**Evaluating the Effect of 20-Hydroxyecdysone (20HE) on
Mechanistic Target of Rapamycin Complex 1 (mTORC1)
Signaling in the Skeletal Muscle and Liver of Rats**

Journal:	<i>Applied Physiology, Nutrition, and Metabolism</i>
Manuscript ID	apnm-2015-0301.R2
Manuscript Type:	Brief communication
Date Submitted by the Author:	12-Sep-2015
Complete List of Authors:	Anthony, Tracy; Rutgers University, Nutritional Sciences Mirek, Emily; Rutgers University, Nutritional Sciences Bargoud, Albert; Rutgers University, Nutritional Sciences Phillipson-Weiner, Lindsey; Rutgers University, Nutritional Sciences DeOliveira, Christopher; Rutgers University, Nutritional Sciences Wetstein, Berish; Rutgers University, Nutritional Sciences Graf, Brittany; Rutgers University, Plant Biology and Pathology Kuhn, Peter; Rutgers University, Plant Biology and Pathology Raskin, Ilya; Rutgers University, Plant Biology and Pathology
Keyword:	leucine, mTOR, phytoecdysteroids, ergogenic aids < nutrition, sports nutrition < nutrition

SCHOLARONE™
Manuscripts

**Evaluating the Effect of 20-Hydroxyecdysone (20HE) on Mechanistic Target of Rapamycin
Complex 1 (mTORC1) Signaling in the Skeletal Muscle and Liver of Rats**

Anthony, Tracy G.¹, Mirek, Emily T.¹, Bargoud, Albert Raouf¹, Phillipson-Weiner, Lindsey¹,
DeOliveira, Christopher M.¹, Wetstein, Berish¹, Graf, Brittany L.², Kuhn, Peter E.², Raskin, Ilya²

¹Department of Nutritional Sciences and ²Department of Plant Biology and Pathology, Rutgers
University, New Brunswick, NJ 08901

Corresponding Author:

Tracy G. Anthony, PhD
Associate Professor
Department of Nutritional Sciences
Foran Hall Room 166
59 Dudley Rd.
New Brunswick, NJ 08901
848-932-6331
tracy.anthony@rutgers.edu

19 ABSTRACT

20 Phytoecdysteroids such as 20HE are nutritional supplements marketed as enhancers of lean body mass. In
21 this study the impact of 20HE ingestion on protein kinase B/Akt-mTORC1 signaling in the skeletal
22 muscle and liver of male rats was found to be limited. Bioavailability of 20HE, whether consumed alone
23 or with leucine, also remained low at all doses ingested. Additional work is necessary to clarify 20HE
24 mechanism of action in vivo.

25

26 KEYWORDS

27 Phytoecdysteroids, PKB/Akt, mTOR, 4E-BP1, leucine

28

Draft

INTRODUCTION

Phytoecdysteroids are a class of polyhydroxylated ketosteroid compounds found in plants and insects. In arthropods, they function as androgens and are involved in reproduction and the molting process (Dinan 2009). In plants, ecdysteroids serve as a defense mechanism in which their ingestion can disrupt an invading insect's hormone balance, resulting in lethal metabolic damage to the invader (Dinan 2009). One of the most commonly studied ecdysteroids, 20-Hydroxyecdysone (20HE), is naturally present in foods such as spinach and quinoa and is marketed as an anabolic agent in various nutritional supplements taken to improve athletic performance (Lafont and Dinan 2003). Ingestion of 20HE reportedly increases the size of muscle fibers in rats (Toth et al. 2008) as well as increased lean body mass in sheep, pigs and quail (Chermnykh et al. 1988; Lafont and Dinan 2003) and is associated with anti-diabetic effects in obese mice (Kizelsztejn et al. 2009). Reports in the literature claim anabolic effects in animals and humans without androgenicity (Chermnykh et al. 1988; Lafont and Dinan 2003; Gorelick-Feldman et al. 2008), but one human trial was unable to substantiate the anabolic potential of 20HE (Wilborn et al. 2006).

The molecular pathway mediating 20HE's anabolic potential remains unknown. Despite being classified as a steroid, 20HE is not structurally close enough to androgens to be able to bind to intracellular steroid receptors in humans (Bathori et al. 2008). Instead, it is suggested that 20HE binds to G protein coupled receptors (GPCR) on the plasma membrane (Bathori et al. 2008). An *in vitro* study showed that ecdysteroids increase protein synthesis in muscle cells through an influx of Ca^{2+} and subsequent activation of protein kinase B/Akt (Gorelick-Feldman et al. 2010). In this study, the effects of 20HE were suppressed by inhibitors of GPCR, phospholipase C (PLC) and phosphoinositide kinase-3 (PI3K).

Separate from its effects in muscle, other work has described anti-obesity and anti-diabetic effects of 20HE in mice via reduced hepatic glucose production in association with increased Akt phosphorylation (Kizelsztejn et al. 2009). It is unknown if reported changes in hepatic glucose

production by 20HE are linked in any way to acute activation of Akt. Furthermore, it is unknown if 20HE activates downstream anabolic signaling separate from metabolic signaling in liver. There are no studies assessing phosphorylation of Akt by 20HE *in vivo*.

Stimulation of muscle protein synthesis by insulin and/or insulin-like growth factor I (IGF-I) is mediated via PI3K-Akt activation of mammalian target of rapamycin complex 1 (mTORC1) assembly and signal transduction (Laplante and Sabatini 2012). Oral ingestion of amino acids, and in particular leucine, also stimulates mTORC1 assembly and signaling but in a PI3K-Akt-independent mechanism, allowing for a convergence of amino acid and insulin/IGF-I signaling on mTORC1 upon meal feeding (Anthony et al. 2000; Anthony et al. 2001a; Anthony et al. 2001b). Downstream effectors of mTORC1 such as eukaryotic initiation factor 4E binding protein 1 (4E-BP1) function to increase the efficiency of mRNA translation initiation. To what extent 20HE alone or in combination with leucine activates mTORC1 signaling in skeletal muscle and liver is unknown. The objective of this study was to assess the ability of 20HE to stimulate mTORC1 signaling in skeletal muscle and liver. A more thorough understanding of 20HE's molecular pathway of action *in vivo* can inform future interventions in humans or animals.

MATERIALS AND METHODS

Animals and Study Design.

All rats were cared for in accordance with the Guide to the Care and Use of Laboratory Animals and all experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Rutgers University. Overnight fasted 4-8 week old male Sprague-Dawley rats (n=5-6 per group) were randomized to one of three study designs: 1) gavaged with 0, 10, 50, 200 mg/kg 20HE and euthanized 30 min post-gavage; 2) gavaged with 200 mg/kg 20HE or excipient and euthanized at pre-gavage or 30 min, 60 min, 120 min, 240 min post-gavage, and 3) administered excipient or 200 mg/kg 20HE alone or in combination with 1.35 g/kg L-leucine and euthanized 30 min post-gavage. In these studies, 3% DMSO

was used as excipient in Study 1 and 2 whereas the emulsifier Labrasol (Hu et al. 2001) was the excipient in Study 3. For all studies the volume administered was 1 ml/100 g body weight. Before food removal, rats were maintained in a temperature-controlled (23-25°C) room with a 12:12h light:dark cycle and freely-provided commercial rodent diet.

Sample collection. Rats were euthanized by decapitation and trunk blood was collected for analysis of serum leucine and 20HE concentrations. The liver and hind-limb muscles (gastrocnemius + plantaris) were quickly extracted, weighed and frozen in liquid nitrogen before storage in a -80°C freezer.

Serum measurements. Serum leucine was analyzed via HPLC using ortho-phthalaldehyde/9-fluorenylmethyl chloroformate (OPA/FMOC) derivatized amino acid analysis. Detection was performed according to Agilent protocol app note: 5990-4547EN using Agilent ZORBAX Eclipse Plus C18 column. To quantify bioavailability of 20HE, 500 µl aliquots of serum samples were extracted with 500 µl of butanol three times. The organic layer was dried and dissolved in 100 µl of 70% ethanol; 5 µl was injected in LC-MS (5-95% acetonitrile gradient). A standard curve of 20HE (5, 10, 50 ng) was run alongside samples in LC-MS for quantification.

Immunoblot Analysis. Tissue samples were prepared for SDS-PAGE followed by immunoblot analysis as previously described (Anthony et al. 2007). Immunoblots included a tissue sample from rats injected with IGF-I as a positive control for Akt phosphorylation. Membranes were incubated with the following primary antibodies: Akt (p-Thr308), mTORC1 (p-Ser2448), and 4E-BP1. Protein expression was visualized using enhanced chemiluminescence (ECL) and signal intensities were digitally captured using a FluorChem M multiplex imager (ProteinSimple) and band densities were quantitated using imaging software.

Statistics. Data are expressed as means ± SEM. Experimental results were analyzed by ANOVA using the STATISTICA software package. Differences between group means were assessed by Tukey's post hoc test. The level of significance was set at $P \leq 0.05$ for all statistical tests.

RESULTS

Study 1: 20HE Dose Response

Young male rats were overnight (12h) fasted then gavaged with doses of 20HE that have been cited in the literature as imparting anabolic or metabolically-favorable properties in rodents. The lowest dose administered was twice the dosage used in a previous study reporting anabolic properties, and the highest dose administered was 200 mg/kg, an amount higher than a commonly suggested dose in humans of 200 mg per day, but substantially below the oral LD50 in mice of >9,000 mg/kg. None of the doses administered significantly altered the phosphorylation states of Akt, mTOR or 4E-BP1 at 30 minutes after ingestion in either the skeletal muscle (Figure 1, A-C) or liver (Figure 2, A-C). Quantification of 20HE from plasma samples showed there were minimal differences in bioavailability among the doses at 30 minutes when delivered in 3% DMSO in saline. Serum 20HE concentrations were detectable only in rats gavaged with 20HE but remained similarly low across all doses (~0.45 ng/μl).

Study 2: 20HE Time Course

To determine if the effect of 20HE on Akt phosphorylation or mTORC1 signaling in skeletal muscle and/or liver was delayed past 30 minutes, rats were overnight fasted and then administered 200 mg/kg 20HE and euthanized at 30 min, 60 min, 120 min and 240 min following gavage. No changes in the phosphorylation status of Akt, mTOR or 4E-BP1 as compared to untreated controls were noted in either skeletal muscle (Figure 1, D-F) or liver (Figure 2, D-F) over the 4 h time course except phosphorylation of 4E-BP1 was reduced at 240 min in skeletal muscle only (Figure 1F). Quantification of 20HE from plasma showed 20HE levels in blood ranged between 0.3-0.5 ng/μl after gavage when delivered in 3% DMSO in saline.

Study 3: Acute Effects of 20HE versus Leucine

Previous work by this lab and others show that oral administration of 1.35 g/kg body weight L-leucine activates mTORC1 signaling in skeletal muscle between 15-60 minutes following ingestion without altering Akt phosphorylation (Anthony et al. 2001a). To compare the impact of 20HE ingestion to that of leucine, mice were orally administered either 20HE (200 mg/kg), leucine (1.35 g/kg), the combination together, or excipient alone. To improve bioavailability of 20HE, Labrasol was used as the gavage excipient instead of 3% DMSO in saline. Average serum concentrations of leucine were statistically similar to average serum concentrations of leucine in rats gavaged with 20HE. Serum leucine concentrations rose significantly in rats gavaged with leucine alone and in mice gavaged with leucine plus 20HE (both $P < 0.05$ as compared to control). While leucine concentrations rose significantly regardless of 20HE consumption, 20HE bioavailability appeared to be improved by Labrasol, though with great variation, for serum levels of 20HE measured 0.74 ± 0.076 ng/ μ l in rats gavaged with 20HE + leucine compared with 1.52 ± 1.23 ng/ μ l in rats gavaged with 20HE alone. In agreement with previous reports, leucine alone significantly increased phosphorylation of mTOR and 4E-BP1 in both skeletal muscle (Figure 1, H-I) and liver (Figure 2, H-I). On the other hand, ingestion of 20HE alone did not alter mTORC1 signaling in skeletal muscle or liver and slightly dampened stimulation of mTORC1 by leucine in skeletal muscle. Neither leucine nor 20HE altered phosphorylation of Akt in liver or muscle at 30 min when gavaged alone or in combination (Figure 1G, 2G).

DISCUSSION

Phytoecdysteroids are one of the most abundant steroid classes in nature, are non-toxic to mammals, yet display pleiotropic effects which are potentially beneficial if harnessed properly. Among the postulated health benefits of this class of compounds, increased muscular mass and improved glucose homeostasis is evidenced in both *in vitro* and *in vivo* studies (Gorelick-Feldman et al. 2008; Toth et al. 2008; Kizelsztejn et al. 2009; Gorelick-Feldman et al. 2010; Seidlova-Wuttke et al. 2010). To understand if mTORC1 signaling may contribute to any of these previously reported outcomes, we embarked on a series of experiments evaluating acute effects of 20HE consumption in the skeletal muscle and liver of

male rats. Based on recent reports describing rapid activation of Akt associated with increased protein synthesis in cultured myocytes (Gorelick-Feldman et al. 2010) and reduced hepatic glucose production and body weight in obese mice (Kizelsztejn et al. 2009), we hypothesized that activation of Akt by 20HE leads to increased signaling via the mTORC1 pathway in skeletal muscle and/or liver. Yet, study results did not support this idea, showing that 20HE does not acutely activate Akt phosphorylation or mTORC1 signaling in hind limb muscle or liver. As such, the mechanism to explain potential health benefits of 20HE remains an open question requiring additional exploration. The following narrative offers alternate approaches and additional considerations.

The bioavailability of 20HE was quite low in these studies and appeared insensitive to increasing doses. While using an excipient such as Labrasol improved bioavailability, this did not result in improved activation of the Akt-mTORC1 pathway at the time points examined. The choice to focus on early time points was based on previous work showing activation of Akt by 20HE within 4 h and activation of mTORC1 by leucine within 1 h (Anthony et al. 2001a; Gorelick-Feldman et al. 2010). A follow-up time course administering 20HE in combination with calories and/or carbohydrates is warranted to test the idea that 20HE functions to augment insulin/IGF-I signaling by meal feeding. Interestingly, in the current study provision of leucine in combination with 20HE results in a dampening of mTORC1 signaling despite elevated circulating leucine. Thus, if 20HE is to augment the action of another nutrient, it is not likely to be an amino acid-driven pathway. Follow up studies exploring how the food matrix impacts 20HE absorption and potential augmentation of macronutrient metabolism is warranted.

This study contributes importantly to the study of phytoecdysteroids and establishes that acute bioavailability of 20HE is limited when consumed alone or with leucine. While one interpretation is that the anabolic signaling effect of 20E is limited by digestibility and absorption, an alternate explanation is that the mechanism of effect is independent of mTOR. Certainly, among the pathways affected by nutritional status, mTORC1 is one of the most important cellular signaling hubs in the body, regulating cell growth, autophagy, and proliferation (Laplane and Sabatini 2012). However, the path to improved body composition does not have to be mediated through mTOR. Alternate mechanisms requiring future

investigation include phospholipase C and cAMP/PKA (Gorelick-Feldman et al. 2010), reducing protein breakdown (e.g., similar to the leucine metabolite β -Hydroxy β -Methylbutyrate (Molfino et al. 2013)), altering vitamin D status or action in muscle (Toth et al. 2010) and increasing lipolysis so as to improve lean body mass. In summary, study results show that ingestion of 20HE does not acutely activate mTORC1 signaling in muscle or liver. Additional work is necessary to clarify the mechanism by which phytoecdysteroids can facilitate muscle growth and liver metabolism in mammals.

Conflict of Interest Disclaimer: The authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the expert technical assistance of Pengxiang She, PhD, Gabriel Wilson, PhD, Rana Al Baghdadi and George Wei. This work was funded by USDA Multistate Project NC1184 and the New Jersey Agricultural Experiment Station at Rutgers University.

REFERENCES

Anthony, J., Anthony, T., Kimball, S., and Jefferson, L. 2001a. Signaling pathways involved in translational control of protein synthesis in skeletal muscle by leucine. *J. Nutr.* **131**: 856S-860S. PMID: 11238774.

Anthony, J., Yoshizawa, F., Anthony, T., Vary, T., Jefferson, L., and Kimball, S. 2000. Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. *J. Nutr.* **130**(10): 2413-2419. PMID: 11015466.

Anthony, T., Anthony, J., Yoshizawa, F., Kimball, S., and Jefferson, L. 2001b. Oral administration of leucine stimulates ribosomal protein mRNA translation but not global rates of protein synthesis in the liver of rats. *J. Nutr.* **131**(4): 1171-1176. PMID: 11285321.

Anthony, T.G., McDaniel, B.J., Knoll, P., Bunpo, P., Paul, G.L., and McNurlan, M.A. 2007. Feeding meals containing soy or whey protein after exercise stimulates protein synthesis and translation initiation in the skeletal muscle of male rats. *J. Nutr.* **137**(2): 357-362. PMID:17237311.

Bathori, M., Toth, N., Hunyadi, A., Marki, A., and Zador, E. 2008. Phytoecdysteroids and anabolic-androgenic steroids--structure and effects on humans. *Curr. Medicinal Chem.* **15**(1): 75-91. doi: 10.2174/092986708783330674#sthash.Ceoi3oxK.dpuf. PMID: 18220764

- 217 Chermnykh, N.S., Shimanovskii, N.L., Shutko, G.V., and Syrov, V.N. 1988. [The action of
218 methandrostenolone and ecdysterone on the physical endurance of animals and on protein
219 metabolism in the skeletal muscles]. *Farmakol. Toksikol.* **51**(6): 57-60. PMID: 3234543.
220
- 221 Dinan, L. 2009. The Karlson Lecture. Phytoecdysteroids: what use are they? *Arch. Insect*
222 *Biochem. Physiol.* **72**(3): 126-141. doi: 10.1002/arch.20334. PMID: 19771554.
223
- 224 Gorelick-Feldman, J., Cohick, W., and Raskin, I. 2010. Ecdysteroids elicit a rapid Ca²⁺ flux
225 leading to Akt activation and increased protein synthesis in skeletal muscle cells. *Steroids*
226 **75**(10): 632-637. doi: 10.1016/j.steroids.2010.03.008. PMID: 20363237.
227
- 228 Gorelick-Feldman, J., Maclean, D., Ilic, N., Poulev, A., Lila, M.A., Cheng, D., and Raskin, I.
229 2008. Phytoecdysteroids increase protein synthesis in skeletal muscle cells. *J. Agric. Food.*
230 *Chem.* **56**(10): 3532-3537. doi: 10.1021/jf073059z. PMID: 18444661.
231
- 232 Hu, Z., Tawa, R., Konishi, T., Shibata, N., and Takada, K. 2001. A novel emulsifier, labrasol,
233 enhances gastrointestinal absorption of gentamicin. *Life Sci.* **69**(24): 2899-2910. PMID:
234 11720093.
235
- 236 Kizelsztejn, P., Govorko, D., Komarnytsky, S., Evans, A., Wang, Z., Cefalu, W.T., and Raskin,
237 I. 2009. 20-Hydroxyecdysone decreases weight and hyperglycemia in a diet-induced obesity
238 mice model. *Am. J. Physiol. Endocrinol. Metab.* **296**(3): E433-E439. doi:
239 10.1152/ajpendo.90772.2008. PMID: 19126784.

240

241 Lafont, R. and Dinan, L. 2003. Practical uses for ecdysteroids in mammals including humans: an
242 update. *J. Insect Sci.* **3**: 7. PMID:15844229.

243

244 Laplante, M. and Sabatini, D.M. 2012. mTOR signaling in growth control and disease. *Cell*
245 **149**(2): 274-293. doi: 10.1016/j.cell.2012.03.017. PMID: 22500797.

246

247 Molfino, A., Gioia, G., Rossi Fanelli, F., and Muscaritoli, M. 2013. Beta-hydroxy-beta-
248 methylbutyrate supplementation in health and disease: a systematic review of randomized trials.
249 *Amino Acids* **45**(6): 1273-1292. doi: 10.1007/s00726-013-1592-z. PMID: 24057808.

250

251 Seidlova-Wuttke, D., Ehrhardt, C., and Wuttke, W. 2010. Metabolic effects of 20-OH-ecdysone
252 in ovariectomized rats. *J. Steroid Biochem. Mol. Biol.* **119**(3-5): 121-126.
253 doi:10.1016/j.jsbmb.2010.01.006. PMID: 20097286.

254

255 Toth, N., Hunyadi, A., Bathori, M., and Zador, E. 2010. Phytoecdysteroids and vitamin D
256 analogues--similarities in structure and mode of action. *Curr. Medicinal Chem.* **17**(18): 1974-94.
257 doi: 10.2174/092986710791163911#sthash.haJDqr9m.dpuf. PMID: 20377509.

258

259 Toth, N., Szabo, A., Kacsala, P., Heger, J., and Zador, E. 2008. 20-Hydroxyecdysone increases
260 fiber size in a muscle-specific fashion in rat. *Phytomed. Intl. J. Phytotherapy Phytopharmacol.*
261 **15**(9): 691-698. doi: 10.1016/j.phymed.2008.04.015. PMID: 18585021.

262

263 Wilborn, C.D., Taylor, L.W., Campbell, B.I., Kerksick, C., Rasmussen, C.J., Greenwood, M., et
264 al. 2006. Effects of methoxyisoflavone, ecdysterone, and sulfo-polysaccharide supplementation
265 on training adaptations in resistance-trained males. *J. Intl. Soc. Sports Nutr.* **3**: 19-27. doi:
266 10.1186/1550-2783-3-2-19. PMID: 18500969.

267

268

Draft

Figure Legend

Figure 1. Phosphorylation of Akt, mTOR and 4E-BP1 in the skeletal muscle of male rats orally administered 20HE. Phosphorylated Akt at threonine 308 and total Akt expression was measured by immunoblot in Study 1 (A), Study 2 (D) and Study 3 (G); Phosphorylated mTOR at serine 2448 and total mTOR was measured by immunoblot in Study 1 (B), Study 2 (E) and Study 3 (H); Gel mobility shift assay showing 4E-BP1 phosphorylation in Study 1 (C), Study 2 (F), and Study 3 (I). Bar graphs represent means $\pm$ SEM of densitometry ratios; n=5-6 per group. In Study 1 and 3, time of euthanasia was 30 min. In Study 3, 20HE dose was 200 mg/kg and leucine dose was 1.35 g/kg. In each bar graph, means not sharing a common letter are different, $P < 0.05$.

Figure 2. Phosphorylation of Akt, mTOR and 4E-BP1 in the liver of male rats orally administered 20HE. Phosphorylated Akt at threonine 308 and total Akt expression was measured by immunoblot in Study 1 (A), Study 2 (D) and Study 3 (G); Phosphorylated mTOR at serine 2448 and total mTOR was measured by immunoblot in Study 1 (B), Study 2 (E) and Study 3 (H); Gel mobility shift assay showing 4E-BP1 phosphorylation in Study 1 (C), Study 2 (F), and Study 3 (I). Bar graphs represent means $\pm$ SEM of densitometry ratios; n=5-6 per group. In Study 1 and 3, time of euthanasia was 30 min. In Study 3, 20HE dose was 200 mg/kg and leucine dose was 1.35 g/kg. In each bar graph, means not sharing a common letter are different, $P < 0.05$.