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Anabolic effect of ecdysterone results in hypertrophy of C2C12 myotubes by an estrogen receptor mediated pathway

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Abstract

Phytoecdysteroids, such as ecdysterone (Figure 1), are structurally similar or identical to insect-moulting hormones and have also been detected in various plant species. Their structures are similar to those of vertebrate steroid hormones. In mammals ecdysteroids are reported to produce a range of effects including the stimulation of protein synthesis and enhancement of physical performance. One of their most interesting properties with respect to sports performance is their anabolic effect, behaving similar to anabolic steroids eventually without the androgenic effect.
Therefore, ecdysterone is available as active component in several over-the-counter supplements and may be misused by athletes to increase muscle mass especially because it may not be covered by the World Anti-Doping Agency's (WADA) prohibited list.
In order to elucidate the molecular mechanism involved in the anabolic activity of ecdysterone, cell culture experiments were conducted using the mouse skeletal muscle cell line C2C12. Differentiation of C2C12 cells towards myotubes was induced. Differentiated myotubes were co-incubated with ecdysterone, dihydrotestosterone (DHT) and antiandrogenic or antiestrogenic substances, such as flutamide (an antiandrogen) and ZK191703 (an antiestrogen).
Cells were fixed and myotube diameter was determined utilizing glutaraldehyde-induced autofluorescence and morphometry. The treatment of the cells with ecdysterone resulted in an increase of the myotube-diameter. This stimulation could be antagonized by an antiestrogen, which suggests an estrogen receptor mediated mechanism. Flutamide could not antagonize this effect, which supports our hypothesis that the anabolic effect of ecdysterone is mediated by an estrogen receptor pathway.

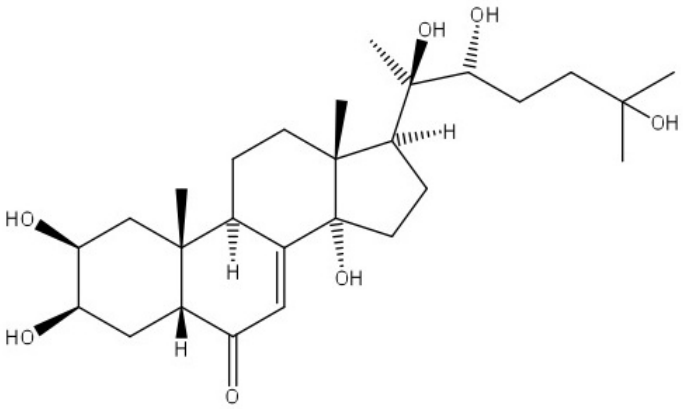

Figure 1: Chemical structure of ecdysterone.

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Introduction

Ecdysteroids are structural analogues of the insect moulting hormone ecdysone, first isolated in 1954 from silkworm pupae (Butenandt & Karlson, 1954). Ecdysterone or 20-hydroxyecdysone has been reported to influence many metabolic pathways such as the lipid and carbohydrate metabolism or protein synthesis (Báthori et al., 2008).
Offered in many over the counter supplements to increase muscle mass, the mechanisms of the cellular mode of action have not been clearly elucidated. Different mechanisms were discussed whether a G-protein coupled receptor is involved or an activation of a nuclear receptor is responsible for the anabolic effect (Gorelick-Feldman et al., 2010).
To investigate the intracellular response that may underlie the anabolic effects of ecdysterone we used the mouse skeletal muscle cell line C2C12.

Experimental

For the cell culture experiments C2C12 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4 nM glutamine, 1,5 g/l sodium bicarbonate, 100 mM sodium pyruvate and 100 units/ml penicillin/streptomycin. Differentiation towards myotubes was induced at confluence by shifting the proliferation medium to differentiation medium (DMEM with 2% horse serum). During the whole experiments the cells were stored at atmospheric conditions by 5% CO_2 and 37 °C.
Differentiated myotubes were co-incubated for 48 h with ecdysterone (Steraloids, Wilton, USA, purity checked by NMR and GC-MS, Figure 2, 1 µM), dihydrotestosterone (DHT, Sigma, Steinheim, Germany, 1 µM), dexamethasone (Sigma, Steinheim, Germany, 1 µM and 10 µM), insulin-like growth factor-1 (IGF-1, Sigma, Steinheim, Germany, 10 ng/ml) or vehicle. For the antagonization study cells were treated with combined solutions of DHT (1 µM) and flutamide (an antiandrogen, Sigma, Steinheim, Germany, 1 µM), ecdysterone and flutamide, (1 µM each), 17β-estradiol (E2, Sigma, Steinheim, Germany, 10 nM) and ZK (an antiestrogen, Bayer, Berlin, Germany, 1 µM) and ecdysterone –ZK (1 µM each).

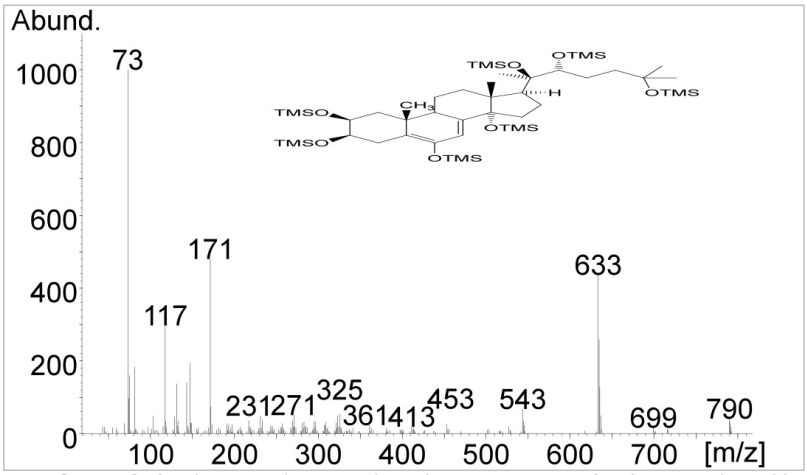

Figure 2: 1H NMR-Spectrum [δ: ppm] of ecdysterone in DMSO-d6 and mass spectrum of ecdysterone heptakis-TMS, M+ = 984.

Cell-cultures were fixed and photographed using glutaraldehyde-induced autofluorescence. Myotube diameters of 50 myotubes per group were measured every 10 to 20 µm along the length of the myotube using Axiovision LE Zeiss software.
All data are expressed as means ± s.d. Statistical significance of differences was calculated using one-way ANOVA followed by Tukey's HSD post hoc test (SPSS Statistical Analysis System, Version 20). Statistical significance was established at $p \leq 0.05$ * and $p \leq 0.01$ **.

Results and Discussion

As reported previously (Stitt et al., 2004) dexamethasone causes atrophy, IGF-1 induces hypertrophy in C2C12 myotubes. The treatment of the cells with ecdysterone resulted in a similar hypertrophic appearance and increased myotube-diameters (Figure 3). This effect could be antagonized with the synthetic glucocorticoid dexamethasone, which suggests a protein increasing effect of ecdysterone. In order to elucidate the molecular mechanism the C2C12 myotubes were co-incubated with an antiandrogen (flutamide) and an antiestrogenic substance (ZK191703, an E2 analogue substance with a lipophilic side chain blocking the estrogen receptor binding site). Hypertrophy caused by ecdysterone could be antagonized with the antiestrogen ZK, which indicates an estrogen receptor mediated mechanism. Flutamide could not antagonize this effect (Figure 4). As shown recently for isoflavones and related compounds (Weigt et al., 2012, Velders et al., 2012) the anabolic effect of ecdysterone could be mediated by estrogen receptor beta pathway.
Our data demonstrate that ecdysterone induced hypertrophy in C2C12 myotubes. As described previously ecdysterone caused an increase of protein synthesis in C2C12 cells most probably mediated by the phosphatidylinositol 3-kinase/ AKT-pathway and inducing AKT phosphorylation (Latres et al., 2005, Gorelick-Feldmann et al., 2010). AKT phosphorylation could also be mediated by estrogen receptor mechanisms (Stoica et al., 2003, Milanesi et al., 2009). Both isoforms, estrogen receptor α and β could be expressed by the C2C12 cell line. Therefore, the data supports our hypothesis that the anabolic effect of ecdysterone is mediated by an estrogen receptor mediated pathway.

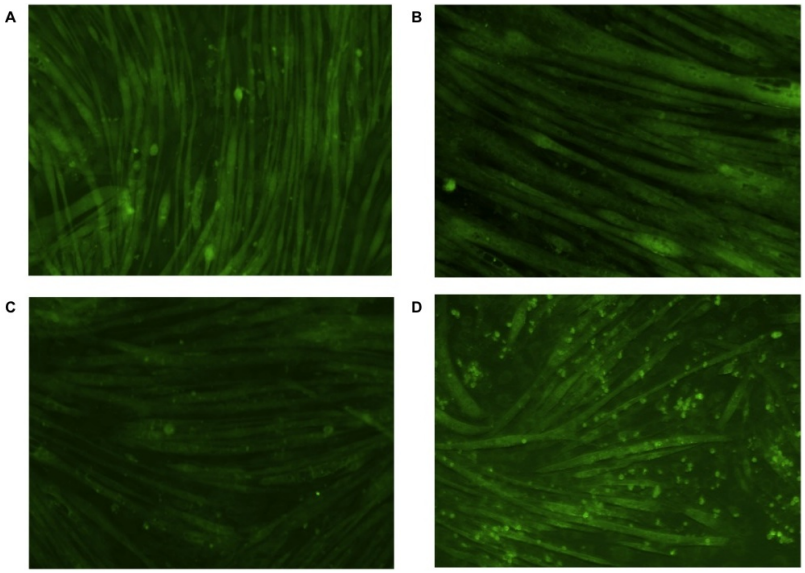

Figure 3: C2C12 myotubes treated with several substances: (A) dexamethasone, (B) ecdysterone, (C) control, (D) ecdysterone + ZK. Cells were fixed and photographed by glutarladehyde-induced autofluorescence. Magnification: 100x.

Conclusions

Our data demonstrate that the anabolic activity of ecdysterone is caused by an estrogen receptor mediated pathway which does not result in an androgenic effect. Our findings may have implications for the improvement of analytical methods for doping control as they assess the influence of anabol active substances into an *in vitro* system.
In summary these observations are relevant for the development of new strategies for the treatment of skeletal muscle injuries such as sarcopenia and cachectic diseases but also to provide scientific data that allow for the classification of ecdysterone in preparations. Currently in Germany a categorization of ecdysterone either as novel food or as pharmaceutical is discussed.

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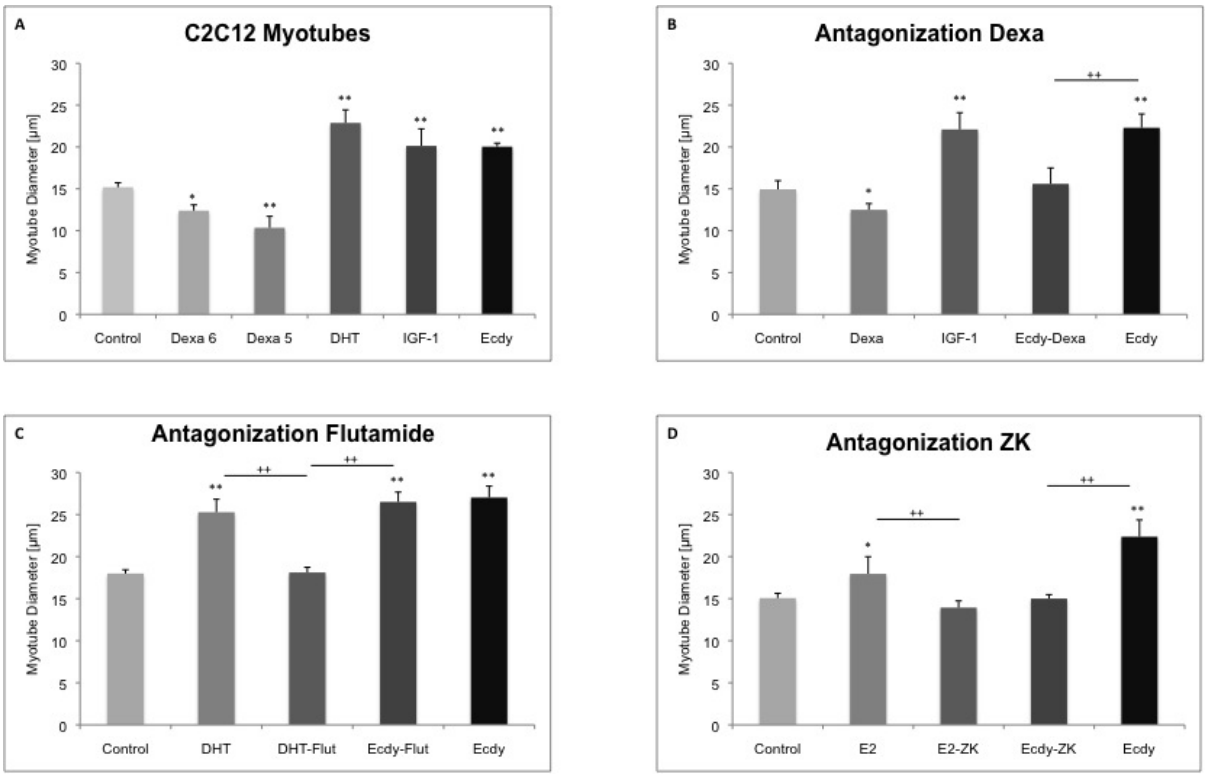

Figure 4: Effects of ecdysterone in C2C12 myotubes (A). (B-C) Antagonization experiments. Control = medium, Dexa = dexamethasone, DHT = dihydrotestosterone, E2 = 17β-estradiol, Ecdy = ecdysterone, Flut = flutamide, IGF-1 = insuline-like growth factor-1, ZK = ZK 191703. Data shown are means + s.d. Statistical significance was established at */+ $p < 0.05$ and **/++ $p < 0.01$. * marks significant differences between control-group and test-groups. + marks significant differences between test groups.

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