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Phytoecdysteroids Increase Protein Synthesis in Skeletal Muscle Cells

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Phytoecdysteroids, which are structurally similar or identical to insect molting hormones, produce a range of effects in mammals, including increasing growth and physical performance. To study the mechanism of action of phytoecdysteroids in mammalian tissue, an in vitro cellular assay of protein synthesis was developed. In C2C12 murine myotubes and human primary myotubes, phytoecdy steroids increased protein synthesis by up to 20%. In vivo, ecdysteroids increased rat grip strength. Ecdysteroid-containing plant extracts produced similar results. The effect was inhibited by a phosphoinositide kinase-3 inhibitor, which suggests a PI3K-mediated mechanism.

KEYWORDS: Ecdysteroid; ajuga; spinach; protein synthesis; skeletal muscle; myotube; C2C12

INTRODUCTION

Ecdysteroids, polyhydroxylated ketosteroids with long carbon side chains, are produced primarily in insects and plants. Although the role of ecdysteroids as insect hormones and their involvement in development and the molting process have been well studied, their role in plants is less obvious. Ecdysteroids found in plants, called phytoecdysteroids, do not elicit any of the classical plant hormone responses; however, they do elicit weak gibberellin-like activity in rice, as well as affect differentiation in alfalfa embryos (*1*). It has been suggested that plants utilize ecdysteroids as a chemical defense against insect herbivory by disrupting the insect hormonal balance and molting process (*2, 3*).

Ecdysteroids have also been reported to have effects in mammals, including lowering cholesterol levels (*4*) and blood glucose (*5*). They have immunomodulating (*6*), antiarrythymic (*7*), and hepatoprotective effects [(*8*); for a review see refs *9* and *10*]. Despite all of these effects, no receptor homologous to the insect ecdysone receptor has been identified in vertebrates. In fact, the transfected ecdysone receptor has been utilized for gene switch systems (*9*).

Anabolic effect is another reported property of ecdysteroids in vertebrates. Ecdysteroids have been shown to increase growth in a wide variety of animals including mice (*11, 12*), rats (*13*), sheep (*14*), pigs (*15*), and quail (*16*). Observed anabolic effects of these compounds are increased physical performance without training, as demonstrated using the forced swim test with rats, and increased synthesis of myofibrillar proteins in both the soleus and extensor digitorum longus (*17*). Increased growth and protein content were also observed in ecdysteroid-treated mouse liver and kidneys (*18*).

Plants are natural sources of ecdysteroids. Although most plants do not contain measurable amounts of ecdysteroids, some plants produce high levels of these compounds. *Ajuga turkestanica*, an herb from the basil family native to Uzbekistan, contains high levels of the C-11 hydroxylated turkesterone, one of the more active ecdysteroids (*19*). The high concentration of this potent ecdysteroid makes *A. turkestanica* a potentially useful medicinal plant. Edible plants, such as *Spinacia oleracea* (spinach), also contain considerable amounts of ecdysteroids, such as 20-hydroxyecdysone (20HE) (*2*), one of the most common plant-derived ecdysteroids. In addition to its use as a food crop, spinach may also have potential therapeutic qualities.

Despite the data showing various in vivo anabolic effects of ecdysteroids, the mechanisms of their cellular mode of action have not been elucidated. One of the barriers has been the lack of a simple in vitro assay to quantify the anabolic effect of ecdysteroids on skeletal muscle. In this study, we developed a cell culture-based method for analyzing the effects of ecdysteroids and report a significant increase in the protein synthesis of muscle cells following ecdysteroid treatment. The model was further used to identify factors that either enhanced or abrogated this anabolic effect. In support of earlier literature, we confirmed that this in vitro effect was translated in vivo as demonstrated by increased grip strength in rats treated with 20HE or with ecdysteroid-containing plant extracts.

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MATERIALS AND METHODS

Materials. 20HE, polypodine B, and ponesterone were purchased from Scitech (Praha, Czech Republic). Methandrostenolone, a synthetic anabolic steroid, was purchased from Steraloids (Newport, RI). Pure turkesterone was a gift from the Tashkent Institute of Cardiology (Uzbekistan). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Plant Extraction. *A. turkestanica* was collected in Uzbekistan, and voucher specimens were cataloged in the Rutgers Herbarium. The dried aerial portion was extracted in 95% ethanol. On the basis of previous phytoecdysteroid extraction protocols (*16*), the ethanolic extract was partitioned with butanol, and the butanolic phase was dried and used for testing.

Locally grown dried spinach powder (*S. oleracea*) was extracted in 95% ethanol for 24 h. After the removal of ethanol, the extract was resuspended in water and partitioned with heptane. The organic phase was removed, and the water phase was partitioned with butanol. The butanolic phase was dried and used for testing.

The ecdysteroid content of plant extracts was determined using (+)ESI LC-MS. A standard curve was generated using increasing amounts of purified turkesterone or 20HE. The typical fragmentation ions of ecdysteroids [(M + H) + (M − H_2O + H) + (M − 2H_2O + H) + (M − 3H_2O + H)] were merged to produce chromatograms for each compound. The fragmentation patterns and retention times of the plant extracts were compared with those of standards to quantify the amount of turkesterone and 20HE present.

Cell Culture. A mouse skeletal muscle cell line, C2C12 (ATCC CRL-1772), was maintained according to the method of Montgomery et al. (*20*). Between passages 3 and 10, cells were seeded at a density of 10^5 cells/cm^2 onto 24-well tissue culture plates. The cells were grown in low-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 6 mM glutamine, 1 mM pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco, Grand Island, NY). Cells were grown for 48 h in 5% CO_2 at 37 °C. After cells reached 80% confluency, the medium was replaced with differentiation medium (DMEM with 2% horse serum). After 5 days, the myoblasts had fused into multinucleated myotubes.

Primary human skeletal muscle cells (a gift from Dr. William Cefalu of the Pennington Biomedical Research Center, Baton Rouge, LA) were seeded at a density of 10^5 cells/cm^2 onto 24-well tissue culture plates. The cells were grown in DMEM supplemented with 10% FBS and a SingleQuot Kit (Lonza, Portsmouth, NH) containing epidermal growth factor, insulin, bovine serum albumin (BSA), fetuin, dexamethasone, and gentamicin/amphotericin-B. Cells were grown for 96 h in 5% CO_2 at 37 °C until they reached 80% confluency, and the medium was replaced with differentiation medium (DMEM with 2% horse serum). After 18 days, myoblasts fused into multinucleated myotubes.

Cell Treatment. For the ecdysteroid dose response, C2C12 cells were washed with serum-free DMEM and treated with increasing concentrations of 20HE, turkesterone, ponesterone, polypodine B, methandrostenolone, or the vehicle, 0.1% ethanol, four wells per treatment. Compounds were added to serum-free medium containing 5 μCi/mL [^{3}H]leucine. Cells were incubated for 4 h before protein measurement. For the 20HE time course study, C2C12 cells were treated with 1 μM 20HE for 0.5−24 h in serum-free medium containing 5 μCi/mL [^{3}H]leucine before protein measurement. For the plant extract study, C2C12 cells were treated with increasing concentrations of spinach extract, *A. turkestanica* extract, or vehicle for 4 h during the radiolabeled exposure period before protein measurement. For the inhibitor study, C2C12 cells were pretreated with either 10 μM of the phosphoinositide kinase-3 (PI3K) inhibitor, LY294002, or vehicle for 30 min. The medium was changed, and the cells were incubated with either 1 μM 20HE, 100 ng/mL insulin-like growth factor-1 (IGF-1), or vehicle for 4 h before protein measurement. For the 24 h 20HE dose response in human muscle cells, the cells were washed and treated with either 1 μM 20HE or vehicle in serum-free leucine containing medium supplemented with 5 μCi/mL [^{3}H]leucine. Cells were then incubated for 24 h before protein measurement.

Protein Synthesis Assay. Protein synthesis was determined by measuring the incorporation of the tritiated amino acid, leucine (*20*). Briefly, following treatment, cells were washed with cold phosphate-buffered saline (PBS), followed by the addition of 5% trichloroacetic acid (TCA) to precipitate protein. After 30 min at 4 °C, the TCA was removed and the precipitate was dissolved in 0.5 M NaOH (500 μL). The dissolved precipitate (400 μL) was added to scintillation vials with 5 mL of scintillation fluid (Ready Safe, Beckman Coulter, Fullerton, CA). Decays per minute (DPM) were measured in a liquid scintillation counter (LS 6500, Beckman Coulter). Total protein was quantified using the bicinchoninic acid (BCA) method following the manufacturer's instructions (Pierce, Rockford, IL). The data were expressed as DPM per milligram of total protein. Each experiment was performed in triplicate. The results were expressed as mean ± SEM. Statistical significance was determined using Student's *t* test ($p < 0.05$).

Binding Assay. Androgen receptor binding assays were performed by MDS Pharma Services, Taiwan, study 1019130. The assay using the androgen receptor was performed as described in ref *21*. Briefly, recombinant rat androgen receptor was combined with [^{3}H]mibolerone in a buffer of 50 mM Tris-HCl, pH 7.5, 0.8 M NaCl, 10% glycerol, 2 mM dithiothreitol, 1 mg/mlL BSA, and 2% ethanol. Increasing concentrations of 20HE, methandrostenolone, or the vehicle (1% DMSO) were added for 4 h at 4 °C. DPM of the incubation buffer was measured to quantify displacement of the labeled ligand. Each treatment was repeated four times, and the results were averaged.

Grip Strength Measurements. Male Sprague–Dawley rats (213–230 g) were subjected to one of four treatments: vehicle (water), 50 mg/kg 20HE, 1000 mg/kg spinach extract, or 10 mg/kg methandrostenolone. Ten animals per group were orally gavaged daily for 28 days. On day 28, grip strength on front limbs was assessed using a Wagner Force Five Digital Force Gage, model FDV-5. After the rat's hind paws grip the screen, the animals were quickly pulled by the base of the tail until the hind paws released from the screen, and the required release force was recorded. Three trials on each animal were performed, and significance was determined using Student's *t* test ($p < 0.05$). Animal care followed the Guide for the Care and Use of Laboratory Animals DHEW of the NIH.

RESULTS AND DISCUSSION

All of the tested ecdysteroids increased protein synthesis in C2C12 myotubes in a dose-dependent manner after 4 h of treatment (**Figure 1**). 20HE and turkesterone elicited the strongest response, increasing protein synthesis to 110% of control at 40 nM. This effect peaked at 0.1 μM, with protein synthesis at 120% of control and was still observed at concentrations up to 10 μM. Ponesterone and polypodine B had less potent activity, requiring 1 μM to produce an increase of 120% of control. At lower concentrations of 10 nM, all of the tested ecdysteroids slightly inhibited protein synthesis, although the results were not statistically significant. Methandrostenolone, an anabolic steroid, had no significant effect on protein synthesis at concentrations up to 10 μM.

In human skeletal myotubes, 20HE produced a similar dose-dependent increase in protein synthesis after 24 h of treatment (**Figure 2**). Treatment with 100 nM 20HE increased protein synthesis by 120% of control. This increase was observed at up to 1 μM.

In the 24 h 20HE study, 20HE increased protein synthesis in C2C12 myotubes by up to 120% of control (**Figure 3**). This increase became statistically significant after 2 h, peaked at 8 h, and remained for the 24 h duration of the experiment.

The most abundant ecdysteroids in the *A. turkestanica* and spinach extracts, prepared as described above, were quantified with (+)ESI LC-MS. The *A. turkestanica* extract contained 2.1% (w/w) turkesterone and 0.9% (w/w) 20HE (**Figure 4**). The spinach extract contained 3.0% (w/w) 20HE. Both extracts contained other ecdysteroids but at much lower

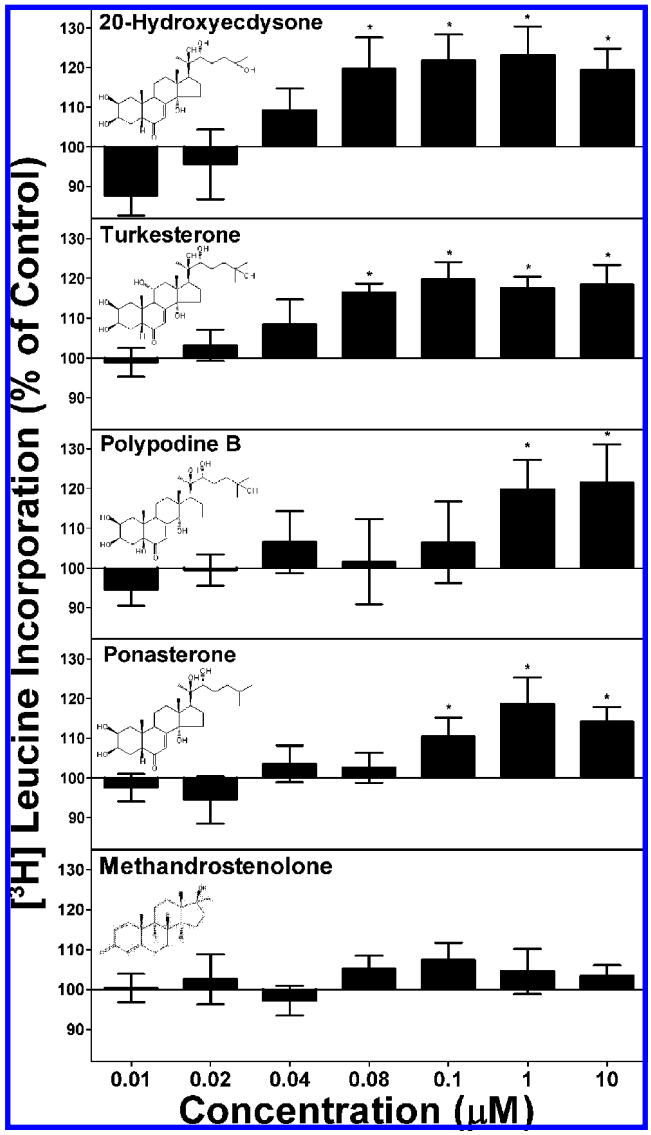

Figure 1. [^{3}H]Leucine incorporation in C2C12 myotubes treated with various ecdysteroids or the androgen, methandrostenolone. Differentiated myotubes were treated for 4 h with increasing concentrations of 20HE, turkesterone, ponastersone, polypodine B, methandrostenolone, or vehicle. DPM were normalized by total protein. The data represent the mean values ± SEM of four samples, each performed in triplicate. ∗ indicates $p < 0.05$ compared with control (Student's *t* test).

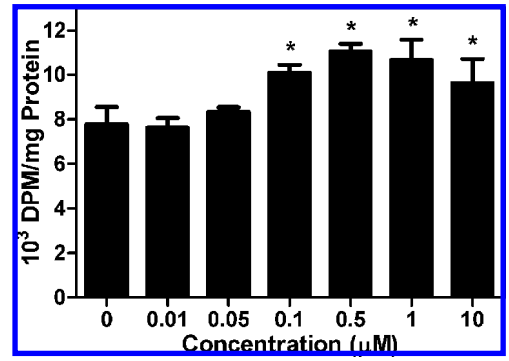

Figure 2. Effect of different concentrations of 20-hydroxyecdysone (20HE) on [^{3}H]leucine incorporation in human skeletal muscle cells. Differentiated myotubes were treated for 24 h with increasing concentrations of 20HE or vehicle. DPM were normalized by total protein. The data represent the mean values ± SEM of four samples, each performed in triplicate. ∗ indicates $p < 0.05$ compared with control (Student's *t* test).

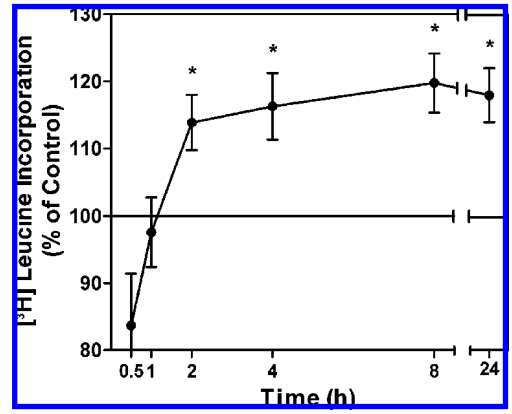

Figure 3. Time course of [^{3}H]leucine incorporation in C2C12 myotubes treated with 20-hydroxyecdysone (20HE). Differentiated myotubes were treated with either 1 μM 20HE or vehicle for 24 h. DPM were normalized by total protein. The data represent the mean values ± SEM of four samples, each performed in triplicate. ∗ indicates $p < 0.05$ compared with control (Student's *t* test).

levels, making quantification difficult (data not shown). Although the concentrations of total ecdysteroids were similar in both plant extracts, the levels found in the original plants varied. The total ecdysteroid contents per tissue found in the dried aerial portion of *A. turkestanica* and spinach were approximately 5 mg/g and 40 μg/g, respectively, comparable with previous studies (*3, 22*).

Both *A. turkestanica* and spinach extracts stimulated protein synthesis in C2C12 myotubes (**Figure 5**). At the low concentration of 0.8 μg/mL, approximately 50 nM total ecdysteroids, both extracts slightly increased protein synthesis, but that increase was not significant at $p = 0.05$. At higher concentrations, the effect was more pronounced and statistically significant. At 1.6 μg/mL, or 100 nM total ecdysteroids, both *A. turkestanica* and spinach extracts significantly increased protein synthesis by 15 and 9%, respectively. This stimulatory effect was observed at up to 8 μg/mL, or 500 nM total ecdysteroids, with increases of 16 and 18% for *A. turkestanica* and spinach, respectively. This effect was similar to that produced by pure ecdysteroids at comparable concentrations.

The PI3K pathway, which activates AKT, has been shown to increase protein synthesis in skeletal muscle (*23*). When C2C12 myotubes were pretreated with 10 μM of the PI3K inhibitor LY294002, the effect of 20HE on protein synthesis was significantly reduced (**Figure 6**). The PI3K inhibitor similarly reduced IGF-1-stimulated protein synthesis.

In the rat androgen nuclear receptor binding assay, methandrostenolone bound to the androgen receptor with an EC_{50} of 24 nM. However, 20HE showed no significant binding from concentrations of 1 to 100 μM (**Table 1**).

To investigate whether the enhanced protein synthesis in the cultured cells translates into enhanced physical performance, rats were daily gavaged with 20HE, spinach extract, or methandrostenolone for 28 days, and the front limb grip strength determined at the end of the treatment period (**Figure 7**). 20HE increased the grip strength by 18% compared to the control ($p < 0.05$), whereas the spinach extract increased grip strength by 24% ($p < 0.005$). Methandrostenolone increased grip strength by 21% ($p < 0.01$).

The ecdysteroids used in this research increased protein synthesis in both murine and human skeletal muscle cells (**Figures 1** and **2**) and had muscle strengthening effects in vivo.

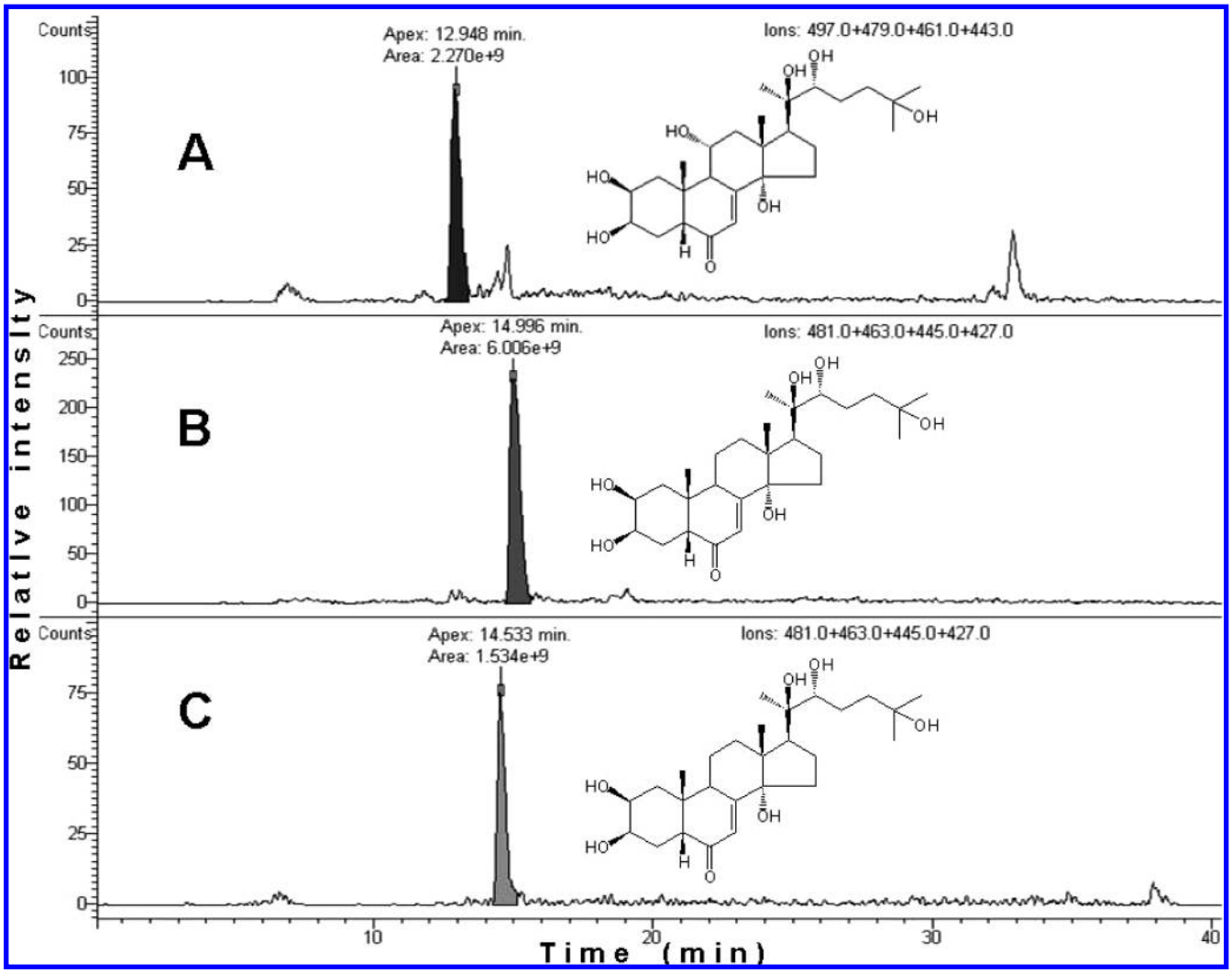

Figure 4. Chromatograms of ecdysteroid-containing plant extracts. Plant extracts were analyzed using (+)ESI LC-MS. Typical fragmentation ions for the ecdysteroids were used to plot the chromatograms of (**A**) turkesterone or (**B**) 20HE present in *A. turkestanica* or (**C**) spinach extract.

The androgenic anabolic steroid methandrostenolone did not have a significant effect on protein synthesis in the experimental model used, confirming a previous study that androgens had no effect on protein synthesis in skeletal myotubes (*24*).

20HE and turkesterone were the most anabolically active among the tested ecdysteroids. The only structural differences between the tested ecdysteroids are the number and placement of hydroxyl groups. The anabolic effect of 20HE became significant after 2 h and was maintained for at least 24 h (**Figure 3**). The effect became significant at 100 nM 20HE, comparable to the affinity of the insect ecdysone receptor (*25*).

Similarly to pure compounds, extracts from plants known to contain ecdysteroids (i.e., *A. turkestanica* and spinach) also enhanced protein anabolism in muscle cells (**Figure 5**). Levels of ecdysteroids present in the extracts were sufficient to explain the observed effects and correlated with the effective concentrations of pure compounds. Similarly to methandrostenolone, 20HE and spinach extract normalized for 20HE dose, were associated with significant increases in muscle strength in rats (**Figure 7**). These results support previous findings that ecdysteroids increase muscle strength in vivo (*17*) and indicate that extracts from both tested plants may stimulate muscle growth and strength.

Anabolic androgenic steroids, structurally and functionally related to testosterone, often produce myotrophic effect in mammals (*26*). Synthetic anabolic androgenic steroids exert their effect mainly through binding to the intracellular androgen receptor, responsible for the mediating effects of its natural ligands—testosterone and dihydrotestosterone. In addition to anabolic effects in muscle tissue, anabolic androgenic steroids cause many adverse side effects: in women, deepening of the voice, acne, and hirsutism; in men, gynecomastia and inhibition of spermatogenesis; and in both women and men, dyslipidemia and associated cardiovascular disease, liver disease, and possible disturbances of mood and behavior. Therefore, separating anabolic and androgenic effects is an important research target in human pharmacology.

The observation that 20HE does not bind to the androgen receptor (**Table 1**) suggests that ecdysteroids may exert their anabolic effect through an androgen-independent mechanism. It is tempting to speculate that ecdysteroids may work through activating the PI3K pathway. The PI3K pathway plays a key role in cell survival and growth in a variety of tissues, including skeletal muscle (*27*). One of the main downstream mediators of this pathway is Akt. Akt, or protein kinase B (PKB), is a serine/threonine-specific protein kinase, which is activated by PI3K. Akt activation leads to cell survival, growth, and proliferation. There is some evidence that ecdysteroids activate Akt (*28*). In the current study, treatment with the PI3K inhibitor,

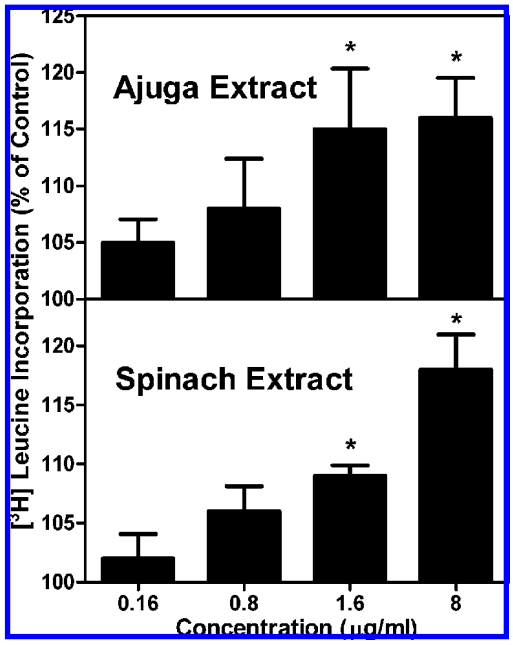

Figure 5. [^{3}H]Leucine incorporation in C2C12 myotubes treated with ecdysteroid-containing plant extracts. Differentiated myotubes were treated for 4 h with increasing concentrations of spinach extract, *A. turkestanica* extract, or vehicle. DPM were normalized by total protein. The data represent the mean values ± SEM of four samples, each performed in triplicate. ∗ indicates $p < 0.05$ compared with control (Student's *t* test).

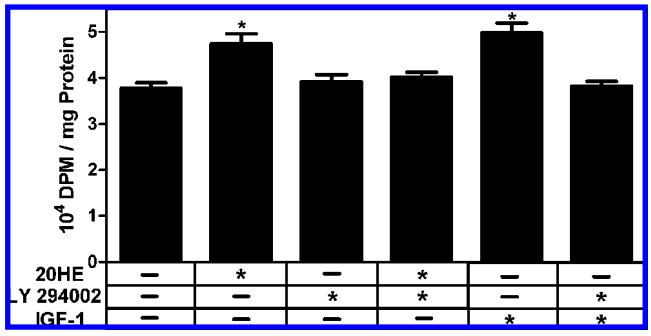

Figure 6. [^{3}H]Leucine incorporation in C2C12 myotubes pretreated with PI3K inhibitor. Differentiated myotubes were pretreated with either 10 μM LY294002 or vehicle for 30 min before treatment with either 1 μM 20HE, 100 ng/mL IGF-1, or vehicle. DPM were normalized by total protein. The data represent the mean values ± SEM of four samples, each performed in triplicate. ∗ indicates $p < 0.05$ compared with control (Student's *t* test).

Table 1. Androgen Receptor Binding of 20-Hydroxyecdysone and Methandrostenolone[a]

20-hydroxyecdysone		methandrostenolone	
concentration (μM)	% inhibition	concentration (μM)	% inhibition
1	1	0.01	32
3	1	0.03	54
10	9	0.10	75
30	−1	0.30	86
100	5	1.00	94

[a] Displacement of labeled ligand after 4 h of incubation of receptor with sample.

LY294002, completely abolished the ecdysteroid effect (**Figure 6**). This finding is consistent with the hypothesis that ecdysteroids act on the PI3K pathway.

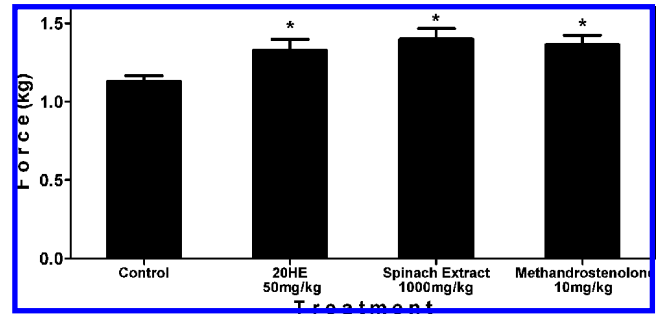

Figure 7. Rat front limb grip strength after 28 days of treatment. Adult male rats were gavaged daily with either 50 mg/kg 20HE, 1 g/kg spinach extract, 10 mg/kg methandrostenolone, or vehicle. On day 28, front limb grip strength was evaluated. ∗ indicates $p < 0.05$ compared with control (Student's *t* test).

In insects, ecdysteroids bind to the nuclear ecdysone receptor, which dimerizes and binds to DNA, activating the transcription of genes involved in molting (*9*). However, in mammals, which seem to lack homologous receptors, the molecular mechanisms of ecdysteroid action are still unknown. Although our findings suggest that ecdysteroids may be mediated by a pathway that converges on the PI3K pathway, rather than on the androgen receptor pathway, more study is needed to confirm or disprove this hypothesis.

ABBREVIATIONS USED

20HE, 20-hydroxyecdysone; BCA, bicinchoninic acid; BSA, bovine serum albumin; DPM, decays per minute. DMEM, Dulbecco's Modified Eagle's Media; FBS, fetal bovine serum; IGF-1, insulin-like growth factor-1; PI3K, phosphoinositide kinase-3; PKB, protein kinase B; TCA, trichloroacetic acid.

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