effect; S = Supplement effect; T x S =Time x Supplement effect; p- = phosphorylated.

Phosphorylated- IGF-1R

Phosphorylated-IGF-1 R expressed as mean ± SD is presented in Figure 4, and table 4. Main effects revealed no statistically significant differences for Time [F (2, 27) = 1.760, p = .179, partial η^2 = .047], Supplement [F (2, 27) = .794, p = .456, partial η^2 = .022], and the Time x Supplement interaction [F (4, 27) = .515, p = .725, partial η^2 = .028]. We failed to reject our hypothesis that no significant difference will exist between groups at baseline. We rejected our hypothesis that IGF-1-R will significantly increase following RE when compared to baseline.

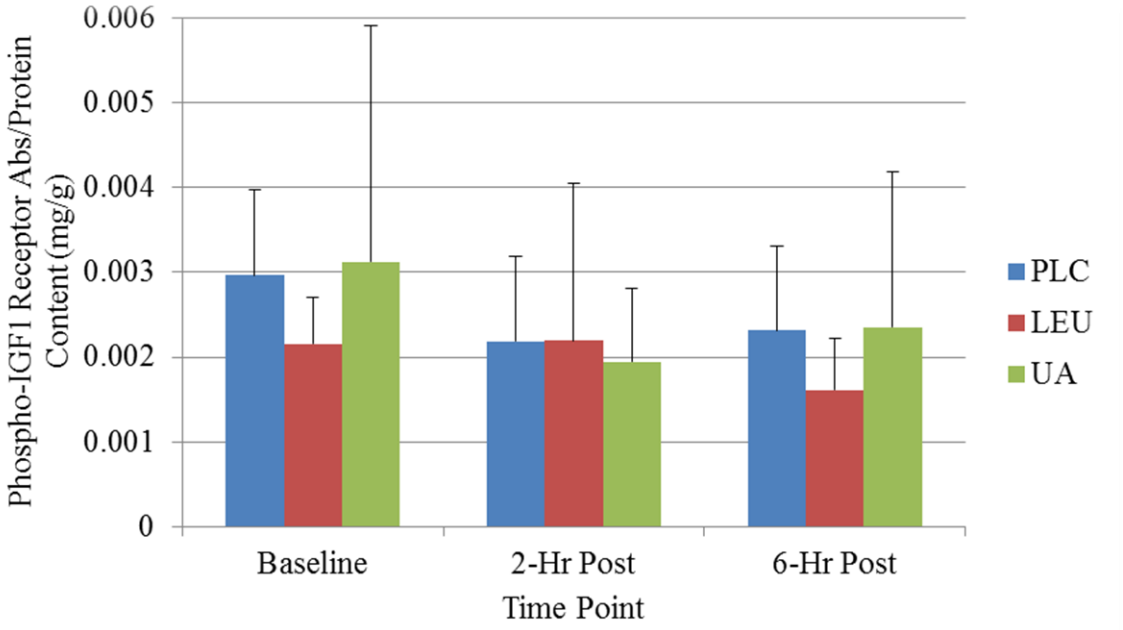

Figure 4: Mean ± SD Phosphorylated- IGF-1R for each time point and supplement. Hr = hours.

Phosphorylated-Akt

Phosphorylated-Akt expressed as mean ± SD is presented in Figure 5, and table 4. Main effects revealed no statistically significant differences for Time [F (2, 27) = .079, p = .924, partial η^2 = .002], Supplement [F (2, 27) = .179, p = .837, partial η^2 = .005], and

the Time x Supplement interaction [F (4, 27) = .760, p = .555, partial η^2 = .041]. We failed to reject our hypothesis that no significant differences will exist between groups at baseline. We reject our hypothesis that Akt will significantly increase following RE when compared to baseline.

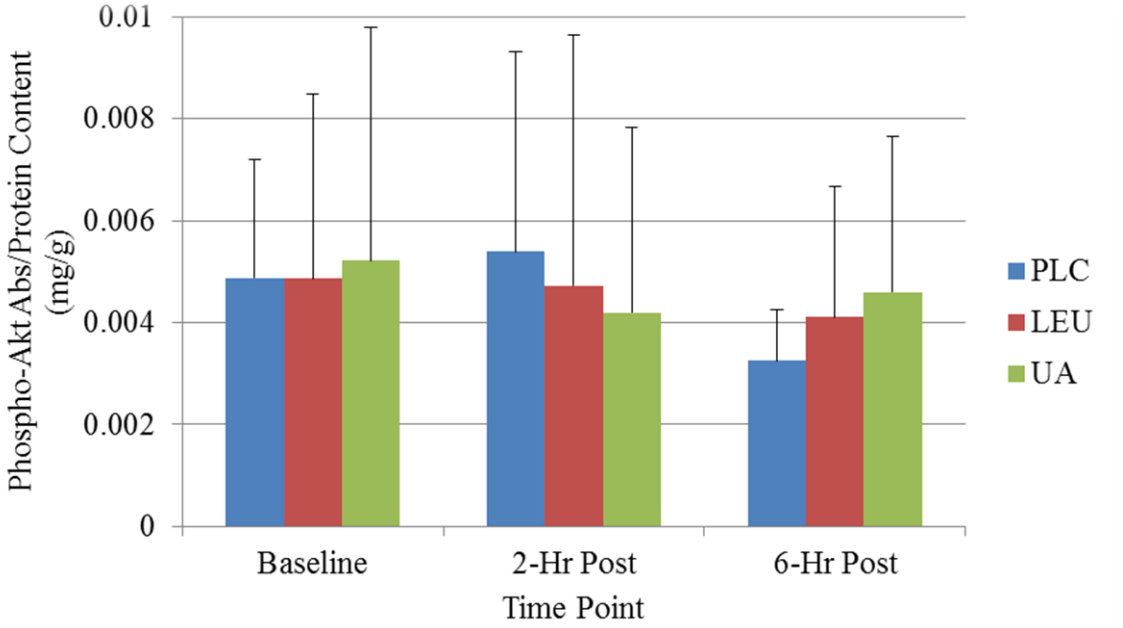

Figure 5: Mean ± SD Phosphorylated-Akt for each time point and supplement. Hr = hours.

Phosphorylated-mTOR

Phosphorylated-mTOR expressed as mean ± SD is presented in Figure 6, and table 4. The main effect for Supplement demonstrated a statistically significant increase for the UA condition when compared to LEU and PLC [F (2, 27) = 7.159, p =.001, partial η^2 =.166]. Post-hoc analysis revealed a significant increase in phosphorylated-mTOR under the UA condition as compared to LEU (p = .001), but not PLC (p= .305). However, analysis of delta scores show demonstrated a significantly increased phosphorylated mTOR content in the LEU condition compared to UA and PLC (p

=0.001). Therefore, we fail to reject our hypothesis that mTOR will not significantly increase compared to baseline in any of the three groups following RE. The main effect for Time revealed no statistically significant difference in [F (2, 27) = .463, p = 0.631, partial η^2 = .013]. Additionally there was no statistically significant interaction between Time and Supplement [F (2, 27) = .692, p = 0.600, partial η^2 = .037]. We reject our hypothesis that mTOR will significantly increase following RE when compared to baseline

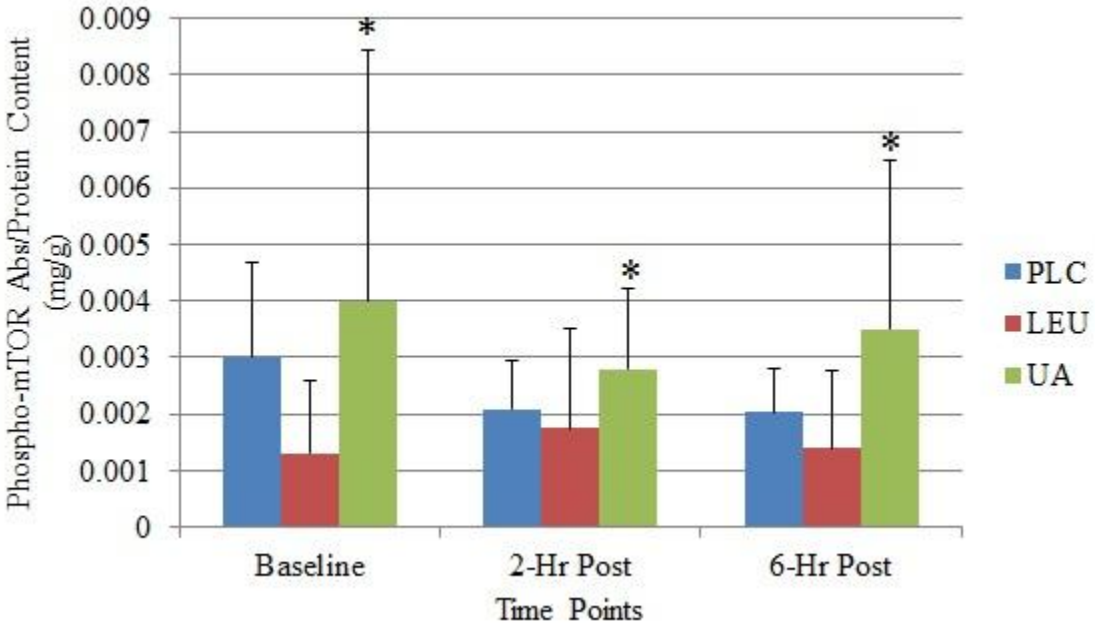

Figure 6: Mean ± SD Phosphorylated-mTOR for each time point and supplement. Hr = hours. * = significantly increased from LEU.

Phosphorylated-P70S6 Kinase

Phosphorylated-P70S6K expressed as mean ± SD is presented in Figure 7, and table 4. Main effects no revealed statistically significant differences for Time [F (2, 27) = 1.327, p = .272, partial η^2 = .036], Supplement [F (2, 27) = 1.643, p = .201, partial η^2 = .044], and the Time x Supplement interaction [F (4, 27) = .552, p = .698, partial η^2 =

.030]. We failed to reject our hypothesis that no significant difference will exist between groups at baseline. We reject our hypothesis that p70S6K will significantly increase following RE when compared to baseline.

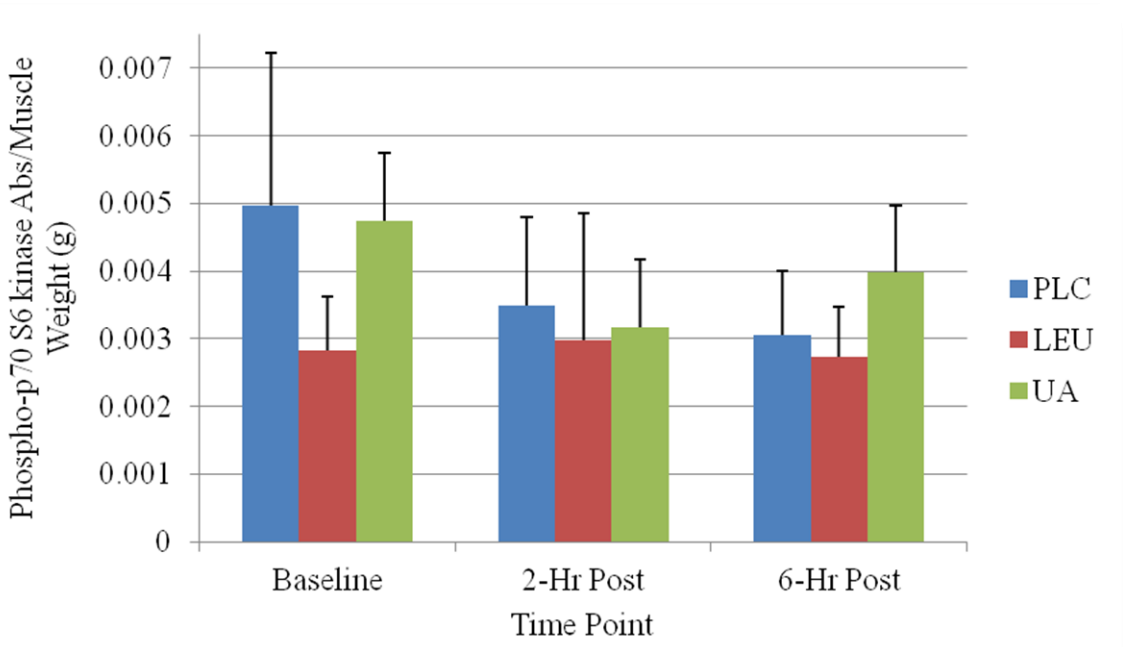

Figure 7: Mean ± SD Phosphorylated-p70S6 Kinase for each time point and supplement. Hr = hours.

CHAPTER FIVE

Discussion

Introduction

Recently, ursolic acid has emerged as a potential natural anabolic agent to prevent muscle atrophy and increase muscle mass and strength by enhancing skeletal muscle insulin/IGF-1 signaling in whole animals (Kunkel et al., 2011). Muscle contractions during RE are known to increase muscle anabolism, with repeated bouts leading to an accretion of muscle proteins (Pasiakos, 2012; Philp, Hamilton, & Baar, 2011). Due to the novel nature of ursolic acid as a supplement, there is a large gap in the literature in regards to how ursolic acid's mechanistic function carries out these effects. In the exercise/sport nutrition industry, UA supplementation is now being marketed as an effective means of increasing muscle mass, comparable or even superior to the effects know to occur with leucine supplementation.

The amount of literature available in regards to altered phosphorylation levels of signaling proteins in response to RE in animal models is lacking, and the amount investigating human models in relation to ursolic acid supplementation is almost non-existent. Due to this void in the literature, the exact intracellular mechanism through which the reported effects of ursolic occur remains unknown; it is speculated to stimulate MPS by activating the Akt/mTOR signaling pathway. Compared to ursolic acid, leucine has been investigated to a greater degree in relation to the mTOR signaling pathway via mTORC1 (Todd & Dee, 2012; Jewell, Russell, & Guan, 2013). As a result, in the present

study this provided the impetus in which to use leucine supplementation as a comparator in our attempts to decipher the mechanism of ursolic acid on the mTOR signaling pathway. The purpose of the present study was to compare the effects of a single 3 gram dose of ursolic acid and leucine supplementation immediately following RE on serum regulators of MPS and to also determine the effects of various markers of skeletal MPS on the Akt/mTOR pathway. In general, we found that the reported effects of UA on Akt/mTOR signaling pathway proteins of previous studies (Kunkel et al., 2011; Ogasawara et al., 2013) were not seen; rather the current study saw similar responses to Ou et al. (2014), whom identified ursolic acid as a direct negative regulator of the mTORC1 pathway.

Participants

Participants in the present study were resistance-trained young men with a mean age of 22.8 ± 4.4 (± SD) years, mean body mass of 83.5 ± 19.0 kg, mean lean mass of 65.3 ± 11.7 kg, and a mean fat mass of 16.15 ± 9.72 kg. Repeated bouts of RE will lead to adaptations of increased muscle proteins, mass, and strength (Pasiakos, 2012; Philp, Hamilton, & Baar, 2011). Therefore, in the current study it was important to ensure that all participants were appropriately resistance-trained. For this study, the participants reported that they had been resistance training with a mean ± SD of 8.1 ± 2.9 years. To better define the resistance training status of the participants, an effort was made to determine the effectiveness of the participants' resistance training history in addition to the duration of resistance training experience. Relative strength may be a suitable indicator of resistance training history because of its ability to measure muscular strength, a known adaptation to resistance training, without the influence of body mass (Baker et

al., 2013). For the current study, relative strength measurements on the leg press (calculated as maximal leg press divided by body weight) demonstrated the average relative strength score to be 4.6 ± 1.1. The Fitness Institute of Texas at The University of Texas at Austin has compiled leg press norms for 18 to 29 year old men using a 45º leg press machine similar to the one employed in the current study (Fitness Institute of Texas, 2011). According to their data, a relative strength value of 4.55 or above ranks a male between 18-29 years of age at the 90th percentile. The lowest score of the current study, 3.6 would rank in between the 50th and 60th percentile. Therefore all participants were above average for relative strength as measured by leg press.

Serum Concentrations of Leucine

The current study was designed to fill the gaps in the literature regarding UA's ability to increase mTOR signaling activity. Even though the UA condition did not receive any type of leucine supplement, at each time point serum leucine concentrations were significantly higher for the UA condition compared to the PLC and LEU conditions. LEU had increased serum concentrations from baseline at 30-min and 2-Hr post, slightly lowered levels at the 6-Hr post compared to baseline, and was significantly increased at the 2-Hr post time point as compared to baseline. PLC exhibited slight decreased serum leucine concentrations from baseline at every time point measured. Despite UA exhibiting statistically significant increased serum leucine concentrations for all time points measured, this was due to the fact that the UA had significantly higher baseline concentrations as compared to PLC ($p < 0.01$) and LEU ($p < 0.01$). Analysis of within supplement group delta scores from baseline, a Tukey post-hoc test revealed the only

supplement condition that caused a significant increase in serum leucine concentrations was LEU (p = .026) at 2-Hr post.

The serum leucine results in the current study are not surprising, as ingestion of leucine should be expected to cause an increase in serum leucine concentrations. There is a paucity of data related to the effects ursolic acid on serum leucine concentrations; although, as expected previous work has shown leucine to cause increased blood leucine levels. Glynn et al., 2010 demonstrated that 3.5 g leucine resulted in significantly increased serum leucine levels as compared to a control of 1.8 g.

Serum Concentrations of IGF-1

No significant changes occurred within or between groups for serum IGF-1 concentrations. A high level of variance was observed for all Supplement and Time factors; the overall mean was 16.03 nmols per mL of serum. While there were no significant changes, all supplement conditions exhibit increased serum IGF-1 levels 30-mins following RE. LEU slightly increased again from 30-min following RE to 2-Hr following RE, and remained elevated above baseline at 6-HR following RE. PLC maintained its level at 2-Hr following RE, and UA dropped below baseline levels at 2-Hr and 6-Hr following RE.

The lack of significant findings is not surprising, as ursolic acid and leucine have not been demonstrated to cause increases in serum IGF-1 concentrations, but rather increased skeletal muscle insulin/IGF-1 signaling and IGF-1 mRNA expression (Kunkel et al., 2011; Kunkel et la., 2012; Ogasawara et al., 2013). The blood serves as the method of transport of hepatically-derived IGF-1 to skeletal muscle, and plasma IGF-1 levels reflect GH-mediated hepatic IGF-1 production (Yakar et al., 1999). Increased IGF-1

serum concentrations would indicate increased mTOR pathway signaling due to IGF-1 being a potent pathway up-regulator through IGF-1R activation (Sakuma & Yamaguchi, 2011).

The results of the current study agree with the work done by Ogasawara et al. (2013), which demonstrated UA alone, or in combination with RE, was unable to elicit a change in serum IGF-1 concentrations. Kunkel et al. (2011) which supplemented a HFD with 0.27% ursolic acid compared to a HFD only, found no significant increases in serum IGF-1 over the course of 7-week period. Crozier et al. (2005) demonstrated LEU can stimulate MPS in the absence of insulin and IGF-1, but in order to maximize MPS in skeletal muscle the presence of circulating IGF-1 and insulin is required. Kunkel et al. (2011), using serum-starved C2C12 skeletal myotubes, demonstrated that IGF-1 and insulin are needed in order for ursolic acid to enhance IGF-1R, Akt, and p70S6K phosphorylation. Data from their study suggest that ursolic acid first enhances the capacity of pre-existing IGF-1 to activate skeletal muscle IGF-1R's. Thus, if the capacity of IGF-1R's is increased, it is reasonable to assume that increased serum IGF-1 concentration would activate its trans-membrane skeletal muscle IGF-1R's. In the current study, UA was associated with decreased serum IGF-1 levels, which may partly explain the observed decrease in IGF-1R phosphorylation/activity.

Phosphorylated- IGF-1R ^{Tyr1131}

Skeletal muscle levels of phosphorylated- IGF-1R did not exhibit any significant changes for Supplement, Time, or Supplement x Time factors. Contrary to the presumption by Kunkel et al. (2011) that ursolic acid supplementation would result in increased phosphorylated- IGF-1R levels, the current study demonstrated decreased IGF-

1R activity at 2-Hr and 6-Hr following RE in the UA condition, compared to baseline. The same trend was seen with the PLC condition; whereas, LEU demonstrated a slight increase at 2-Hr following RE. However, 6-Hr following RE the LEU condition had dropped below baseline levels. As noted by both Kunkel et al. (2011) in their myotubes model, increased IGF-1 R phosphorylation did not occur in the absence of IGF-1. Therefore, the lack of increased phosphorylated IGF-1 R in the current study may partly be explained by the lack of increased IGF-1 serum concentrations.

Although the current study reported conflicting data in regards to UA condition increasing IGF-1R activity compared to that reported by Kunkel et al. (2011), differences in study design between the two studies may, in part, account for this disagreement. The current study used resistance-trained male human participants, whereas Kunkel et al. (2011) utilized an *in-vitro* C2C12 myotube model to examine phosphorylated- IGF-1R. The probable causes for the difference in outcome occurring between *in-vivo* human models and *in-vitro* models are quite abundant and may be explained by self-reported dietary logs, lack of control over environmental stressors from one participant to the next, circadian rhythms, etc. Currently, it is hard to know if chronic ursolic acid supplementation will cause increased ursolic acid levels in the body. A study by Zhu et al. (2013) demonstrated plasma ursolic acid concentrations did not significantly increase in patients (whose levels were significantly lower than healthy volunteers) with advanced solid tumors due to 14 days of intravenous injection of 74 mg/m^2.

Ogasawara et al. (2013) utilized a RE protocol in their rodent model study; however, unlike the current study a non-weight bearing method was used. An isometric protocol was used where rats were stimulated with electrodes place subcutaneously for

ten 3-second contractions, with a 7-second interval between contractions, per set for 5 sets, with 3-minute rest intervals (Ogasawara et al., 2013). The current study utilized a resistance protocol that incorporated load-bearing concentric and eccentric contractions.

Phosphorylated-Akt ^{Thr308}

No significant differences in the Supplement and Time factors occurred for phosphorylated Akt levels in skeletal muscle. In the current study, the results observed for the PLC condition are in agreement with the findings of Kunkel et al. (2011) and Ogasawara et al. (2013). Similar to IGF-1R, UA resulted in lower levels at 2-Hr and 6-Hr following RE as compared to baseline levels. Observed values for LEU were similar to the effects that occurred in phosphorylated- IGF-1R activity. The results of the current study disagree with the findings of Ogasawara et al. (2013) and Kunkel et al. (2011); both studies demonstrated UA to increase phosphorylated Akt at Thr^{308}. Specifically, Ogasawara et al. (2013) reported increased Akt levels at 1-Hr and 6-Hr following RE, whereas Kunkel et al. (2011) demonstrated that a HFD enriched with 0.27% ursolic acid for 16-weeks was able to increase phosphorylated skeletal muscle Akt levels by 1.8 fold.

Similar to the case of phosphorylated IGF-1R, the difference in study designs as previously mentioned is a plausible explanation for the difference in results. Non-human participant models allow for a much tighter control of the intramuscular environment as compared to human models. Of further note, the current study administered ursolic acid orally immediately following RE; however, Ogasawara et al. (2013) injected ursolic acid intraperitoneally immediately after RE. Intraperitoneal delivery is a parenteral route of administration; absorbed by the mesenteric vessels which drain into the portal vein and

through the liver (Lukas, Brindle, & Greengard, 1971; Turner, Brabb, Pekow, & Vasbinder, 2011).

The difference in routes of administration is a conceivable explanation for the difference in signaling response between Ogasawara et al. (2013), and the current study. Both methods allow for first pass hepatic clearance, and tissue distribution data from Chet et al. (2011) suggest ursolic acid is absorbed in the liver; which may indicate a first-pass effect. However, intraperitoneal administration will bypass the stomach and intestines, and as Turner et al. (2011) states, "Parental routes also circumvent some of the unpredictability associated with enteral absorptive processes." Ursolic acid has similar molecular properties to midazolam: low bioavailability, lipophilic, and potential first pass effect (Chet et al., 2011; Lau et al., 1996). Pharmacokinetic data of midazolam demonstrated that intraperitoneal injection enhanced the bioavailability and shorten time to peak concentration as compared to oral administration (Chen et al., 2011; Lau et al., 1996; Zhu et al., 2013). Intraperitoneal administration of ursolic acid in Ogasawara et al. (2013), compared to oral administration in the current study, is a plausible explanation for some of the differences between the two studies.

The improved bioavailability, and increased rate to maximum concentration is an important consideration due to hormonal responses to RE, one of which being GH. GH acts as a potent regulator of hepatic IGF-1 stimulation, and does not remain elevated for prolonged periods following RE. A recent study demonstrated that the half-life of endogenous GH in men to be 18.9 ± 0.8 (±SE) minutes by monoexponential analysis (Faria, Veldhuis, Thorner, & Vance, 2013). If ursolic acid does in fact arrive to muscle within GH's half-life, ~19 minutes (Faria et al., 2013); intraperitoneal administration of

midazolam resulted in max concentration being reach in less than 7 minutes, then it is plausible to assume that the effects observed Ogasawara et al. (2013) could involve a GH-mediated mechanism.

Recent data has been published utilizing C2C12 myotubes observed significantly increased phosphorylated p70S6K, 4E-BP1, and Akt due to insulin treatment, which was then suppressed by ursolic acid treatment (Ou et al., 2014). This corresponds with earlier work by Kunkel et al. (2011), which also demonstrated insulin was necessary for ursolic acid to significantly increase insulin receptor activity. In the current study, participants ingested an Atkins© Advantage™ caramel chocolate nut roll (which claims three grams of net carbohydrates and 7 grams of protein) 1-Hr prior to both the baseline and 6-Hr following RE muscle biopsy sample collection time points. The significance being, participants in the current study would have had low blood sugar levels due to the fasted state, and thus low circulating insulin levels. This may have been the cause for the lack of response seen in Akt/mTOR pathway activity in response to both ursolic acid and leucine supplementation immediately following an acute bout of RE (Crozier et al., 2005, Kunkel et al., 2011). However, in the current study we wanted a low insulin state; in order to investigate the effects of only the three supplement conditions in combination with a RE session. This was based on the premise that phosphorylation of mTOR is able to occur through an insulin-induced activation of the PI3K/Akt pathway (Byfield, Murray, & Backer, 2005). Previous work has shown that exogenous AA supplements can cause a more robust anabolic response which generally exceeds those elicited by exercise performed in the absence of nutrition (Drummond et al., 2009). Therefore, prevention of high insulin levels was necessary to compare the effects of the UA and LEU conditions

on their own. By using participants in a fasted state, the current study was able to provide insight to the possible mechanism through which ursolic acid effects occur. Based off data from the current study it appears that ursolic acid's reported effects do not occur on its own, but rather in a hormone-mediated mechanism.

Ogasawara et al. (2013) states of both the Kunkel et al. (2011 & 2012) studies: "chronic ursolic acid injection led to an increase in the phosphorylation of at Akt at Ser^{473}, we can infer that accumulative ursolic acid stimulation may be required to phosphorylate the Ser^{473} site of Akt *in- vivo*." The current study did not examine the Ser^{473} phosphorylation site of Akt, only the Thr^{308} site. Phosphorylation at Thr^{308} is necessary for Akt activation, but complete activation of Akt requires phosphorylation of Akt Ser^{473}. Despite Ogasawara et al. (2013) and Kunkel et al. (2011) demonstrating different results as compared to the current study, the difference in the route of ursolic acid administration and the current study not measuring Akt Ser^{473} phosphorylation site may explain some of these differences.

Phosphorylated-mTOR^{Ser2448}

Phosphorylated mTOR at Ser^{2448} resulted in a significant increase for the Supplement and Time factors for the UA condition. The levels of mTOR for the UA condition were elevated at every time point as compared to LEU and PLC. However, this was due to the UA condition starting with significantly higher baseline values as compared to both PLC and LEU conditions, which remained elevated throughout the subsequent time points, yet did not change. Closer examination of the delta scores from baseline provides greater insight as to the actual changes for mTOR activity. The PLC condition demonstrated decreases from baseline at 2-Hr and 6-Hr following RE,

indicating decreased mTOR activity post-exercise. However, the LEU condition, despite having the lowest baseline levels, resulted in the greatest increase in mTOR activity at 2-Hr and 6-Hr post-exercise. These results indicate that LEU was the only supplemental condition which resulted in increased mTOR activity following RE. Viewing delta scores demonstrates that the UA in conjunction with an acute bout of RE did not elicit increased activity the Akt/mTOR pathway, seen in previous studies (Kunkel et al., 2011; Kunkel et al., 2012; Ogasawara et al., 2012). This can be attributed to the lack of increased Akt activity in the UA seen in the current study; as Akt can activate mTOR through two separate signaling pathways (Byfield, Murray, & Backer, 2005).

However, differences in study designs continues to provide plausible reasons as to why the current study did not demonstrate the findings of similar studies (Kunkel et al., 2011; Ogasawara et al., 2013). Another conceivable explanation as to why the UA condition was unable to increase mTOR signaling is, much like Akt, an acute bout of RE is unable to provide enough of a stimulus in humans for ursolic acid's effectiveness. This assertion is supported by Hamilton et al. (2010) who demonstrated that an acute RE bout did not enhance PI3K/Akt/mTORC1 signaling. As such, multiple bouts of RE may be necessary in order for ursolic acid to have a significant effect on mTOR signaling (Pasiakos, 2012; Philp, Hamilton, & Baar, 2011). The higher protein content that comes about due to repeated bout of RE could increase ursolic acid effectiveness; due to the poor bioavailability of ursolic acid in the body. The accretion of the mTOR signaling pathway proteins would provide an increased number of targets for ursolic acid.

Phosphorylated-p70S6K^{Thr389}

Phosphorylated p70S6K did not reveal any significant changes for the Supplement and Time factors, and was similar to IGF-1R and Akt activity for all supplement conditions. PLC and UA conditions decreased p70S6K activity at 2-Hr and 6-Hr following RE as compared to baseline. The LEU condition slightly increased at the 2-Hr time point, which is consistent with data demonstrating peak MPS occurring at 2-hr post leucine ingestion (Bohe, Low, Wolfe, & Rennie, 2001; Atherton et al., 2010). The current study agrees with the results found by Ogasawara et al. (2013) which demonstrated UA alone did not increase the phosphorylation of p70S6K.

In the current study, LEU and UA conditions combined with an acute bout of RE did not result in significant increases in p70S6K activity as compared to PLC. Previous worked performed by Miyazaki et al. (2011) demonstrated p70S6K activity can be increased with an acute bout of RE. Kunkel et al. (2011) reported that UA enhanced IGF-1- and insulin-mediated p70S6K activity. However, West et al. (2009) demonstrated that a 10-fold increase in IGF-1 was unable to increase p70S6K phosphorylation and MPS as compared to a control group. This is in accordance with earlier work performed by Hornberger et al. (2004) demonstrating that p70S6K activation can occur due to RE with IGF-1 inhibition, and that p70S6K activation is not directly IGF-1-dependent. This taken all together rules out the possibility that the lack of increased IGF-1R activity or IGF-1 serum concentrations were the reason that, in the current study, we did not witness increased p70S6K activity. The lack of increased p70S6K signaling in the current study is most likely due to the lack of increased Akt/mTOR signaling seen, and the associated explanations.

Conclusions and Future Directions

Ursolic acid supplementation has been demonstrated to cause a number of beneficial effects including but not limited to: antioxidant, anti-inflammatory, trypanocidal, anti-heumatic, antiviral, and anti-tumoral properties. The focus of the current study was to try and replicate the effects seen in previous non-human models, which demonstrated increased Akt/mTOR pathway signaling due to ursolic acid (Kunkel et al., 2011; Kunkel et al., 2012; Ogasawara et al., 2013).

The current study did demonstrate significant increase serum leucine concentration for all measured time points in the UA condition compared to PLC (p = .000) and LEU (p = .000). We also observed a significant increase in phosphor-mTOR skeletal muscle content due in the UA condition compared to LEU (p = .001), but not PLC (p= .305). However, both of these significant findings are misleading, which is revealed by an analysis of delta scores. UA condition demonstrated decreased serum leucine concentrations and phosphorylated mTOR skeletal muscle content across all the time points measured. The significant result was due to significantly increased levels at baseline. The LEU condition demonstrated increased serum leucine concentrations and phosphorylated mTOR from baseline to the 2-Hr following RE; significance occurred at 2-Hr following RE.

The difference in study designs and the associated issues that occur when using humans as subjects instead of rats and myotubes provide plausible reasoning for the lack of increased signaling in the current study. Ou et al. (2014) demonstrated that ursolic acid supplementation inhibited leucine-stimulated mTOR activation in C2C12 myotubes, which occurred by inhibiting mTOR from targeting the lysosome. To observe the effects

of ursolic acid alone, pre-treatment of the myotubes with ursolic acid had little effect on Raptor, Rictor, and mTOR protein levels. These results together indicate that the mechanism reported in Ou et al. (2014) may have played a role in what prevented a UA-induced increase in mTOR pathway signaling in the current study.

As mentioned previously, the amount of literature investigating the effects of ursolic acid on Akt/mTOR pathway activity in humans is sparse. Consequently, results from the current study suggest that the UA condition, at the dose provided, does not increase mTOR signaling. However, currently this is the only study that has examined the effects of ursolic acid supplementation after RE on the mTOR signaling pathway in human participants. Therefore, it is premature to rule out the possibility of ursolic acid supplementation directly increasing mTOR pathway signaling altogether, as more research in this area is warranted. There remains a plethora of possible routes through which ursolic acid might exhibit the effects reported by Kunkel et al. (2011) and Ogasawara et al. (2013), and these routes also warrant further investigation.

APPENDICES

APPENDIX A

Informed Consent Form

BAYLOR UNIVERSITY

Department of Health, Human Performance, & Recreation

Informed Consent Form

Title of Investigation: A Comparison of the Effects of Ursolic Acid and L-Leucine Supplementation on Markers of Muscle Protein Synthesis via Akt/mTOR signaling in Response to Resistance Exercise

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Sponsors: Exercise and Biochemical Nutrition Lab (Baylor University)

Rationale:

Resistance exercise (RE) stimulates skeletal MPS to occur during post-exercise recovery. For example, the mammalian target of rapamycin (mTOR) signaling pathway is activated during and after a single bout of RE, which was associated with a significant increase in MPS. Essential amino acids (EAAs) in nutrient supplements serve as substrates for new protein synthesis, stimulate insulin release, and largely independent of insulin, directly activate the nutrient-sensitive mTOR signaling pathway. However, of the EAAs, leucine independently stimulates MPS by activating components of the mTOR signaling pathway.

Recent research has discovered a natural compound which also appears to stimulate MPS by activating the mTOR signaling pathway, just as with the amino acid, leucine. This compound is called ursolic acid, which is a natural pentacyclic triterpenoid carboxylic acid that is widely found in apple (a major compound found in apple skin) and other fruits such as cranberries. It is known to exhibit a wide range of biological functions such as anti-oxidative, anti-microbial, anti-inflammatory, and anti-cancer. A recent study based on microarray analysis identified ursolic acid as an inhibitor of muscle atrophy in humans and rodents, and also revealed that muscle hypertrophy in rodents occurs by daily ursolic acid consumption. A more recent study has shown that ursolic acid given immediately after isometric exercise in rats was effective in increasing the activity of mTORC1, a key signaling regulator in MPS.

Therefore, the purpose of this study is to compare the effects of ursolic acid and leucine supplementation given immediately after RE on serum regulators of MPS and to also determine the effects on various markers of MPS involved in the Akt/mTOR signaling pathway.

Description of the Study:

I will be one of 10 apparently healthy, resistance-trained males between the ages 18 to 30 who will participate in this study. I understand that I will be required to visit the laboratory on 7 separate occasions in the following manner: visit 1 = entry/familiarization session, visit 2 = testing/ RE session 1, visit 3 = 6 hour follow-up for session 1, visit 4 = testing/ RE session 2, visit 5 = 6 hour follow-up for session 2, visit 6 = testing/ RE session 3, visit 7 = 6 hour follow-up session for session 3. Relative to the testing sessions (visits 2, 4, 6), I will perform a RE session involving the angled leg press, and knee extension exercises on three occasions separated by two weeks. One session will constitute the control/placebo session and the other two will be the experimental sessions involving either ursolic acid or leucine. At each of the seven visits, I will have my heart rate and blood pressure assessed. At visits 2 – 7, I will also have blood and muscle samples obtained.

During an initial familiarization session (visit 1), I will be informed of the requirements of the study and sign an informed consent statement in compliance with the Human Subjects Guidelines of Baylor University and the American College of Sports Medicine. Trained study personnel will examine me to determine if I am qualified to participate in this study. If I am cleared to participate in the study, I will be familiarized to the testing procedures and will then undergo assessments for body composition and muscle strength on the squat, angled leg press, and knee extension exercises. This session will take approximately 90 minutes to complete. Once I complete the familiarization session, I will be scheduled for the first RE session (visit 2).

During the familiarization session, I understand that I will have my maximum muscle strength determined on the angled leg press, and knee extension exercises. I will warm up by completing 5 to 10 repetitions with a very light weight and then complete three to five repetitions with a heavier weight. The weight will then be increased conservatively, and I will attempt to lift the weight for one repetition. If the lift is successful, I will rest for two minutes before attempting the next weight increment. This procedure will be continued until I fail to complete the lift. My maximum strength will be recorded as the maximum weight I am able to lift for one repetition.

In addition, I will have my body composition (body fat and muscle mass) determined using dual-energy x-ray absorptiometry (DEXA). The DEXA body composition test will involve me lying down on my back in a comfortable position in a pair of shorts/t-shirt. A low dose of radiation will then scan my entire body for approximately 6 minutes. Radiation exposure from the DEXA is approximately 1.5 mR per scan. This is similar to the amount of natural background radiation I would receive in one month while living in Waco, TX. The maximal permissible x-ray dose for non-occupational exposure is 500 mR per year. Total radiation dose will be less than 5 mR for the entire study.

Following the familiarization session, I will be instructed to refrain from exercise for 48 hours prior to each RE session. I will also be informed to eat a light breakfast on the morning of, and two hours before, each testing session. I will be provided with a dietary analysis form that I am to complete for 4 days prior to each RE testing session (including the light breakfast on the morning prior to the testing session). Once I report to the lab for each testing session, I will turn in my dietary analysis form.

I understand that I will be required to participate in a three separate testing sessions (visits 2, 4, 6) consisting of the angled leg press, and knee extension exercises where I will perform 4 sets of 8-10 repetitions at 75% - 80% of my maximum strength.

During the study, I understand that I will donate about 20 milliliters (4 teaspoons) of venous blood from a vein in my arm using sterile techniques by an experienced phlebotomist involving standard procedures, just as if I were to have my blood drawn by my physician. This procedure may cause a small amount of pain when the needle is

inserted into the vein as well as some bleeding and bruising. However, proper pressure will be applied upon removal to reduce bruising. I understand that I may also experience some dizziness, nausea, and/or faint if I am unaccustomed to having blood drawn. I understand that personnel who will be performing the blood draws are experienced in phlebotomy (procedures to take blood samples) and are qualified to do so under guidelines established by the Texas Department of Health and Human Services. At visits 2, 4, and 6 (RE /testing sessions 1, 2 and 3) I will have blood obtained at three different time points. In addition, 6 hours after each session (visits 3, 5, and 7) I will have one blood sample obtained.

In addition to the blood draws, I will undergo the muscle biopsy. I understand that I will have the biopsy location identified on the thigh. The biopsy area will be shaved clean of leg hair, washed with antiseptic soap and cleaned with rubbing alcohol. In addition, the biopsy site will be further cleansed by swabbing the area with Betadine (fluid antiseptic). I understand that a small area of the cleaned skin approximately 2 cm in diameter will be anesthetized with a 1.0 mL subcutaneous injection of the topical anesthetic Lidocaine. Once the local anesthesia has taken effect (approximately 2-3 minutes) the biopsy procedure will only take 15-20 seconds. Once anesthetized, I understand that 14 gauge fine needle biopsy needle will be used to puncture the skin. Due to the localized effects of the anesthetic, however, I should feel no pain during this process. At this point, I understand that the biopsy needle will be advanced into the puncture approximately 1 cm and during this part of the procedure I may feel pressure in my thigh area. Once the muscle sample has been obtained, pressure will be immediately applied to the incision. With the puncture being so small, bleeding is slight; therefore, only an adhesive bandage is needed to cover the puncture. I understand that I will be provided verbal and written instructions for post-biopsy care. I understand that I will be instructed to leave the adhesive bandage in place for 12 hours. I will be further advised to refrain from vigorous physical activity with my leg during the first 24 hours post-biopsy. I understand that if I feel it necessary I may take a non-prescription analgesic medication such as acetaminophen to relieve pain if needed and that some soreness of the area may occur for about 24 hours after the biopsy. I will also be advised to avoid such medications such as aspirin, Advil, Bufferin, Nuprin, or Ibuprofen as they may lead to bruising at the biopsy site. I understand that I will be asked to undergo the muscle biopsy procedure twice at each of the three testing sessions, and once after each testing session. As a result, I understand that I will undergo a total of nine muscle biopsies throughout the study. I understand that personnel who will be performing the muscle biopsies are experienced in the procedure and are qualified to do. At visits 2, 4, and 6 (RE /testing sessions 1, 2 and 3) I will have a muscle biopsy obtained at two different time points (before and 2 hours following RE). In addition, 6 hours after each session (visits 3, 5, and 7) I will have one muscle biopsy sample obtained.

I understand that when I report to the laboratory for the three testing/ RE sessions on visits 2, 4, and 6, I will turn in my 4-day dietary records. In addition, I will have my heart rate and blood pressure determined, and will also provide blood and muscle samples. I understand that if clinically significant side effects are reported from my participation in the study, I will be referred to discuss the problem with Darryn Willoughby, Ph.D. Upon his discretion, I may be referred to discuss the matter with my primary care physician to determine whether any medical treatment is needed and/or whether I can continue in the study. I understand that if I fail to report my progress and health status to the research assistant I may be removed from the study.

I agree to do my best to: 1) follow the instructions outline by the investigators; 2) show up to all scheduled testing times; and 3) put forth my best effort as instructed. I agree not to take any other nutritional supplements or performance enhancing aids during this study (i.e. vitamins/minerals, creatine, HMB, androstenedione, DHEA, etc). In addition, I agree not to take any non-medically prescribed medications and to report any medication that is prescribed for me to take during this study. I understand that if I take any nutritional supplements or medications during the course of the study that I will be removed from the study.

Exclusionary Criteria

I understand that in order to participate in the study, trained research personnel will examine me to determine whether I qualify to participate. I understand that I will not be allowed to participate in this study if: 1.) I have any known metabolic disorder including heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism; 2.) I have a history of pulmonary disease, hypertension, liver or kidney disease, musculoskeletal disorders, neuromuscular or neurological diseases, autoimmune disease, cancer, peptic ulcers, or anemia; 3.) I am taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic (ie, thyroid, insulin, etc), psychotropic, neuromuscular/neurological, or androgenic medications; 4.) I have any bleeding disorders; 5.) I have any chronic infections (e.g., HIV); 6) I have a known allergic reaction to topical anesthetics.

I have reported all nutritional supplements, medically prescribed drugs, and non-medically prescribed drugs that I am presently taking. I have reported whether I have had any prior allergic reactions to topical anesthetics. I have completed medical history questionnaires and am not aware of any additional medical problems that would prevent me from participating in this study. I agree to report all changes in medical status, nutritional and/or pharmacological agents (drugs) that I take during the course of the investigation to Darryn Willoughby, Ph.D. (254-710-3504). I understand that if I experience any unexpected problems or adverse events from participating in this study I

may be referred to discuss the problem with my primary care physician to determine whether any medical treatment is needed and/or whether I can continue in the study.

Risks and Benefits

I understand that there are minor risks of muscular pain and soreness associated with the resistance training protocol required in this study which are not uncommon to any exercise program especially for individuals who do not resistance train on a regular basis.

On 12 separate occasions during this study, I understand that I will have approximately four teaspoons (20 milliliters) of blood drawn from a vein in my forearm using standard procedures. All blood sampling will be performed by an experienced phlebotomist. This procedure may cause a small amount of pain when the needle is inserted into my vein as well as some bleeding and bruising. I may also experience some dizziness, nausea, and/or faint if I am unaccustomed to having blood drawn.

On nine separate occasions during this study (2 at each testing/exercise session and one 24 hours after each session), I understand that I will undergo a muscle biopsy in which a small sample of muscle will be obtained from the thigh of my exercised leg. I understand that Darryn Willoughby, Ph.D. or Neil Schwarz, M.S. will perform all of the biopsies and that a local anesthetic (Lidocaine) will be injected into the skin of my thigh prior to the biopsy, which will help prevent any pain and discomfort during the procedure. I understand that I will have a small puncture made in my skin and a biopsy needle introduced 1 cm into the puncture. I also understand that the puncture is so small that it will not require any stitches and will be simply covered with an adhesive bandage (band-aid). After the anesthetic wears off within 3-4 hours, I understand that the sensation at the biopsy site is comparable to that of a bruise and may persist for 12-24 hours after the procedure. I understand that I am required to inform the study investigators if I have had any prior allergic reactions to anesthesia (e.g. while in the hospital or during a dental visit).

Alternative Treatments

This is not a medical treatment. Therefore, if medical treatment is needed, I must obtain treatment for any medical problem I might have from my personal physician.

Costs and Payments

If I am a Baylor University student, I will not receive any academic credit for participating in this study. I understand that if I am an intercollegiate scholarship athlete I may not be eligible to receive payment to participate in this study. I will be paid $150 for completing the study. I also understand that I will be given free blood pressure, nutrition, and strength assessments during the course of the study as described above and may receive information regarding results of these tests if I desire.

New Information

Any new information obtained during the course of this research that may affect my willingness to continue participation in this study will be provided to me. In addition, I will be informed of any unusual/abnormal clinical findings in which medical referral to my personal physician may be warranted. If I desire, I may request that this information be provided to my physician.

Confidentiality

I understand that any information obtained about me in this research, including medical history, laboratory findings, or physical examination will be kept confidential to the extent permitted by law. However, I understand in order to ensure that FDA regulations are being followed, it may be necessary for a representative of the FDA to review my records from this study which may include medical history, laboratory findings/reports, statistical data, and/or notes taken about my participation in this study. In addition, I understand that my records of this research may be subpoenaed by court order or may be inspected by federal regulatory authorities. I understand that data derived may be used in reports, presentations, and publications. However, I will not be individually identified unless my consent is granted in writing. Additionally, that confidentiality will be maintained by assigning code numbers to my files, limiting access to data to research assistants, locking cabinets that store data, and providing passwords to limit access to computer files to authorized personnel only. I understand that once blood and muscle samples are analyzed that they will be discarded.

Right to Withdrawal

I understand that I am not required to participate in this study and I am free to refuse to participate or to withdraw from the study at any time. Further, that my decision to withdraw from the study will not affect my care at this institution or cause a loss of benefits to which I might be otherwise entitled. If there is concern about my medical safety, I may be referred to seek medical attention.

Compensation for Illness or Injury

I understand that if I am injured as a direct result of taking part in this study, I should consult my personal physician to obtain treatment. I understand that the cost associated with the care and treatment of such injury will be the responsibility of me or my insurance carrier. In some cases, insurers may not reimburse claims submitted for a research-related injury resulting from medical procedures or treatments performed as part of a research study. I understand that Baylor University, the investigator's institutions, and the grant sponsor have not budgeted funds to compensate me for injury or illness that may result from my participation in this study and thus will not be accountable for illness

or injury acquired during the course of this study. However, I may be referred to my personal physician if any clinically significant medical/psychological findings are observed during the course of this study. I agree to indemnify and hold harmless Baylor University, its officers, directors, faculty, employees, and students for any and all claims for any injury, damage or loss I suffer as a result of my participation in this study regardless of the cause of my injury, damage or loss.

Statement on Conflict of Interest

I understand that this study is funded by the Exercise and Biochemical Nutrition Laboratory at Baylor University, and that the researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

Voluntary Consent

I certify that I have read this consent form or it has been read to me and that I understand the contents and that any questions that I have pertaining to the research have been, or will be answered by Darryn Willoughby, Ph.D. (principal investigator, Department of Health, Human Performance & Recreation, 120 Marrs McLean Gymnasium, Baylor University, phone: 254-710-3504) or one of the research associates. My signature below means that I am at least 18 years of age and that I freely agree to participate in this investigation. I understand that I will be given a copy of this consent form for my records. If you have any questions regarding your rights as a participant, or any other aspect of the research as it relates to you as a participant, please contact the Baylor University Committee for Protection of Human Subjects in Research, Dr. David W. Schlueter, Ph.D., Chair Baylor IRB, Baylor University, One Bear Place #97368 Waco, TX 76798-7368. Dr. Schlueter may also be reached at (254) 710-6920 or (254) 710-3708.

Date _____ Participant's Signature _____

I certify that I have explained to the above individual the nature and purpose of the potential benefits and possible risks associated with participation in this study. I have answered any questions that have been raised and have witnessed the above signature. I have explained the above to the volunteer on the date stated on this consent form.

Date _____ Investigator's Signature _____

APPENDIX B

IRB Proposal

Application to the Baylor IRB

For Review of Research/Activity Proposal

Part 1: Signature Page

1. Name Darryn S. Willoughby, Ph.D., FACSM, FISSN
2. Email Address (optional) Darryn_Willoughby@baylor.edu
3. Complete Mailing Address P.O. Box 97313
4. Position Associate Professor
5. Faculty Advisor (if researcher is Graduate Student) _____
6. Department/School HHPR/SOE & Biomedical Science Institute
7. Telephone # x3504 FAX # x3527
8. Are you using participants in research (***Y*** or N) or in teaching exercises (Y or ***N***)?
9. Title of the research project/teaching exercise:

A Comparison of the Effects of Ursolic Acid and L-Leucine Supplementation on Markers of Muscle Protein Syntheis via Akt/mTOR Signaling in Response to Resistance Exercise.

10. Please return this signed form along with all the other parts of the application and other documentation to the University Committee for Protection of Human Subjects in Research: Dr. David Schlueter, Department of Communication, P.O. Box 97368, Waco, Texas 76798, phone number (254) 710-6920.

_____ _____

Signature of Principal Investigator Date

Signature of Faculty Advisor (required if researcher is a Graduate Student)

Departmental Review: _____

Department Chair or the Chair's Designate

Part 2: Introduction & Rationale

Resistance exercise (RE) stimulates skeletal MPS during post-exercise recovery [Dreyer et al., 2007]. For example, the mammalian target of rapamycin (mTOR) signaling pathway is activated during and after a single bout of RE, which was associated with a signficant increase in MPS [Dreyer et al., 2006]. Essential amino acids (EAAs) in nutrient supplements serve as substrates for new protein synthesis, stimulate insulin release, and largely independent of insulin, directly activate the nutrient-sensitive mTOR signaling pathway [Dennis et al., 2011]. However, of the EAAs, leucine independently stimulates MPS by activating components of the mTOR signaling pathway [Crozier et al., 2005].

Recent research has discovered a natural compound which also appears to stimulate MPS by activating the mTOR signaling pathway, just as with the amino acid, leucine. This compound is called ursolic acid, which is a natural pentacyclic triterpenoid carboxylic acid that is widely found in apple (a major compound found in apple skin) and other fruits such as cranberries. It is known to exhibit a wide range of biological functions such as anti-oxidative, anti-microbial, anti-inflammatory, and anti-cancer [Ikeda et al., 2008]. A recent study based on microarray analysis identified ursolic acid as an inhibitor of muscle atrophy in humans and rodents, and also revealed that muscle hypertrophy in rodents occurs by daily ursolic acid consumption [Kunkel et al., 2011]. A more recent study has shown that ursolic acid given immediately after isometric exercise in rats was effective in increasing the activity of mTORC1, a key signaling regulator in MPS [Ogasawara et al., 2013].

Therefore, the purpose of this study is to compare the effects of ursolic acid and leucine supplementation given immediately after RE on serum regulators of muscle protein syntehsis and to also determine in muscle the effects on various markers of MPS involved in the Akt/mTOR signaling pathway.

Part 3: Methodology

Methods

Experimental Approach

In a randomized, cross-over design, participants will visit the laboratory on 7 separate occasions in the following manner: visit 1 = entry/familiarization session, visit 2 = testing/ RE session 1, visit 3 = 6 hour follow-up for session 1, visit 4 = testing/ RE session 2, visit 5 = 6 hour follow-up for session 2, visit 6 = testing/ RE session 3, visit 7 = 6 hour follow-up session for session 3. Relative to the testing sessions (visits 2, 4, and 6), participants will perform a RE session involving the angled leg press, and knee extension exercises on three occasions separated by two weeks. One session will constitute the control/placebo session and the other two will be the experimental sessions involving either ursolic acid or leucine (Figure 1). This approach is based on the premise that since RE is known to increase MPS, the proposed experimental model will allow for the determination in the ability of ursolic acid and/or leucine to augment MPS when ingested immediately after RE.

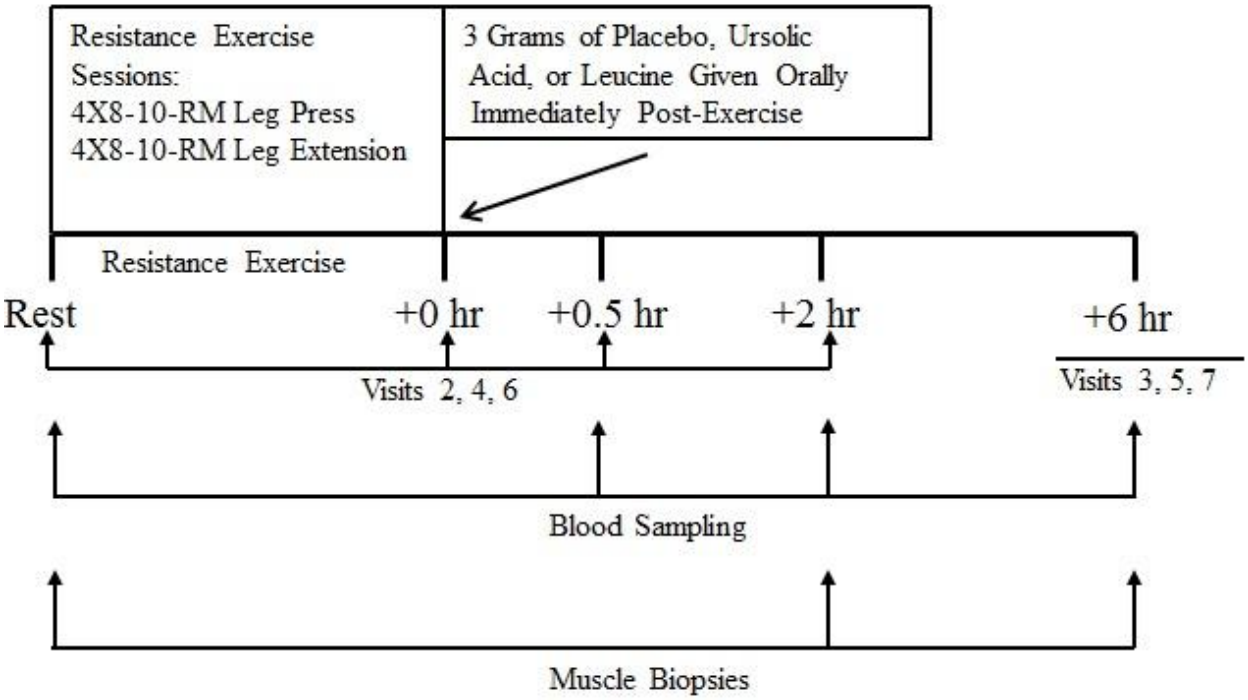

Figure 1. An illustration of the experimental protocol for the testing sessions during visits 2-7. Blood and muscle samples will be obtained at rest, +0.5 hr, +2 hr at visits 2, 4, 6, and at +6 hr for visits 3, 5, 7.

Participants

Ten apparently healthy, resistance-trained [regular, consistent resistance training (i.e. thrice weekly) for at least 1 year prior to the onset of the study], men between the ages of 18-30 will volunteer to serve as participants in this study. Enrollment will be open to men of all ethnicities. Only participants considered as low risk for cardiovascular disease and with no contraindications to exercise as outlined by the American College of Sports Medicine (ACSM), and who have not consumed any nutritional supplements (excluding multi-vitamins) one month prior to the study will be allowed to participate. All eligible participants will sign university-approved informed consent documents and approval will be granted by the Institutional Review Board for Human Subjects. Additionally, all experimental procedures involved in the study will conform to the ethical consideration of the Helsinki Code.

Study Site

All supervised testing and supplement assignment will be conducted in the Resistance Training and Assessment Laboratory (RTAL) at Baylor University. All sample analyses will be completed in the Exercise and Biochemical Nutrition Laboratory (EBNL) at Baylor University.

Independent and Dependent Variables

The independent variable will be the RE /supplementation protocol (placebo, ursolic acid, and leucine). Dependent variables in serum will include: ursolic acid, leucine, and IGF-1. In skeletal muscle, the variables will include: IGF-1 R, Akt, mTOR, p70S6k, and myofibrillar protein content.

Entry and Familiarization Session (Visit 1)

Participants expressing interest in participating in this study will be interviewed on the phone to determine whether they appear to qualify to participate in this study. Participants believed to meet eligibility criteria will then be invited to attend an entry/familiarization session. Once reporting to the lab, participants will complete a medical history questionnaire and undergo a general physical examination to determine whether they meet eligibility criteria. Participants meeting entry criteria will be familiarized to the study protocol via a verbal and written explanation outlining the study design and will undergo assessments for body composition and muscle strength assessments for the three lower-body exercises involved in the study. At the conclusion of the familiarization session, participants will be given an appointment in which to attend their first testing session. In addition, each participant will be instructed to refrain from exercise for 48 hours prior to each testing session, eat a light breakfast two hours prior to reporting for each testing session, and record their dietary intake for four days (including the light breakfast the morning of testing) prior to each of the three testing sessions involved in the study.

Muscle Biopsies (Visits 2-7)

Percutaneous muscle biopsies (50-70 mg) will be obtained from the middle portion of the vastus lateralis muscle of the dominant leg at the midpoint between the patella and the greater trochanter of the femur at a depth between 1 and 2 cm. After the initial biopsy, for the remaining biopsies attempts will be 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 successive incision that will be made approximately 0.5 cm to the former from medial to lateral. After removal, adipose tissue will be trimmed from the muscle specimens and will be immediately frozen in liquid nitrogen and then stored at -80°C for later analysis. Two muscle samples will be obtained at each of the three RE sessions and one 6 hours after each session, for a total of nine muscle samples being obtained during the course of the study. Muscle samples will be obtained: immediately prior to the commencing the testing session and 2 hours after exercise at visits 2, 4, and 6, and 6 hours after exercise at visits 3, 5, and 7.

Blood Sampling (Visits 2-7)

Venous blood samples will be obtained into 10 ml vacutainer tubes from a 20 gauge intravenous catheter inserted into the antecubital vein. Blood samples will be allowed to stand at room temperature for 10 min and then centrifuged. The serum will be removed and frozen at -80°C for later analysis. Three blood samples will be obtained at each of the three RE sessions and one 6 hours after each session, for a total of 12 blood samples being obtained during the course of the study. Blood samples will be obtained: immediately prior to the commencing the testing session, 0.5 hour after exercise, 2 hours after exercise at visits 2, 4, and 6, and 6 hours after exercise at visits 3, 5, and 7.

Body Composition Testing (Visit 1)

At the entry and familiarization session, total body mass (kg) will be determined on a standard dual beam balance scale (Detecto). Total body water (total, intracellular, and extracellular) will be determined with bioelectrical impedance [(BIA) Xitron 4200, San Diego, CA]. Percent body fat, fat mass, and fat-free mass, will be determined using using a calibrated Hologic 4500W dual-energy x-ray absorptiometry (DEXA). The DEXA will segment regions of the body (right arm, left arm, trunk, right leg, and left leg) into three compartments.

Dietary Analysis (Visits 2, 4, 6)

Participants will be required to record their dietary intake for four days prior to each of the two RE sessions. The participants' diets will not be standardized and participants will be asked not to change their dietary habits during the course of the study. The dietary recalls will be evaluated with the Food Processor dietary assessment software program to determine the average daily macronutrient consumption of fat, carbohydrate, and protein in the diet for the duration of the study.

Muscle Strength Assessments (Visit 1)

In order to determine muscular strength, participants will perform one-repetition maximum (1-RM) tests on the angled leg press, and knee extension exercises while attending the familiarization session. Participants will warm up by completing 5 to 10 repetitions at approximately 50% of the estimated 1-RM. The participant will rest for 1 minute, and then complete 3 to 5 repetitions at approximately 70% of the estimated 1-RM. The weight will then be increased conservatively, and the participant will attempt to lift the weight for one repetition. If the lift is successful, the participant will rest for 2 minutes before attempting the next weight increment. This procedure will be continued until the participant fails to complete the lift. The 1-RM will be recorded as the maximum weight that the participant is able to lift for one repetition.

Assessment of Heart Rate & Blood Pressure (Visits 1-7)

At visits 1-7, heart rate and blood pressure will be assessed. At the entry and familiarization session, these variables will be obtained as part of the health history assessment. At visits 2 and 4, heart rate and blood pressure will be obtained at each of the 7 time point where blood samples are obtained. Heart rate and blood pressure will also be obtained at visits 3 and 5. Heart rate will be determined by palpation of the radial artery using standard procedures. Blood pressure will be assessed in the supine position after resting for 5-min using a mercurial sphygmomanometer using standard procedures.

Résistance Exercice Protocol (Visits 2, 4, 6)

During each of the three RE sessions, participants will perform four sets of 8-10 repetitions with 75%-80% of the 1-RM on the angled leg press, and knee extension exercises. If muscle fatigue/failure occurs during a set, a spotter will provided assistance until the participant completes the remaining repetitions and resistance will be reduced for subsequent sets. In all cases, two minutes of rest will separate sets and exercises. All RE sessions will be supervised by study personnel.

Nutrient Supplementation Protocol (Visits 2, 4, 6)

In a randomized, doubled-blind fashion one of three nutrient supplements will be orally ingested in capsule form immediately after the completion of each RE session. Within eight gelatin capsules, all of the same size, shape, and color, the control/placebo supplement will consist of three grams of cellulose (Nutricology, Alameda, CA) and the two experimental supplements will consist of three grams of leucine and three grams of ursolic acid. Since the purity of many over-the-counter nutrient supplements may be questionable, both L-leucine and ursolic acid will be 99% pure, confirmed by analytical GC/MS analyses by the manufacturer (Sigma Chemical Company, St. Louis, MO).

Serum IGF-1 and GH Assessments

From the 12 blood samples obtained at the three RE sessions, GH and IGF-1 levels will be determined using commercially available enzyme-linked immunoabsorbent assays (ELISA) kits (Alpha Diagnostic Laboratories, San Antonio, TX). All samples will be run in duplicate and the assays will be performed at 450 nm wavelength with a microplate reader (iMark, Bio-Rad, Hercules, CA). Hormone concentrations will be determined using data reduction software (Microplate Manager, Bio-Rad, Hercules, CA).

Plasma L-Leucine and Ursolic Acid Assessments

The levels of ursolic acid and L-leucine will be determined by HPLC. Plasma samples will be outsourced to Quest Diagnostics and the levels of L-leucine will be assessed in duplicate. For ursolic acid, plasma samples will be outsourced to an independent research laboratory yet to be determined.

Assessment of Skeletal Muscle mTOR Signaling Pathway Intermediates

From the nine muscle tissue samples obtained at the three RE sessions, the phosphorylated levels of IGF-1 R, protein kinase B (Akt), mTOR, and p70S6 kinase will be determined by phosphoprotein ELISA kits (Active Motif, Carlsbad, CA). All samples will be run in duplicate and the assays will be performed at 450 nm wavelength with a microplate reader (iMark, Bio-Rad, Hercules, CA). Protein concentrations will be determined using data reduction software (Microplate Manager, Bio-Rad, Hercules, CA), and the final concentration expressed relative to muscle protein content.

Assessment of Skeletal Muscle Myofibrillar Protein Content

The content of myofibrillar protein from each muscle sample will be determined spectrophometrically at a wavelength of 595 nm using bovine serum albumin as the standard. The final concentration will be expressed relative to muscle wet-weight.

Statistical Analyses

Statistical analyses will be performed by utilizing separate 3 x 4 [Session (placebo, ursolic acid, leucine) x Test (pre, post, 2-hour post, 6-hour post)] factorial analyses of variance (ANOVA) with repeated measures for blood variables. For muscle variable, a 3 x 3 [Session (placebo, ursolic acid, leucine) x Test (pre, 2-hour post, 6-hour post)] factorial ANOVA will be employed. Further analysis of the main effects will be performed by separate one-way ANOVAs. Significant between-group differences will then be determined involving the Tukey's Post Hoc Test. All statistical procedures will performed using SPSS 20.0 software and a probability level of < 0.05 will be adopted throughout.

References

1. Ogasawara R, Sato K, Higashida K, Nakazato K, Fufita S. Ursolic acid stimulats mTORC1 signaling after resistance exercise in rat skeletal muscle. *Am J Physiol Endocrinol Metab* 2013, doe:10.1152, epub ahead of print.
2. Kunkel S, Suneja M, Ebert S, Bongers K, Fox D, Malmberg S, Alipour F, Shields F, Adams C. mRNA expression signatures of human skeletal muscle atrophy identify a natural compund that increases muscle mass. *Cell Metab* 2011, 13:627-38.
3. Dreyer H, Fujita S, Cadenas J, Chinkes D, Volpi E, Rasmussen B. Resistance exercise increases AMPK activity and reduces 4e-BP1 phosphorylation and protein synthesis in human skeletal muscle. *J Physiol*, 2006 576:614-24.
4. Dreyer H, Drummond M, Pennings B, Fujita S, Glynn E, Chinkes D, Dhanani S, Volpi E, Rasmussen B. Leucine-enriched essential amino acid and carbohydrate ingestion following resistance exercise enhances mTOR signaling and protein synthesis in human muscle. *Am J Physiol Endocrinol Metab* 2008, 294:E392-E400.
5. Dennis M, Baum J, Kimball S, Jefferson L. Mechanisms involved in the coordinate regulation of mTORC1 by insulin and amino acids. *J Biol Chem* 2011, 286:8287-96.
6. Crozier S, Kimball S, Emmert S, Anthony J, Jefferson L. Oral leucine administration stimulates protein synthesis in rat skeletal muscle. *J Nutr* 2005, 135:376-82.
7. Ikeda Y, Murakami A, Ohigashi H. Ursolic acid: an anti- and pro-inflammatory triterpenoid. Mol Nutr Food Res 2008, 52:26-42.

Research Team

Darryn S. Willoughby, PhD, FACSM, FISSN. Dr. Willoughby will serve as the co-principal investigator. He is an Associate Professor of Exercise and Muscle Physiology and Biochemistry in the Department of Health, Human Performance, & Recreation at Baylor University. He is also an Associate Professor of Baylor's Biomedical Science Institute. Dr. Willoughby is an internationally recognized exercise biochemist and molecular physiologist. He has conducted a vast amount of research focusing on the biochemical and molecular regulatory mechanisms regarding exercise performance and nutrition. Dr. Willoughby will be the principal supervisor of the project. He will perform blood sampling and oversee all aspects of the study and perform the majority of the biochemical and clinical chemistry assays involved in the project.

Paul LaBounty, PhD. Dr. LaBounty will serve as the co-principal investigator. He is an Associate Professor of Exercise Physiology in the Department of Health, Human Performance, & Recreation at Baylor University. Dr. LaBounty will assist in providing oversight in data collection, strength and body composition testing, and performing blood draws and clinical laboratory assessments.

Brian Leutholtz, Ph.D., FACSM. Dr. Leutholtz is a Professor of Exercise Physiology in the Department of Health, Human Performance, & Recreation at Baylor University. Dr. Leutholtz will assist in providing oversight, in data collection, strength and body composition testing, and performing clinical laboratory assessments.

Neil Schwarz, M.S. Mr. Schwarz is an exercise physiologist pursuing his Ph.D. in Exercise, Nutrition, and Preventative Health and serves as a research assistant in the EBNL. He will perform blood draws and muscle biopsies and assist in all areas involved in the project.

Sarah McKinley, M.S.Ed. Ms. McKinley is an exercise physiologist pursuing her Ph.D. in Kinesiology, Exercise Nutrition, and Preventative Health and serves as a research assistant in the EBNL. She will assist in all areas involved in the project.

Dave Church, B.A. Mr. Church is an exercise physiologist pursuing his Master's in Exercise Physiology and serves as a research assistant in the EBNL. He will assist in all areas of the project in order to fulfill his thesis requirement.

Procedures

Medical Monitoring.

Interested participants will be invited to attend familiarization sessions. During this time, participants will sign consent forms and complete medical history information. Participants will then undergo a general exam to determine whether they meet entry criteria to participate in the study. This exam will include evaluating the medical and training history questionnaires and performing a general physical examination according to ACSM exercise testing guidelines. Based on this examination, participants will be assessed for their risk of cardiovascular disease and contraindications to exercise and then a recommendation will be made on whether the participant meets entry criteria and may therefore participate in the study. Trained, non-physician exercise physiologists certified in CPR will supervise participants undergoing testing and assessments. A telephone is in the laboratory in case of any emergencies, and there will be no less than two researchers working with each participant during testing sessions. In the event of any unlikely emergency one researcher will check for vital signs and begin any necessary interventions while the other researcher contacts Baylor's campus police at extension 2222. Instructions for emergencies are posted above the phone in the event that any other research investigators are available for assistance. Participants will be informed to report any unexpected problems or adverse events they may encounter during the course of the study to Darryn S. Willoughby, Ph.D. If clinically significant side effects are reported, the participants will be referred to their physician for medical follow-up. New findings and/or medical referrals of unexpected problems and/or adverse events will be documented, placed in the participants research file, and reported to the Baylor IRB committee.

Screening for Cardiopulmonary Disease Risk and Exercise Contraindications.

All participants will have their risk of cardiopulmonary disease and their possible contraindications to exercise assessed by trained exercise specialists in accordance to standard procedures described by the American College of Sports Medicine (ACSM) (*ACSM's Guidelines for Exercise Testing and Prescription, 8th ed. Williams & Wilkins Publishers, 2010).* Only those participants considered as low risk for cardiovascular disease with no contraindications to exercise will be considered as eligible to participate in the study. These guidelines are outlined and presented below:

ACSM Risk Stratification Criteria for Cardiovascular Disease

Low Risk

Younger individuals (men < 45 years of age; women < 55 years of age) who are asymptomatic for cardiovascular disease and possess no more than one positive cardiovascular disease risk factor.

Moderate Risk

Older individuals and/or those who are asymptomatic for cardiovascular disease and possess no more than two cardiovascular disease risk factors.

High Risk

Individuals who have known cardiovascular, pulmonary, or metabolic disease or one or more signs/symptoms suggestive of such disease.

ACSM Criteria for Signs and Symptoms Suggestive of Cardiovascular Disease

1. Pain, discomfort in the chest, neck, jaw, arms, or other areas that may be due to myocardial ischemia.
2. Shortness of breath at rest or with mild exertion.
3. Dizziness or syncope.
4. Orthopnea or paroxysmal nocturnal dyspnea.
5. Ankle edema.
6. Palpitations or tachycardia.
7. Intermittent claudication.
8. Known heart murmur.
9. Unusual fatigue or shortness of breath with usual activities.

ACSM Absolute and Relative Contraindications to Exercise

Absolute Contraindications

1. Unstable angina.
2. Uncontrolled dysrhythmias.
3. Recent EKG changes and cardiac events.

4. Acute myocarditis or pericarditis.
5. Acute pulmonary embolism or acute myocardial infarction.
6. Severe aortic stenosis.
7. Dissecting aneurysm.
8. Acute infections.

Relative Contraindications
1. Left main coronary stenosis.
2. Severe hypertension (> 200/115).
3. Tachycardia or bradycardia.
4. Uncontrolled metabolic disease.
5. High-degree AV block.
6. Chronic infectious disease.
7. Cardiomyopahty and outflow obstructions.
8. Stenotic valve disease.
9. Ventricular aneurysm.

Muscle Biopsies.

Percutaneous muscle biopsies (~50 mg) will be 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 based on our previously-approved procedures. The leg to be biopsied for the first testing session will be chosen at random with the opposite leg being used for the second testing session. The biopsy area will be shaved clean of leg hair, washed with antiseptic soap and cleaned with rubbing alcohol. In addition, the biopsy site will be further cleansed by swabbing the area with Betadine (fluid antiseptic). A small area of the cleaned skin approximately 2 cm in diameter will be anesthetized with a 1.0 mL subcutaneous injection of the topical anesthetic Lidocaine. Once the local anesthesia has taken effect (approximately 2-3 minutes), the biopsy procedure will only take 15-20 seconds. Once anesthetized, a 16-gauge fine needle aspiration biopsy (Tru-Core I Biopsy Instrument, Medical Device Technologies, Gainesville, FL) will be inserted into the skin at an approximate depth of 1 cm to extract the muscle sample. After the initial biopsy, the next biopsy attempts will be made to extract tissue from approximately the same location as the initial biopsy by using the pre-biopsy markings and depth markings on the needle. After removal, adipose tissue will be trimmed from the muscle specimens. Specimens will be immediately frozen in liquid nitrogen and then stored at -80°C for later analysis. Once the local anesthesia has taken effect (approximately 2-3 minutes) the biopsy procedure will take approximately 15-20 seconds. Written instructions for post-biopsy care will be given to the subjects. The participant will be instructed to leave the bandages on for 24 hours (unless unexpected bleeding or pain occurs) and asked to report back to the lab within 24 hours to have the old bandages removed, the incision inspected and new bandages applied. The participant will be further advised to refrain from vigorous physical activity during the first 48 hours post-biopsy. These suggestions will minimize pain and possible bleeding of the area. If needed, the subject may take non-prescription analgesic medication such as acetominophen to relieve pain if needed. However, medications such as aspirin, Advil, Nuprin, Bufferin, or Ibuprophen will be discouraged as these medications may lead to

ecchymosis at the biopsy site. Soreness of the area may occur for about 24 hours post-biopsy. Participants will be given a written protocol for caring for the muscle biopsy site.

Participants

Recruitment

Ten apparently healthy, resistance-trained [regular, consistent resistance training (i.e. thrice weekly) for at least one year prior to the onset of the study], men between the ages of 18-30 will volunteer to participate in the study. Enrollment will be open to men of all ethnicities. A recruitment flyer that will be posted on campus and at area fitness centers is attached.

Selection Criteria

Participants will not be allowed to participate in the study if they:

1. Have not been involved in a habitual resistance training program (minimum of 3 hours/week for at least 1 year);
2. Use tobacco products;
3. Have orthopedic limitations that would limit participation in resistance training;
4. Have a known allergy to topical anesthetics;
5. Have any known metabolic disorder including heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism;
6. Have a bleeding disorder, history of pulmonary disease, hypertension, hepatorenal disease, musculoskeletal disorders, neuromuscular/neurological diseases, autoimmune disease, cancer, peptic ulcers, anemia, or chronic infection (e.g., HIV);
7. Are taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic (e.g,thyroid, insulin, etc), emotional/psychotropic (e.g., Prednisone, Ritalin, Adderall), neuromuscular/neurological, or androgenic medications (anabolic steroids);
8. Have taken ergogenic levels of nutritional supplements that may affect muscle mass (e.g., creatine, HMB) or anabolic/catabolic hormone levels (e.g., androstenedione, DHEA, etc) within one month prior to the start of the study.
9. Have any absolute or relative contraindication for exercise testing or prescription as outlined by the American College of Sports Medicine;
10. Report any unusual adverse events associated with this study that in consultation with the supervising physician recommends removal from the study.

Compensation or Incentives

Participants completing all familiarization and testing sessions as well as turning in all required materials (i.e., dietary logs) in the study will be paid $150. Participants may receive information regarding results of these tests if they desire. If subjects are Baylor students, they will not receive any academic credit for participating in this study.

Potential Risks

Participants who meet eligibility criteria will be exposed to a very low level of electrical current that will be passed through each subject's body using a bioelectrical impedance analyzer. This analyzer is commercially available and has been used in the health care/fitness industry as a means to assess body composition and body water for over 20 years. The use of the BIA analyzer has been shown to be safe methods of assessing body composition and total body water and is approved by the FDA.

Participants who meet eligibility criteria will be subjected to strength testing sessions involving dynamic muscle contractions. Participants in this study will not be experienced resistance trainers, and will be instructed to only perform the prescribed RE protocol throughout the duration of the study. As a result of the exercise protocol, participants will most likely experience short-term muscle fatigue. In addition, they will likely experience muscle soreness in their thigh area for up to 24 to 48 hours after exercise. This soreness is normal and should be commensurate with the type of muscle soreness participants may have felt after doing unaccustomed physical activity. Muscle strains/pulls resulting from 1-RM testing and the dynamic exercise protocol are possible. During the familiarization session, participants will be informed of the resistance training program and correct lifting technique for each exercise demonstrated. Therefore, potential injury due to exercise will be minimized since all participants will be instructed on how to adhere to correct lifting technique. In addition, only Darryn Willoughby, Ph.D., Paul LaBounty, Ph.D., Brian Leutholtz, Neil Schwarz, M.S., Sarah McKinley, M.S.Ed. or Dave Church, B.S. will conduct the testing and exercise procedures. Participants will be made aware of the intensity and duration of the expected soreness due to the exercise sessions. However, there are minor risks of muscular pain and soreness associated with the resistance training protocol required in this study which are not uncommon to any exercise program especially for individuals who do not resistance train on a regular basis. Participants will donate about approximately 20 milliliters of venous blood a total of 16 times during the study by way of an intravenous catheter and standard phlebotomy using sterile techniques by an experienced phlebotomist using standard procedures. These procedures may cause a small amount of pain when the needle is inserted into the vein as well as some bleeding and bruising. However, proper pressure will be applied upon removal to reduce bruising. The subject may also experience some dizziness, nausea, and/or faint if they are unaccustomed to having blood drawn.

Complications resulting from the muscle biopsy are rare, especially in this case where the biopsy is similar to receiving a routine intramuscular injection. As with the blood draw, however, there is a risk of infection if the subject does not adequately cleanse the area for approximately 48-72 hours post biopsy. While leaving the butterfly bandage in place, participants will be instructed to cleanse the biopsy area with soap and water every 4-6 hours, pat the area dry and reapply a fresh adhesive bandage. The participant will be instructed to leave the bandages on for 24 hours (unless unexpected bleeding or pain occurs) and asked to report back to the lab within 24 hours to have the old bandages removed, the incision inspected and new bandages applied. The participant will be further advised to refrain from vigorous physical activity with the affected leg for 24 hours after

the biopsy. There is a potential risk of an allergic reaction to the Lidocaine. All subjects will be asked if they have known allergies to local anesthetics (e.g. Lidocaine, Xylocaine, etc.) that they may have been previously given during dental or hospital visits. Participants with known allergies to anesthesia medications will not be allowed to participate in the study. Darryn Willoughby, Ph.D. or Neil Schwarz, M.S. are trained in muscle biopsy techniques and will perform all muscle biopsies. Researchers involved in collecting data represent trained, non-physician, exercise specialists. All personnel involved in collecting data will be certified in CPR, which is also a condition to holding these professional certifications. A telephone and automated electronic defibrillator (AED) is located in the laboratory in case of any emergencies and there will be no less than two researchers working with each subject during testing. In the event of any unlikely emergency one researcher will check for vital signs and begin any necessary interventions while the other researcher contacts Baylor's campus police at extension 2222. Instructions for emergencies are posted above the phone in the event that any other research investigators are available for assistance.

Potential Benefits

The main benefit that participants may obtain from this study is how the body may increase the rate of MPS resulting from a single dose of ursolic acid and/or leucine in response to RE. In addition, participants may gain insight into how they can enhance muscle mass and performance that typically occurs in conjunction with resistance training as well as improved health profiles. Participants may also gain insight about their health and fitness status from the assessments to be performed.

Assessment of Risk

While there are risks associated with the muscle biopsy and blood sampling, both of these procedures are done so often in the EBNL, that they are considered routine. Both Dr. Willoughby and Mr. Schwarz have performed countless numbers of both procedures and they are skilled and competent. To date, there have been hundreds of both procedures performed in the EBNL with no untoward events. All three supplements are naturally-occurring in many foods that humans consume daily. The greatest risk associated with participating in this study will likely be from the muscle soreness participants will experience from participating in the RE protocol. However, the intensity of the exercise protocol will be no more than when individuals engaged heavily in a new or different form of physical activity. Therefore, the potential benefits of subjects participating in this study outweigh the potential risks.

Compensation for Illness or Injury

Each participant will agree to indemnify and hold harmless Baylor University, its officers, directors, faculty, employees, and students for any and all claims for any injury, damage or loss suffered as a result of participation in this study regardless of the cause of injury, damage, or loss.

Confidentiality

Information obtained from this research (including questionnaires, medical history, laboratory findings, or physical examination) will be kept confidential to the extent permitted by law. However, according to FDA regulations, records will be open to FDA representatives to review if necessary. This may include questionnaires, medical history, laboratory findings/reports, statistical data, and/or notes taken throughout this study. Records of the research may also be subpoenaed by court order or may be inspected by federal regulatory authorities. Data derived from this study may be used in reports, presentations and publications. Participants in this study will not be individually identified unless they give their written consent. All participants will have a number to identify their results. Only the study personnel will know the participant numbers. Only study personnel will have access to the data. All data will be stored in a locked cabinet in the Exercise and Biochemistry Laboratory and only Darryn Willoughby, Ph.D. will have access to the key. Additionally, that confidentiality will be maintained by assigning code numbers to the files, limiting access to data to research assistants, locking cabinets that store data, and providing passwords to limit access to computer files to authorized personnel only. All evidence of primary data will be stored for exactly three years after the completion of the study. At this time data will be destroyed in a manner that instills complete privacy to all participants of the study. Analyzed muscle and blood samples will be discarded in an appropriately-labeled biohazard waste disposal container. However, unused muscle and blood samples will be kept in a locked freezer for no longer than one year. If any subsequent analysis occurs with the samples, they will be re-coded to further instill confidentiality.

Data Presentation & Publication

Data will be presented at an appropriate scientific conference (e.g., American College of Sports Medicine, International Society of Sports Nutrition, etc.) and published in a peer-reviewed scientific journal (e.g., Medicine & Science in Sport and Exercise, Nutrition Research, etc.).

Statement on Conflict of Interest

Funding for this study will be provided by the Exercise and Biochemical Nutrition Laboratory of Baylor University. Researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

APPENDIX C

Recruitment Flyer

Want to get Paid to Workout?

Resistance-Trained Men Needed for a Weight Lifting/Supplement Study

*Researchers in the Exercise & Biochemical Nutrition Lab at Baylor University are recruiting 10 healthy**, resistance-trained** men between the ages of **18-30** to participate in a study designed to evaluate and compare the effects of the amino acid L-Leucine and Ursolic Acid on markers of muscle protein synthesis. Participants will be required to engage in 3 resistance exercise/supplement sessions separated by 2 weeks. Participants will be required to undergo strength and body composition testing, and to also submit to providing blood samples and muscle biopsies. Eligible subjects will receive **$150** for completing the study and free muscle strength and body fat testing.*

For more information contact:

Exercise & Biochemical Nutrition Lab

Department of HHPR

Rena Marrs McLean Gymnasium Room 120

254-710-3504

Dave_Church@baylor.edu

Darryn_Willoughby@baylor.edu

APPENDIX D

Medical History Inventory

Directions. The purpose of this questionnaire is to enable the staff of the Exercise and Biochemical Nutrition Laboratory to evaluate your health and fitness status. Please answer the following questions to the best of your knowledge. All information given is **CONFIDENTIAL** as described in the **Informed Consent Statement.**

Name:_____ Age _____Date of Birth_____

Name and Address of Your Physician:_____

MEDICAL HISTORY

Do you have or have you ever had any of the following conditions? (Please write the date when you had the condition in blank).

____	Heart murmur, clicks, or other cardiac findings?	____	Asthma/breathing difficulty?
____	Frequent extra, skipped, or rapid heartbeats?	____	Bronchitis/Chest Cold?
____	Chest Pain of Angina (with or without exertion)?	____	Cancer, Melanoma, Skin Lesions?
____	High cholesterol?	____	Stroke or Blood Clots?
____	Diagnosed high blood pressure?	____	Emphysema/lung disease?
____	Heart attack or any cardiac surgery?	____	Epilepsy/seizures?
____	Leg cramps (during exercise)?		
____	Rheumatic fever?		
____	Chronic swollen ankles?	____	Scarlet fever?
____	Varicose veins?	____	Ulcers?
____	Frequent dizziness/fainting?	____	Pneumonia?
____	Muscle or joint problems?	____	Anemias?
____	High blood sugar/diabetes?	____	Liver or kidney disease?
____	Thyroid Disease?	____	Autoimmune disease?
____	Low testosterone/hypogonadism?	____	Nerve disease?
____	Gluacoma?	____	Psychological Disorders?

Do you have or have you been diagnosed with any other medical condition not listed?

_____
_____

Please provide any additional comments/explanations of your current or past medical history.

_____
_____

Please list any recent surgery (i.e., type, dates etc.).

_____
_____

List all prescribed/non-prescription medications and nutritional supplements you have taken in the last 3 months.

_____

What was the date of your last complete medical exam?

_____

Do you know of any medical problem that might make it dangerous or unwise for you to participate in this study (including strength and maximal exercise tests) _____ If yes, please explain:

_____

Have you been involved in any type of weight loss program within the past 6 months? ____ If yes, please explain:

Recommendation for Participation

____ No exclusion criteria presented. Subject is ***cleared*** to participate in the study.

____ Exclusion criteria is/are present. Subject is ***not cleared*** to participate in the study.

APPENDIX E

Exercise History Questionnaire

Baylor University

Exercise and Biochemical Nutrition Laboratory

Personal Information
Name:
Address:
City: _____ State: _____ Zip Code _____
Home Phone: (___) _____ Work Phone: (___)_____
Cellular: (___) _____ E-mail address: _____
Birth date: _____ / _____ / _____ Age: _____ Height: _____ Weight: _____

Exercise & Activity Questionnaire

1. Describe your typical occupational activities.

2. Describe your typical recreational activities (non-structured exercise activities, i.e. basketball, other sports). Approximately how many hours do you spend doing these activities per week?

3. Do you currently do any aerobic exercise training (i.e. running, cycling, elliptical, etc.)? If so, approximately how many days per week do you train? On average, approximately How many hours of aerobic exercise do you perform per week?

4. Do you currently do any resistance exercise training (i.e. weight lifting)? If so, approximately how many days per week do you train? On average, approximately how many hours of resistance exercise do you perform per week?

5. If you do resistance exercise, do you exercise both upper and lower body? Approximately how many times do you train each body part per week?

6. How long (years/months) have you been consistently training (aerobic and/or resistance exercise)?

APPENDIX F

Diet Logs

Baylor University
Exercise & Biochemical Nutrition Laboratory

NAME _____ **Date** _____

INSTRUCTIONS

1. Record everything you eat for 3 days. If you eat pretzels, record how many. If you eat a bag of chips, record the number of ounces. For drinks, record the number of cups or ounces. Record everything you drink except water.
2. Record the Food, Amount, Brand Name, and Preparation Methods. For example: baked vs. fried chicken; 1 cup of rice; 2 teaspoons of margarine; 1 cup of 2% milk; McDonald's, Healthy Choice, or Frosted Flakes.
3. Record immediately after eating. Waiting until that night may make it difficult to remember all foods and quantities.

Food (include brand)	Method of Preparation	Quantity (cups, oz., no.)
BREAKFAST:		
LUNCH:		
DINNER:		
SNACKS:		

APPENDIX G

Entry Session Data Collection Form

Subject Code		
Session		
	Inches	cm
Height		
	LBS	KGS
Weight		
Resting HR		
Resting BP		
DEXA		
Fat Mass		
Lean Mass		
BMC		
BIA		
Total Body Water		
Intracellular		
Extracellular		
Notes		

Supplement Code			
Leg Press	Positions		
	Set	Reps	Load
Warm up	1		
	1 Minute Rest		
Warm up	2		
	2 Minutes Rest		
	3	1	
	2 Minutes Rest		
	4	1	
	2 Minutes Rest		
	5	1	
	2 Minutes Rest		
	6	1	
Knee Extension	Positions		
	Sets	Reps	Load
Warm up	1		
	1 Minute Rest		
	2		
Warm up	2 Minutes Rest		
	3	1	
	2 Minutes Rest		
	4	1	
	2 Minutes Rest		
	5	1	
	2 Minutes Rest		
	6	1	

APPENDIX H

Resistance Exercise Session Data Collection For m

Subject Code	
Session	

Resting HR	
Resting BP	

BIA	
Total Body Water	

Leg Press			
	Set	Reps	Load
	1		
	2 Minute Rest		
	2		
	2 Minutes Rest		
	3		
	2 Minutes Rest		
	4		

Knee Extension			
	Sets	Reps	Load
	1		
	2 Minute Rest		
	2		
	2 Minutes Rest		
	3		
	2 Minutes Rest		
	4		

Supplement	

Blood Sampling	
Timepoint	
Rest	
+ 0.5 Hr	
+ 2 Hr	
+6 Hr	

Muscle Biopsies	
Timepoint	
Rest	
+ 2 Hr	
+6 Hr	

Timepoint BP & HR		
	BP	HR
Timepoint		
+ 0.5 Hr		
+ 2 Hr		
+6 Hr		

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