Effect of phytoecdysteroids on protein synthesis and Akt/mTOR signaling after downhill running in skeletal muscle of mice

by

Kevin Hans Goslen

Honors Thesis
Appalachian State University
Submitted to The Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science
May, 2017

Approved by:

Kevin A. Zwetsloot, Ph.D., Thesis Director
Shea Tuberty, Ph.D., Second Reader
Ted Zerucha, Ph.D., Interim Director, The Honors College
Abstract

Phytoecdysteroids are natural plant steroids synthesized by a variety of hardy plants. Phytoecdysteroids, such as 20- hydroxyecdysone (20E), possess anabolic properties and previous research indicates that 20E stimulates protein synthesis in cultured muscle cells and increases grip strength in young rats after 28 days of supplementation. The purpose of this study was to investigate the extent to which 20E stimulates protein synthesis and Akt/mTOR signaling in mouse skeletal muscle after an acute bout of downhill running (DHR). Male C57BL6 mice (3-6 mo old) were randomly assigned to eight groups (n=8-10/group). Mice in the DHR groups performed an acute bout of DHR to exhaustion on a rodent treadmill at 17 m•min-1 and negative 20° decline. After completion of the DHR bout and recovery, mice received an oral gavage treatment of either 50 mg•kg-1 body mass (BM) of 20E (DHR + 20E) or vehicle (DHR + vehicle), and then treated daily for the next one (2-day post DHR) or four consecutive days (5-day post DHR). Groups that did not perform DHR, but were treated for two or five consecutive days with either 50 mg•kg-1 BM of 20E (No DHR + 20E) or vehicle (No DHR + vehicle), were used as controls. On the second or fifth day post-DHR, mice were not treated with 20E or vehicle; however, an IP injection of puromycin (0.040 μmol•g-1 BM) was administered 30 min prior to sacrifice to assess protein synthesis. Skeletal muscles were harvested and activation of protein synthesis was assessed using the SUnSET method and Western blot and Akt/mTOR signaling activation was measured via Western blot. At the 2-day time point, puromycin incorporation was significantly higher in 20E-treated mice, compared to vehicle-treated mice when no DHR was performed (p=0.011). Also, mice that were treated with 20E for two days, but did not perform DHR, had significantly higher puromycin incorporation, compared to the 2-day No DHR + vehicle mice (p<0.001). At the 5-day time point, no significant interaction was found
with DHR and treatment (p=0.965). No significant interactions were found in the states of signaling proteins at either time point: phospho-Akt 2-day (p=0.283), 5-day (p=0.767); phospho-p70S6K 2-day (p=0.060); phospho-4EBP1 2-day (p=0.202), 5-day (p=0.080); phospho-rpS6 2-day (p=0.104), 5-day (p=0.962). It appears that 20E was unable to enhance puromycin incorporation or activation of the Akt/mTOR signaling pathway after an acute bout of DHR. Limitations of this study include the possibility of low bioavailability and rapid metabolic half-life of 20E, and that 20E was not administered on the day of sacrifice.

Introduction

Skeletal muscle allows for the voluntary movement of the body and is the most abundant tissue in the body. Skeletal muscle is composed of different types of muscle fibers, which are divided by their twitch, force-generating, and metabolic capacities: type I fibers are slow-twitch and oxidative, type IIa fibers are fast-twitch and oxidative, type IIb and IIx fibers are fast-twitch and glycolytic. Muscle fibers themselves are composed of sarcomeres, the functional unit of muscle contraction. In sarcomeres, ATP-consuming myosin proteins generate force by cross-bridge cycling with actin, bringing the Z-disks at the ends of the sarcomere closer to the central M-line, which results in sarcomere shortening during muscle contraction (Douglas, Pearson, Ross, & McGuigan, 2017). The contractile force produced at the whole-muscle scale results from the combined shortening of these sarcomeres across the muscle fiber.

Different kinds of contraction can occur upon muscle loading. In concentric contractions, the muscle produces sufficient force to lift its load and the muscle shortens. Isometric contractions occur when the muscle generates a force equal to its load and the muscle’s length remains constant. Lastly, with eccentric contractions, the muscle does not produce sufficient
force to lift its load and the muscle actively lengthens. Eccentric contractions have been widely used to induce muscle damage due to their lengthening nature. Eccentric exercise is thought to induce damage by the mechanical detachment of myosin heads from actin and is marked by Z-disk streaming as sarcomeres become disorganized (Douglas et al., 2017). One commonly-used model to induce eccentric muscle damage in rodent hind limbs is downhill running (DHR) (Hody et al., 2013; Marqueste, Giannesini, Fur, Cozzone, & Bendahan, 2008). Muscles connected to more than one joint, such as the gastrocnemius, are particularly vulnerable to eccentric damage (Souza & Gottfried, 2013).

Muscle damage activates repair processes and invokes a local inflammatory response within the tissue. Multipotent satellite cells are activated to generate new myoblasts through mitosis that fuse to damaged myofibrils to aid in the repair process (Douglas et al., 2017). The proliferation of satellite cells is also upregulated in eccentric exercise in comparison to concentric exercise, mostly due to the degree of damage caused by eccentric exercise (Douglas et al., 2017). Neutrophils and macrophages are recruited as part of the inflammatory response, releasing growth factors and chemokines, as well as removing cellular debris left from the injury. Inflammatory cytokines released by activated immune cells and the injured muscle tissue also upregulate protein synthesis in myocytes, allowing them to replace damaged cellular machinery.

One such cell signaling pathway activated by the muscle damage process is the phosphatidylinositol 3-kinase (PI3K)/Protein kinase B (Akt)/mammalian Target of Rapamycin (mTOR) pathway. The mTOR pathway stimulates protein synthesis, specifically the initiation of translation. Upregulation of translation is achieved when mTOR phosphorylates (and inhibits) eukaryotic initiation factor 4E (eIF4E) binding protein-1 (4EBP-1), which results in unbound eIF4E. Once free, eIF4E can then facilitate translation via assembly of the translation apparatus
to the ribosome (Fingar et al., 2004). Additionally, mTOR phosphorylates (and activates) ribosomal protein S6 kinase (also known as p70S6K), which phosphorylates (and activates) several proteins associated with translation, for example ribosomal protein S6 (rpS6) (Hannan et al., 2003). Chronic activation of this pathway has also been shown to induce muscle hypertrophy, or an increase in the cross-sectional area of muscle fibers (Bodine et al., 2001). mTOR activation through PI3K has been shown to be activated after eccentric exercise by mechano growth factor (MGF), a skeletal muscle-specific variant of insulin-like growth factor (IGF-1), in mice (Hornberger et al., 2004; Ochi, Ishii, & Nakazato, 2010) and humans (Roschel et al., 2011). Other studies suggest that mTOR may also be activated by PI3K/Akt-independent mechanisms (Jacobs et al., 2017; O'Neil, Duffy, Frey, & Hornberger, 2009). In human skeletal muscle, p70S6K has been shown to be activated in an mTOR-independent manner after eccentric exercise (Eliasson et al., 2006).

Phytoecdysteroids are a class of anabolic steroids found in hearty plant species, such as spinach. These compounds are thought to compose a defense system in plants that results in the premature molting of feeding insects. In mammals, the phytoecdysteroid 20-hydroxyecdysone (20E), has been shown to activate Akt/mTOR signaling pathway and increase protein synthesis in cultured skeletal muscle cells and increase grip strength in rats after 28 days of supplementation (Gorelick-Feldman et al., 2008). 20E is thought to work through a cell surface G-protein coupled receptor mechanism, as opposed to the canonical nuclear receptor mechanism common to steroids (Gorelick-Feldman, Cohick, & Raskin, 2010). However, it has also been reported that the low bioavailability of 20E in rodents may be a limiting factor in their biological effects (Anthony et al., 2015).

It is unknown if phytoecdysteroid supplementation after an acute bout of DHR will
enhance skeletal muscle protein synthesis and activation of the Akt/mTOR pathway in rodent skeletal muscle. The purpose of this study was to investigate if 20E enhances protein synthesis and activation of the Akt/mTOR pathway in the gastrocnemius of young mice after an acute bout of DHR. I hypothesized that supplementation with 20E after an acute bout of DHR would increase total protein synthesis and increase the activation of the Akt/mTOR pathway in the gastrocnemius of young mice, compared to control groups.

Methods

Mouse Model

All study procedures were approved by the Institutional Animal Care and Use Committee at Appalachian State University. Male C57BL/6 mice, 3-6 months of age, were kept under a reverse 12-hour light and 12-hour dark cycle and randomly assigned to 8 groups: 4 control (no DHR) and 4 DHR groups (See Figure 1). All mice in the 4 DHR groups were acclimated to the treadmill over five consecutive days, reaching a maximum speed of 11 m•min⁻¹ with no decline. On the day following completion of the acclimation protocol, all mice in the 4 DHR groups performed an acute bout of DHR, to exhaustion, on a rodent treadmill at 17 m•min⁻¹ and -20° decline. After completion of the DHR bout and recovery, mice received an oral gavage treatment of either 50 mg•kg⁻¹ body mass (BM) of 20E in vehicle (rodent liquid diet; BioServ AIN-76) or vehicle only and then returned to their cage, where they had ad libitum access to chow and water. Mice were treated daily for the next one (2-day treatment groups) or four consecutive days (5-day treatment groups) and weighed to monitor health. The four control (no DHR) groups were
treated for two or five consecutive days with either 50 mg•kg\(^{-1}\) BM of 20E in vehicle or vehicle only, but never performed DHR. This is represented in schematic form in figure 1. On the day of sacrifice, mice were not treated with 20E or vehicle; however, an IP injection of puromycin (0.040 μmol•g\(^{-1}\) BM) was administered 30 minutes prior to sacrifice and utilized as a marker to assess skeletal muscle protein synthesis using the SUnSET technique (Goodman and Hornberger (2013); Goodman et al. (2011). Mice were anesthetized with 4% isoflurane inhalation and the right gastrocnemius was dissected after euthanasia by cervical dislocation. Tissue samples were weighed before being flash frozen in liquid nitrogen and stored at -80 °C until further analysis.

![Figure 1. Protocol Schematic](image)

**Tissue Homogenization, Protein Quantification, and Sample Preparation**

Frozen muscle samples were pulverized before approximately 25 mg of tissue was
homogenized on ice with a polytron in ice-cold RIPA buffer (150 mM NaCl, 1.0% Triton-X, 0.5% Sodium Deoxycholate, and 10 mM Tris-HCl, pH 7.4), containing phosphatase and protease inhibitors (Sigma). Homogenates were cleared by centrifugation at 14,000 x g for five minutes at 4 °C. The supernatant was transferred to a new tube and stored overnight at -80 °C.

A protein assay was performed, in triplicate, for each sample and 8 BSA standards using the BCA assay kit (ThermoFisher Pierce), according to the manufacturer’s instructions. A BioTek Eon microplate reader was used to determine the absorbance at 540 nm. Standard curves and protein concentration of each sample was determined based on average absorbance values of each triplicate set. Based on these concentrations, samples were uniformly prepared with Laemmli buffer in reducing conditions (β-mercaptoethanol) to 1 µg/µL and denatured by boiling.

**SUUnSET Technique and Western Blot Analysis**

Prepared muscle homogenate samples (10-30 µg of protein) were loaded into each well and separated in TGX-stain free 4-15% gradient gels (BioRad) using SDS poly-acrylamide gel electrophoresis (PAGE) at 200v for ~30 minutes or until the dye front reached the bottom of the gel. A Trans-Blot Turbo transfer apparatus (BioRad) was used to transfer the proteins to a PVDF membrane, according to the manufacturer’s instructions. Membranes were imaged under ultraviolet light to ensure equal loading and transferring of proteins, as well as to quantitate total protein for normalization before blocking with 5% nonfat dry milk in wash buffer (1X tris-buffered saline (TBS) with 0.1% Tween20) for 1 hour at room temperature on a rocking platform. Membranes were washed twice, for 5 min each, and then immediately incubated with the following primary antibodies overnight @ 4°C: anti-puromycin [MillporeSigma; Billerica, MA, USA – MABE343, clone 12D10; 1:5000 in 1% bovine serum albumin (BSA)], as well as
phosphorylated Akt\textsuperscript{Ser473} (Cell Signaling; Danver, MA, USA – 9271); phosphorylated p70S6k\textsuperscript{Thr389} (Cell Signaling – 9234); phosphorylated 4EBP1\textsuperscript{Thr37/46} (Cell Signaling – 2855); and phosphorylated rpS6\textsuperscript{Ser235/236} (Cell Signaling – 2211), (all signaling antibodies = 1:1000 in 5% BSA). After primary antibody incubation, membranes were washed 5 times for 5 minutes each in wash buffer and then incubated with anti-rabbit IgG secondary antibodies for 1 hour at room temperature (1:50,000 for puromycin and 1:20,000 for signaling antibodies). Next, the membranes were washed 5 times for 5 minutes each in wash buffer. Membranes were exposed to SuperSignal West Dura ECL chemiluminescent detection HRP reagents (Pierce; Thermo Fisher Scientific, Inc.; Rockford, IL, USA) mixed 1:1 for 5 minutes. Membranes were then imaged and densitometry analyzed using the ChemiDoc XRS+ molecular imaging system and analysis software (BioRad).

**Statistical Analyses**

A two-tailed, independent Student’s t-test was used to determine if differences existed in DHR run time between groups for both 2-day and 5-day experiments. A 2-way ANOVA (treatment x DHR) was used to determine if differences existed in muscle to body mass ratio and Western blot data (puromycin and signaling protein phosphorylation) between groups for both 2-day and 5-day experiments. After a significant F-ratio, Fisher LSD post-hoc tests were performed to determine differences between individual treatment groups. Significance was set at \( \alpha = p \leq 0.05 \) and data are presented as Mean + SD.
Results

_Muscle mass to body mass ratio and DHR run times_

Muscle mass was normalized to body mass as a ratio (mg tissue • g BM\(^{-1}\)) and analyzed to determine if differences existed between treatments groups at each time point (Table 1). At the two-day time point, no statistically significant interaction was found (p=0.410), nor was there a significant interaction at the five-day time point (p=0.319). DHR run time to exhaustion was compared between 20E and vehicle groups at each time point to assess uniformity. In the 2-day experiment, the Vehicle group ran significantly longer, compared to the 20E group (78.4 + 14.5 minutes vs. 54.5 + 7.8 minutes, respectively; p<0.001). In the 5-day experiment, the Vehicle group ran 65.8 + 15.5 minutes, while the 20E group ran 59.3 + 7.6 minutes. No statistically significant difference was found between run times for mice in the 5-day groups (p=0.255).

Table 1. Muscle Tissue Wet Mass to Body Mass Ratio (mg tissue • g BM\(^{-1}\))

<table>
<thead>
<tr>
<th></th>
<th>2-day</th>
<th>5-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No DHR</td>
<td>DHR</td>
</tr>
<tr>
<td>Vehicle</td>
<td>4.94 ± 0.19</td>
<td>4.73 ± 0.33</td>
</tr>
<tr>
<td>20E</td>
<td>5.00 ± 0.28</td>
<td>4.72 ± 0.31</td>
</tr>
<tr>
<td>ANOVA:</td>
<td>p=0.410</td>
<td>ANOVA: p=0.319</td>
</tr>
</tbody>
</table>

_Protein synthesis measurements by SUnSET_

Relative quantification of puromycin incorporation into all polypeptides over a 30-minute time period was assessed by Western blot. In the 2-day experiment, there was a significant overall interaction (p=0.041; Figure 2A) with puromycin incorporation. Post-hoc analyses revealed that puromycin incorporation was significantly higher in 20E-treated mice, compared to vehicle-treated mice when no DHR was performed (p=0.011). Also, mice that were treated with 20E for two days, but did not perform DHR, had significantly higher puromycin incorporation,
compared to the 2-day Vehicle No DHR mice (p<0.001). At the 5-day time point, no significant interaction was found with DHR and treatment (p=0.965; Figure 2B).
Figure 2. Puromycin incorporation in skeletal muscle at the 2-day (A.) and 5-day (B.) time points. Data are presented as Mean + SD; * - Significantly different from No DHR + Vehicle (p = 0.011); # - Significantly different from No DHR + 20E (p < 0.001); AU – Arbitrary Units.

A. 2 day phospho-Akt

B. 2 day phospho-p70S6K

C. 2 day phospho-4EBP1

D. 2 day phospho-rpS6

Figure 3. Phosphorylation status of key molecules in the Akt/mTOR signaling pathway in skeletal muscle at the 2-day time point: Akt phosphorylation (A.), p70S6K phosphorylation (B.), 4EBP1 phosphorylation (C.), and rpS6 phosphorylation (D.). Data are presented as Mean + SD; AU – Arbitrary Units.
Discussion

Overall, my hypothesis that 20E supplementation after DHR would increase protein synthesis, using puromycin incorporation as a marker, via the Akt/mTOR pathway was not supported. In fact, the only significant results would indicate that the effects of 20E to stimulate protein synthesis are inhibited, rather than enhanced, by DHR. There is also no indication of stimulating protein synthesis by DHR or 20E through the Akt/mTOR pathway. These results are surprising in that they are inconsistent with in vitro studies on the effects of 20E and grip-strength studies in rats (Gorelick-Feldman et al., 2008). However, previous studies from Dr. Zwetsloot’s lab (unpublished data) indicates that the same dosage of 20E (50 mg•kg BM⁻¹) was
also unable to stimulate protein synthesis or signaling in skeletal muscle of young mice with normal cage activity. These previous findings and my results suggest that 20E is unable to stimulate protein synthesis in the skeletal muscle of young mice with or without eccentric exercise-induced muscle damage. However, Dr. Zwetsloot’s lab has previously shown that 28 days of daily 50 mg•kg BM\(^{-1}\) of 20E supplementation was able to increase muscle fiber cross sectional area and Akt/mTOR signaling in skeletal muscle of 21-month-old sarcopenic mice (Lawrence et al. – in preparation).

One potential explanation for this may be that, in young mice, the damage caused by eccentric exercise is not comparable to the effects of sarcopenia, which is the loss of muscle size/mass associated with aging. Sarcopenia has been shown to involve anabolic resistance, in which anabolic pathways, like the Akt/mTOR pathway, show decreased sensitivity to anabolic stimuli, such as exercise and amino acids (Walker et al., 2011). It is possible that low bioavailability of orally administered 20E may also be a limiting factor, preventing the concentration of the phytoecdysteroid from reaching levels necessary to stimulate protein synthesis, regardless of dosage. However, continuous infusion of 20E into the blood of mice at the rate of 5 mg/kg BM/day was also unable to demonstrate an anabolic effect (Cheng et al., 2013). The metabolic half-life of 20E may also play a role in preventing its effects in vivo, as 90% of a 50 mg•kg BM\(^{-1}\) dose of 20E has been shown to be eliminated from blood plasma in 30 minutes (Dzhukharova et al., 1987), before it is fully able to effect skeletal muscle protein synthesis. Since mice were not treated with either 20E or vehicle on the day of sacrifice, concentrations of the phytoecdysteroid in the plasma of the mice should have been negligible. The liquid diet solution used as vehicle in this study contains 14.6 gm/kg of the amino acid leucine, which has been shown to stimulate the Akt/mTOR pathway, however the effects of
leucine and other amino acids on this pathway have been shown to only last one to two hours (Walker et al., 2011).

Previous studies in Dr. Zwetsloot’s lab conclude that 20E supplementation does not acutely or chronically stimulate skeletal muscle protein synthesis or Akt/mTOR signaling in young sedentary mice, but does appear to be effective in skeletal muscle of aged mice. Contrary to the hypothesis, my results suggest that 20E is not an effective treatment to stimulate skeletal muscle protein synthesis after an acute bout of DHR. Taken together with previous results from our lab suggests that 20E may not be an effective treatment to stimulate skeletal muscle protein synthesis or Akt/mTOR signaling in young skeletal muscle, regardless of whether mice performed an acute bout of DHR or not. In the current study, mice were sacrificed and muscles dissected 24 hours after the last dose of 20E to evaluate the chronic effects of 20E on muscle repair processes after eccentric muscle damage. The limitations of 20E listed above may cause the stimulation of protein synthesis by 20E to be short lived, and that after 24 hours, the effects of 20E are no longer present. Future studies, therefore, should assess protein synthesis and Akt/mTOR signaling immediately after the combination of eccentric exercise and 20E is administered. Perhaps administering 20E more than once per 24 hours could counteract the decrease in concentration caused by catabolism of the phytoecdysteroid.
REFERENCES


