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Continuous Infusion of 20-Hydroxyecdysone Increased Mass of Triceps Brachii in C57BL/6 Mice

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Phytoecdysteroids have been attributed with numerous pharmacological properties in animals, including increasing muscle mass, and 20-hydroxyecdysone (20E) is one of the most abundant phytoecdysteroids produced by plants. In this study, the physiological and gene expression effects of 20E were analyzed in C57BL/6 mice given a continuous infusion of saline or 20E (5 mg/kg/day) for 5 or 15 days using subcutaneously implanted Alzet® osmotic pumps. The masses of the total body, muscle groups and organs were determined. There was a significant increase ($p = 0.01$) in the mass of triceps brachii in mice treated with 20E for 5 days (115 ± 8 mg) compared with mice treated with saline for 5 days (88 ± 3 mg), however, there were no differences in the other measured parameters. To determine potential mechanisms of 20E in skeletal muscle, Illumina's Mouse Whole Genome-6 v2.0 Expression BeadChips were used to evaluate changes in gene expression of the triceps brachii after 20E infusion. Ingenuity Pathways Analysis was used to identify genes with the most evidence for differential expression, of which, 16 genes involved in the skeletal and muscular system were identified. Overall, the data suggest that 20E does not have potent anabolic properties, however, a muscle-specific increase was observed and genes were identified to provide an explanation for the muscle accretion. **Copyright © 2012 John Wiley & Sons, Ltd.**

Keywords: 20-hydroxyecdysone; infusion; mice; microarray; phytoecdysteroids; skeletal muscle.

Supporting information may be found in the online version of this article (Supplementary Material)

INTRODUCTION

Phytoecdysteroids (PE) are a diverse class of plant triterpenoids that contain a steroidal skeleton and vary in the number, position and orientation of hydroxy substituents as well as conjugated moieties. Phytoecdysteroids are plant-produced analogues of ecdysteroids, insect molting hormones that control cell proliferation, growth and developmental cycles (Dinan, 2001). They are widely distributed in the plant kingdom and there is strong evidence to support their role as plant defense compounds against non-adapted insects and nematodes (Schmelz *et al*., 2000; Soriano *et al*., 2004). 20-Hydroxyecdysone (20E; Fig. 1) is the most abundant PE produced by several plant families and the main PE to which PE-containing herbal preparations are standardized (Báthori and Kalász, 2001; Báthori *et al*., 2008). The medicinal herb *Rhaponticum carthamoides* (Willd.), traditionally valued in Siberia for its muscle and strength enhancing properties, contains up to 0.81%, 1.22% and 1.51% 20E of dry weight of roots, aerial portion and seeds, respectively (Kokoska and Janovska, 2009). Botanical supplements containing 20E include Magnum Thrust® from Magnum Nutraceuticals (British Columbia, Canada) and Ecdysten® from ThermoLife (Phoenix, Arizona, USA) and are often marketed as potent energy and muscle enhancers. Additionally, spinach (*Spinacia oleracea*) and quinoa (*Chenopodium quinoa*) are two cultivated crops reported to accumulate high levels of phytoecdysteroids; upwards of 100 μg/g fresh weight 20E and 380 μg/g seed 20E equivalents, respectively (Bakrim *et al*., 2008; Kumpun *et al*., 2011). The consumption of 20E and other PE in dietary supplements and foods warrants investigation into their potential biological function for humans.

Phytoecdysteroids have been attributed with numerous pharmacological properties in animals, including humans. Growth-promoting effects have been reported for mice, rats, sheep, pigs and Japanese quail after ingestion of PE, implying a potentially valuable application in improving livestock production (Lafont and Dinan, 2003). The enhancement of strength, stamina and muscle mass in mammals are among the main purported bioactive properties and commercial use of PE and PE-containing extracts (Báthori and Kalász, 2001). 20-Hydroxyecdysone stimulated protein synthesis *in vitro* in C2C12 murine myotubes as well as *in vivo* in rats given a single daily dose of 5 mg/kg for 10 days (Gorelick-Feldman *et al*., 2008; Syrov, 2000). Additionally, 20E increased the mass (tibialis anterior) and size (soleus and extensor digitorum longus) of skeletal muscle and stimulated muscle fiber growth in regenerating soleus muscles (Syrov, 2000; Tóth *et al*., 2008), demonstrating the potential of 20E as a therapeutic agent for muscle atrophy. As a relatively new class of medicinally active compounds, comprehensive reviews of the purported

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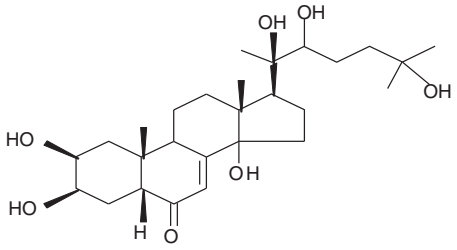

Figure 1. 20-Hydroxyecdysone.

biological effects of phytoecdysteroids have been published only within the past decade (Dinan, 2001; Lafont and Dinan, 2003; Báthori and Pongrácz, 2005; Báthori *et al.*, 2008).

This study focuses on the purported muscle-enhancing properties of 20E in healthy mice. Because the *in vivo* bioactivity and the mechanism of action of PE in mammals remain ambiguous, we examined the effects of 20E on mammalian gene expression and physiology. Mice were administered a steady infusion of phytochemical during treatment using subcutaneously implanted Alzet® osmotic pumps for 5 or 15 days, after which total body, extracted muscle groups and organs were weighed. Global gene expression changes were evaluated in skeletal muscle and potential molecular targets of 20E were identified using the Illumina BeadArray platform.

MATERIALS AND METHODS

Animals. All experimental protocols were approved by the University of Illinois Institutional Animal Care and Use Committee. Adult male mice (12 weeks old) strain C57BL/6J were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Animals were individually housed in plastic cages in a temperature-controlled room (22 °C) with 12:12 hour light–dark cycles and given *ad libitum* access to food (AIN-93 G) and water.

Following a 1 week acclimation period, mice were weighed and randomly assigned to weight balanced treatment groups ($n = 9$–10). Alzet® osmotic pumps (model 2002; Cupertino, CA, USA) were subcutaneously implanted according to manufacturer specifications to provide a continuous infusion of the test compound. 20-Hydroxyecdysone from Bosche Scientific (95% purity, New Brunswick, NJ) was further purified through a silica gel column as described in Cheng *et al.* (2008). Pumps supplied a dose of 5 mg/kg/day of 20E dissolved in saline (0.9% NaCl) or saline alone (control), with a flow rate of 0.5 μL/h. During surgery, animals were anesthetized with isoflurane from a gas vaporizer maintained between 2 and 4%. After pumps were implanted, incisions were sealed with surgical clamps and animals were given a subcutaneous injection of carprofen (5 mg/kg).

Mice were euthanized by CO_2 asphyxiation followed by cervical dislocation on the fifth and fifteenth day after implantation. Blood was drawn immediately postmortem by cardiac puncture, plasma was separated by centrifugation at $14000 \times \mathbf{g}$ for 15 min at 4 °C, and samples were stored at −20° C. Plasma samples were treated with β-glucuronidase (Helix Pomatia β-glucuronidase Type H1; Sigma) and incubated overnight at 37 °C as described by Seidlova-Wuttke *et al.* (2010). Hydrolyzed samples were partitioned with hexane followed by ethyl acetate (three times), lyophilized, redissolved in 0.5 mL ethanol and filtered through a 0.2 μm membrane prior to HPLC analysis as described in Cheng *et al.* (2008).

The following muscle groups and organs were extracted and weighed: biceps femoris, triceps brachii, tibialis anterior, gastrocnemius, kidney, liver and heart. All tissues were immediately frozen and stored at −80° C. Statistical significance was determined by comparing the 20E treatment group with saline control at each time point by analysis of variance (ANOVA) using SAS version 9.2 for Windows (SAS Institute Inc., Cary, NC, USA). A probability of $p < 0.05$ was considered statistically significant.

RNA isolation. The triceps muscles were selected for RNA isolation and microarray analysis because an increase in mass was demonstrated in this muscle group with 20E treatment after 5 days. Total RNA was extracted from triceps (ca. 100 mg) by homogenization with 1 mL TriReagent (St Louis, MO) for 3 min at 30 Hz using a Tissuelyser II (Qiagen, Gaithersburg, MD). Total RNA was treated with DNase I and purified using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Quantity and purity of RNA were determined using the NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA). Total RNA was pooled into groups containing three individual biological replicates (except 15 days saline infusion, which had two replicates). Each pool contained an equal quantity of RNA (1 μg) from each individual animal. Three pools were analyzed per treatment using Illumina's MouseWG-6 v2.0 Expression BeadChips (San Diego, CA, USA).

Microarray hybridization and labeling reactions. For Illumina microarray analysis, samples were prepared and analyzed by the W. M. Keck Center for Comparative and Functional Genomics in the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign. RNA quality was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Three-hundred nanograms of high quality total RNA were primed with an oligo(dT) primer bearing a T7 promoter, and reverse transcribed in the first-strand cDNA synthesis using the Illumina® TotalPrep RNA Amplification Kit (Ambion, Inc., Austin, TX). Single stranded cDNA was then converted into double stranded cDNA according to the manufacturer's instructions. The double-stranded cDNA was purified and served as a template in the 14 h *in vitro* transcription reaction. Prior to hybridization, the synthesized biotin labeled cRNA was cleaned up using the same Amplification Kit. After the quality control assessment, 1.5 μg of cRNA from each experimental sample along with hybridization controls were hybridized for 16 h to the MouseWG-6 v2.0 Expression BeadChips (Illumina, Inc., San Diego, CA, USA) in the 58 °C Illumina Hybridization Oven. Washing, staining with streptavidin-Cy3 (GE Healthcare Bio-Sciences, Piscataway, NJ), and scanning were performed according to the Illumina Whole-Genome Gene Expression Direct Hybridization Assay Guide (revision A). Two BeadChips were used in this study, each containing six arrays. The arrays were scanned using an Illumina BeadArray Reader. Each array image was visually screened to discount for signal artifacts, scratches or debris.

Data processing and analysis. The raw bead-level files were processed with Illumina® BeadStudio 3.1.3, Gene Expression Module v3.4.0 (Illumina), without background correction or normalization, to obtain one value per beadtype for each array. These beadtype values were normalized and analyzed in R (R Development Core Team, 2008) using the beadarray package (Dunning *et al*., 2007) from the Bioconductor Project (Gentleman *et al*., 2004). Background fluorescence was corrected by subtracting the mean of the negative control beadtypes; negative and zero values after background correction were set to 0.5. Quantile normalization was performed and data were log2 transformed. Differential expression was assessed using a linear model using the limma package (Smyth, 2005), which uses an empirical Bayes correction (Smyth, 2004) that helps to improve power by borrowing information across beadtypes. BeadStudio's Detection *p*-values were used to filter out 21572 beadtypes that were not detected ($p > 0.05$) above the negative controls in any of the 12 samples. The remaining 23709 beadtypes were input to Ingenuity Pathways Analysis (IPA, Ingenuity® Systems, www.ingenuity.com) as the reference set. For each pairwise comparison (20E vs. saline) at each time point, beadtypes with raw *p* values < 0.01 from the limma analysis were selected for functional analysis, pathway mapping, and network generation.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al*., 2002) and are accessible through GEO Series accession number GSE23121 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23121).

RESULTS

Body tissue analysis

To determine whether 20E conferred an anabolic effect on skeletal muscle, muscle groups were weighed in mice infused with 20E or saline (Table 1). There was a significant increase in the mass of the triceps brachii in the mice treated with 20E for 5 days (115 ± 8 mg) compared with mice treated with saline for 5 days (88 ± 3 mg) ($p = 0.01$). However, there were no differences in masses of the other muscle groups (tibialis anterior, biceps femoris and gastrocnemius) and also no differences in mice treated with 20E compared with saline for the longer duration of 15 days. Additionally, results showed no significant differences in body mass, organ mass or average daily feed intake between the treatment groups. Administration of 20E by infusion was confirmed by measuring the volume of 20E solution or saline remaining in the osmotic pump reservoirs; approximately 160 and 90 μL for the 5- and 15-day treatments, respectively. However, 20E was not detected in the hydrolyzed plasma samples possibly due to the low plasma sample volume (< 155 μL) and concentration of 20E that was below the limit of detection.

Microarray analysis

To determine potential leads for the biological action of 20E, RNA was extracted from the triceps brachii, where there was an observed increase in muscle mass with 20E treatment, for whole-genome microarray analysis. Neither the response to 20E (20E vs. saline) at 5 days nor the response to 20E at 15 days had any beadtypes on the array that were significantly different after False Discovery Rate (FDR) correction (Benjamini and Hochberg, 1995). This did not mean there were no genes with differential expression due to 20E treatment, just that the model was not able to distinguish them from the expected false positives of testing 23709 beadtypes. Instead, genes with the most evidence for differential expression were evaluated using IPA to identify candidate genes or overall trends. A raw *p*-value cutoff of 0.01 was used, which resulted in 159 beadtypes for the response to 20E at 5 days and 224 beadtypes for the response to 20E at 15 days. All genes are listed in Supplementary Table 1. Beadtypes meeting the raw *p*-value cutoff of 0.01 were input into IPA, which mapped them to 104 network eligible genes for the response to 20E at 5 days and 147 network eligible genes for the response to 20E at 15 days.

Table 1. Body masses, average daily feed intake, muscle group masses and organ masses of mice infused with saline (control) or 20-hydroxyecdysone (20E) for 5 days or 15 days reported as the mean ± standard error

	Treatment			
	5 days		15 days	
Variables	Saline	20E	Saline	20E
Initial body weight (g)	23.1 ±0.5	23.0 ± 0.5	23.3 ± 0.6	23.1 ± 0.6
Final body weight (g)	24.4 ± 0.8	24.3 ± 0.7	23.8 ± 0.6	23.4 ± 0.5
Average daily feed intake (g)	3.0 ± 0.2	3.2 ± 0.1	3.1 ± 0.1	3.1 ± 0.1
Muscle groups (mg):				
tibialis anterior	61 ± 4	63 ± 3	66 ± 5	62 ± 4
gastrocnemius	137 ± 4	135 ± 3	128 ± 4	131 ± 4
triceps	88 ± 3	115 ± 8[a]	94 ± 8	89 ± 5
biceps	173 ± 10	161 ± 16	169 ± 12	170 ± 8
Organs (mg):				
kidney	361 ± 18	355 ± 8	357 ± 15	386 ± 15
heart	142 ± 7	158 ± 18	141 ± 7	145 ± 10
liver	913 ± 89	953 ± 73	1037 ± 44	1058 ± 49

[a]Significantly different from control, $p < 0.05$.

In addition to providing gene-level information, IPA organized genes into biological functions and tested whether each function is significantly overrepresented in the selected list of genes; overrepresentation may indicate that a particular biological process was affected by the 20E treatment. In the categories of skeletal and muscular system development and function and skeletal and muscular disorders, IPA identified 16 genes presented in Table 2. The *p* value associated with each gene indicates the probability of the biological function assigned to the dataset by chance alone. A list of all biological functions identified by IPA can be found in Supplementary Table 2.

DISCUSSION

In this study, physiological and gene expression effects were analyzed in mice given a continuous infusion of 20E. Total body, individual muscle groups and organ masses were recorded, and carcass composition was determined to evaluate changes in nutrient partitioning. Results showed a significant increase in the mass of the triceps brachii in mice treated with 20E for 5 days compared with mice treated with saline for 5 days and no differences in masses between treatment groups in the other parameters (Table 1). Overall, the data suggest that 20E does not have potent anabolic properties. Statistical analysis of microarray beadtypes with False Discovery Rate correction did not identify any genes as differentially expressed. The IPA identified genes involved in skeletal muscle development and function that may be lead candidates for further investigation.

Although there are numerous reports on the growth-promoting effects of 20E in mammals, studies do not consistently demonstrate an increase in muscle and/or body mass (Stopka *et al*., 1999; Gao *et al*., 2008; Tóth *et al*., 2008). The variation in results could be due to varying routes of administration, doses and rodent strains. In this study, 20E infusion (5 mg/kg/day) for 5 or 15 days did not result in an increase in total body mass. Tóth *et al.* (2008) reported an increase in body mass with daily subcutaneous injections of 20E (5 mg/kg) for 8 days in rats. Conversely, others have indicated no difference in body mass with 20E treatment with daily veinous injections of 20E (1 mg/kg) for 3 weeks in mice (Gao *et al*., 2008). An increase in the mass of triceps brachii was demonstrated in this study, and other muscle specific increases in the soleus, extensor digitorum longus and tibialis anterior have been reported for rats given daily administration of 20E orally or by subcutaneous injection (Syrov, 2000; Tóth *et al*., 2008). Further research involving the optimization of dose, duration and route of administration of phytoecdysteroids, to demonstrate reproducible responses *in vivo*, would be required to show anabolic efficacy.

In this study, 20E was administered via an osmotic pump implant to provide a well-defined and continuous infusion to address issues of bioavailability and as well as to eliminate the stress from daily handling and injections. Elimination of 20E in mice is rapid, which may explain the discrepancies in muscle mass accretion. After caudal vein injection of 20E at 50 mg/kg, approximately 90% of 20E was eliminated in 30 min and the effective

Table 2. Ingenuity Pathways Analysis of genes with the most evidence for differential gene expression in triceps brachii after 5 days of 20-hydroxyedysone infusion. The *p* value associated with each gene indicates the probability of the biological function or disease assigned to the dataset by chance alone

Skeletal and muscular	Genes	*p* value	Function annotation
Disorders	GDF5	0.0067	Brachydactyly, type C
	SOCS1	0.0067	Destruction of synovial joint
	CCL24	0.0067	Hyperplasia of airway smooth muscle
	SOCS1	0.0067	Lymphoma of bone marrow
	CACNA2D1, EFEMP2, GDF5	0.0088	Osteoarthritis
	ABCC1	0.0266	Damage of bone marrow
	SLC8A3	0.0266	Necrosis of myofibre
System development and function	GDF5	0.0067	Size of cartilage tissue
	GDF5, TBX3	0.0121	Morphogenesis of forelimb
	ACVR2A	0.0134	Growth of gastrocnemius
	ACVR2A	0.0134	Growth of quadriceps femoris
	ACVR2A	0.0134	Growth of triceps brachii muscle
	ACVR2A	0.0134	Patterning of axial skeleton
	TOR2A	0.0200	Mitogenesis of vascular smooth muscle cells
	PTEN	0.0200	Recruitment of vascular smooth muscle cells
	PTEN, SOCS1, TRAF6	0.0227	Differentiation of osteoclasts
	GDF5	0.0266	Chondrogenesis of mice
	INHA	0.0266	Formation of bone cell lines
	INHA	0.0331	Formation of colony forming unit osteoblasts
	GDF5, KCNC3, SOCS1	0.0330	Skeletal and muscular process of mice
	TBX3	0.0331	Morphology of forelimb
	TBX3	0.0331	Morphology of hindlimb
	ACVR2A, GHR, HOXC9, INHA, TBX3, TRAF6	0.0377	Development of skeleton

half-time was 8.15 min (Dzhukharova *et al*., 1987). Continuous exposure of 20E was employed to allow for biological effects to develop, and resulted in an increase in the mass of the triceps brachii for the 5 day infusion of 20E but not the 15 day infusion. Likewise, infusion of clenbuterol, a potent anabolic drug, demonstrated anabolic action after 2 days, however Choo *et al*. (1992) noted that a decline was detected after about the tenth day of drug exposure. It is possible that a similar response occurred in this study in which there was an increase followed by a decrease in skeletal muscle mass due to adaptive changes from prolonged continuous administration.

A whole genome mouse array was used to screen for molecular targets in the triceps, the muscle group that demonstrated growth after 20E infusion. No genes were differentially expressed after FDR correction and IPA was used to identify genes with the greatest probability for differential expression and organize the genes in biological context. Absence of differentially expressed genes may be due to the complexity of muscle tissue, individual variation, timing and hormonal interferences that may confound the mild influence of 20E on skeletal muscle *in vivo*. *In vitro* studies using C2C12 murine myotubes suggested that 20E may induce an intracellular calcium flux to activate Akt and increase protein synthesis through a G-protein coupled receptor–phospholipase c–phosphoinositide-3-kinase (GPCR-PLC-PI3K) pathway (Gorelick-Feldman *et al*., 2010). The murine skeletal muscle genes revealed by IPA, such as ACVR2A, involved in muscle growth, may be targets for further investigation of mechanisms in defined *in vitro* systems.

The potential therapeutic applications and commercial market for phytoecdysteroid-containing supplements illustrate the importance of discerning their specific biological activities, clarifying claims of anabolic potency and providing insight on *in vivo* mechanisms of action in mammals. Administration of 20E by continuous infusion did not demonstrate potent anabolic properties, however, there was a statistically significant increase in the mass of the triceps brachii. Genes and biological pathways identified by IPA provide potential leads on the mechanism of 20E influence.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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