PHYTOECDYSTEROIDS

UNDERSTANDING THEIR ANABOLIC ACTIVITY

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ABSTRACT OF THE DISSERTATION

Phytoecdysteroids – Understanding their Anabolic Activity

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Phytoecdysteroids, polyhydroxylated ketosteroids, are the plant analogues of insect growth hormones. Although their role in insect molting is well characterized, their function in plants is less clear. Lacking the properties of classic plant hormones, phytoecdysteroids may be involved in plant growth and defense. One of the main benefits of phytoecdysteroids may be their therapeutic effects on mammals, including humans. Their claimed medicinal properties include anabolic, adaptogenic, hepatoprotective, and hypoglycemic activity. Although ethnobotanical use has been supported by some evidence, the research is quite limited, lacking the scientific rigor necessary to be convincing.

Two ecdysteroid containing plants, *Ajuga turkestanica*, and *Spinacia olearaceae* (Spinach), were selected as beneficial sources of phytoecdysteroids. Cultivation, analysis of ecdysteroid content, and characterization of anabolic activity were performed to support future medicinal use.

Phytoecdysteroids' anabolic activity, one of their most interesting properties due to the claimed lack of androgenic effect, was studied. Anabolic activity was confirmed in animal studies and a cellular model of skeletal muscle. The cellular model was used to characterize ecdysteroids' effect on protein incorporation and to elucidate the signal transduction pathway involved. Ecdysteroid's lack of androgenic activity was confirmed in vivo and in vitro, with ecdysteroids showing no specific binding to the androgen receptor.

Identification of mammalian nuclear receptors homologous with the insect nuclear ecdysone receptor led to binding and activation assays of potential receptors using ecdysteroids. The discovery of a lesser known membrane bound G Protein Coupled Receptor (GPCR) insect ecdysone receptor, DoEcR, suggested the existence of a hypothetical mammalian membrane bound GPCR ecdysone receptor.

Use of specific inhibitors supported the involvement of G protein signaling, Phospholipase C (PLC), Inositol Phosphate 3 Receptor (IP_3R), and Akt. Ecdysteroid stimulated activation of Akt confirmed its role in the anabolic effect. Ecdysteroid generated increases in intracellular calcium were also characterized, with the rapid flux in Ca^{2+} linked with Akt activation and anabolic activity. The evidence produced suggests the involvement of a putative mammalian GPCR ecdysteroid receptor mediating the anabolic effect through the rapid activation of the PLC/IP_3R pathway, generating Ca^{2+} flux which leads to activation of the Phosphoinositide 3 Kinase/Akt pathway, eventually causing increases in protein incorporation.

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As the holiday of freedom approaches, I wish everyone freedom from their own personal bondage and the ability to appreciate the real truth which fills the world.

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Introduction

Ecdysteroids, polyhydroxylated ketosteroids with long carbon side chains, are produced primarily in arthropods and plants, but are also present in fungi, and even in marine sponges. Although they are found throughout the living world, their discovery was only made within the last 60 years (Butenandt and Karlson 1954), and at first, their significance was not appreciated. While their effects on mammalian cells were first documented in 1961 (Burdette and Richards 1961), research on the actual role of ecdysteroids in mammals has been lacking, with studies recording interesting biological activity but no characterization of the various effects. Over time, the accumulation of these interesting observations on the potential importance of ecdysteroids has justified a closer look at the role and function of ecdysteroids in animals and plants.

Origin

The term ecdysone, referring to the first ecdysteroid described, comes from the Greek, *ecdysis*, meaning to molt. This name dates back to the original discovery and isolation of ecdysone from silkworms in 1954 (Butenandt and Karlson 1954). At the time, large amounts of insect material were required to isolate milligrams of purified ecdysone, the first ecdysteroid identified. Since then, over 300 different ecdysteroid analogues have been identified from animal and plant sources (Structures can be found in the ecdysone database Ecdybase, http://ecdybase.org).

Structure

All ecdysteroids have a steroid backbone containing a Cis A/B ring junction, a 7-ene-6-one chromophore, and a 14α-hydoxyl group (Fig 1) (Lafont and Horn 1989). The major variations among ecdysteroids are due to the length and structure of the carbon side chain at C17, and the number and placement of the hydroxyl groups. The total carbon number may be between 19 and 29.

Arthropods

Occurrence

Although first discovered in insects, ecdysteroids have been identified in many different invertebrates. Zooecdysteroids, or ecdysteroids present in animals, are found primarily in arthropods, including insects (Insecta), spiders (Chelicerata), and crustaceans (Crustacea) (Spindler 1989), but have also been found in some non-arthropods including some nematodes, snails, and even soft coral (Cnidaria) (Franke and Kauser 1989).

The two primary ecdysteroids found in arthropods are α-ecdysone and 20-hydroxyecdysone (Baltaev and Shangaraeva 2001). Other related ecdysteroids, including 2-dehydroxyecdysone and 25-dehydroxyecdysone, are also present but in much lower concentrations (Rudolph et al. 1992).

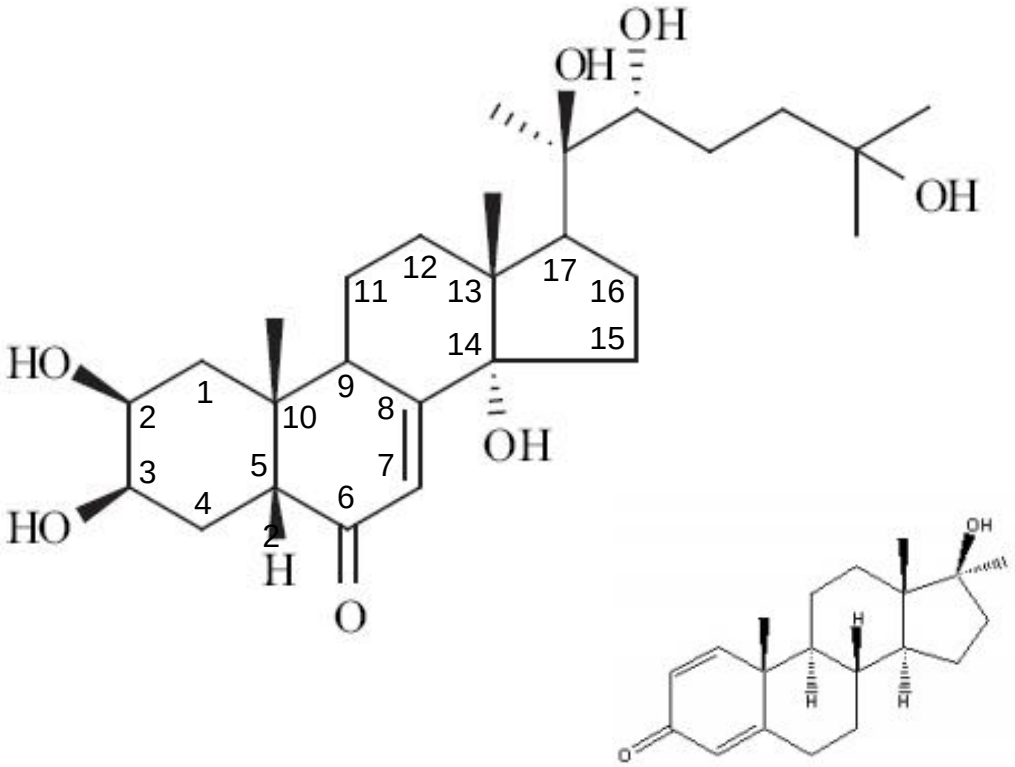


Fig. 1. Ecdysteroid structure. Structure of 20-hydroxyecdysone. Note the required 7-en-6-one moiety, the characteristic hydroxylation at C14, and the long alkyl side chain at C17. In the inset, methandrostenolone, an androgen analogue is used for comparison. Note the lack of a long alkyl chain at C17, hydroxylation sites, or the 7-en-6-one moiety.

Mode of Action

In insects and other arthropods, ecdysteroids elicit their main response through a nuclear Ecdysone Receptor (EcR) (Laudet, 1997). This response has been well defined with ecdysone response elements (EcRE) identified, and ecdysone induced genes characterized (Lafont and Dinan 2003). The response requires a partner, the nuclear receptor called Ultraspiracle (USP), to dimerize with EcR to be activated by ecdysteroids.

Plants

Occurrence

Within a few years of the isolation of ecdysone in insects, it was discovered that plants also contain ecdysteroids, known as phytoecdysteroids. Their occurrence is distributed throughout the plant kingdom, with approximately 6% of all plants surveyed containing detectable levels of ecdysteroids (Dinan 2001).

There is evidence using Radio Immuno Absorption (RIA) that most plants contain very low levels, and may retain the biosynthetic capability to produce ecdysteroids in higher levels (Dinan et al. 2001a). Although their distribution is across the plant kingdom, certain families have many species high in ecdysteroids, including Asteraceae and Lamiaceae. While most plants contain only small amounts, some species can contain high concentrations of ecdysteroids, reaching concentrations of 1–2% of the plant dry weight.

Structure

Phytoecdysteroids, although varying somewhat in their chemical structure, are all derived from C27, C28, or C29 sterols (Grebenok et al. 1994). Unlike insects, which are unable to synthesize ecdysteroids and must consume dietary phytosterols that are then converted into ecdysteroids, plants can completely produce ecdysteroids from mevalonic acid and cholesterol (Adler and Grebenok 1995). The sterol structure is modified to produce ecdysteroids. The *trans* A/B ring juncture in sterols is converted to a *cis* A/B ring juncture in ecdysteroids, and a 7-en-6-one chromophore and a 14 α-hydroxy group are added (Fig. 1) (Davies et al. 1980). Additional hydroxylations at C2, C20, C22, and C25 are required to produce the most common phytoecdysteroid, 20-hydroxyecdysone (20HE).

Although the most common ecdysteroid found in plants is 20-hydroxyecdysone, the main insect ecdysteroid, over 300 different phytoecdysteroids have been identified (Baltaev 2000). The various analogues differ in the number and site of hydroxylations, as well as the length and structure of the carbon side chain. Glycosylated and acetylated ecdysteroids have been described both in nature and in the laboratory (Maria et al. 2005).

Taxonomy

Even though only a small percentage of all plants have been tested for ecdysteroids, there is some taxonomic distinction among those species sampled. Although ecdysteroids are present in many different plant families, certain groups contain many ecdysteroid

containing species (Lafont and Horn 1989). The largest number of ecdysteroid containing species is among the angiosperms, although there are some found among non-flowering plants, including twenty families of ferns, Polypodiophyta, and nine families of gymnosperms, Pinophyta. In the angiosperms, seventy eight families possess ecdysteroid containing species. A majority of these families are within eleven orders: Liliales, Ranunculales, Urticales, Malvales, Violales, Capparales, Rosales, Sapindales, Polemoniales, and Scrophulariales.

Role

Plant Hormone

The role of phyotecdysteroids in plants is not clearly understood, as there is conflicting data supporting ecdysteroids as plant hormones, signaling molecules produced in plants which travel to targeted locations, regulating specific cellular responses. Although initial reports showed ecdysteroids did not elicit any of the classical responses of known plant hormones (Machackova et al. 1995), there are also data supporting ecdysteroids being physiologically active in plants.

Golovatskaya (2004) showed that 20HE in nanomolar concentrations affected metabolic processes, including α-amylase activation and retardation of leaf senescence. 20HE appeared to modulate classical hormonal responses, having a synergistic effect with auxin on coleptile elongation, while antagonizing the effects of gibberelic acid (Golovatskaya 2004). 20HE also altered germination, shoot growth, and root elongation in tomato (Bakrim et al. 2007). Ecdysteroids have been shown to affect the algae, *Chlorella*

vulgaris (Beijerinck), producing a stimulatory effect on growth and metabolism (Bajguz and Koronka 2001). DNA, RNA, protein, sugars, chlorophylls, and phaeophytins were all stimulated by 1 nM ecdysteroid treatment. (Bajguz and Dinan 2004).

Although there are examples of ecdysteroid induced growth and proliferation, it is difficult to consider ecdysteroids as plant hormones. No plant receptor for ecdysteroids is known, and documented responses are not well characterized. Ecdysteroids may have a general growth promoting effect on plants, although further study is needed to elucidate its mode of action.

Defense

Phytoecdysteroids may play a role in plant defense. Production of 20HE in spinach is elicited by both mechanical wounding and insect feeding. Both exogenous 20HE and plant produced ecdysteroids produced abnormal molting, immobility, reduced invasion, impaired development, and death in insects (Schmelz et al. 2002). Phytoecdysteroids were found to protect spinach from plant-parasitic nematodes and may confer a mechanism for nematode resistance (Soriano et al. 2004). Although not conclusive, ecdysteroid's role in plants may be similar to the defensive role of lignins, where production increases in response to pathogens.

Mammals

Occurrence

Although no biosynthesis of ecdysteroids has been documented in mammals, low levels of ecdysteroids have been found in mammals. This has been attributed to dietary intake of ecdysteroid containing plants, flora in the gut, or helminth infections (Graham 2002).

Role

Although the role of ecdysteroids as insect hormones and their involvement in development and the molting process has been well studied, their role in mammals is less obvious. The nuclear ecdysone receptor responsible for classical edysteroid responses in insects is absent in mammals. Their absence has been exploited with the development of gene switches. Mammalian cells can be transfected with a gene of interest linked to an EcRE and a modified EcR. Since mammals do not produce ecdysteroids, the gene will only be switched on when an ecdysteroid agonist is introduced (No et al. 1996). Many studies have been performed using gene switch technology. However, the authenticity of their results is predicated on the assumption that mammals lack an endogenous ecdysteroid signaling system.

There are some studies that may undermine this assumption. Constantino et al. (2001) showed that treatment with muristerone or ponasterone potentiated the IL-3-dependent activation of PI 3-kinase/Akt pathway in a pro-B lymphocyte cell line. Ecdysteroids inhibited Fas and TRAIL induced apoptosis in human colon carcinoma (Oehme et al.

2006), and RKO colon cancer cells treated with 3 μM muristerone for 48 h showed an increase in anti-apoptotic proteins including BCL-XL and Akt. Although these studies were surprising, there is a significant bulk of evidence supporting the biological activity of ecdysteroids in mammals. This includes a long history of traditional use of ecdysteroid containing medicinal plants, as well as a number of animal and cellular studies, starting in 1961, describing various activities of ecdysteroids in mammals. However, little molecular work has been performed studying ecdysteroids' effects on mammalian cells, which may be the main reason for the assumption that ecdysteroids produce no effects and are suitable ligands for gene switch technology.

Therapeutic use

These surprising studies on the bioactivity of ecdysteroids are supported by the ethnobotanical tradition that many ecdysteroid containing plants are beneficial in many ways. Historically, ecdysteroid containing plants have been used as adaptogens, to increase energy, and to reduce stress and fatigue. They have also been used to treat diabetes and aid in wound healing. More recently, ecdysteroids are used to increase muscle. This is clearly illustrated by the large online market of ecdysteroid and ecdysteroid containing plants.

Traditional Use

There is a long history of use of ecdysteroid containing plants for many different therapeutic effects (Table 1). *Leuzea* (= *Rhaponticum*) *carthamoides* (Asteraceae), also

Species	Family	Local Name	Part Used
Ajuga turkestanica	Lamiaceae	Sanabor	Whole Plant
Leuzea (Rhaponticum) carthamoides	Asteraceae	Maral	Roots, Seeds
Serratula coronata	Asteraceae		
Pfaffia paniculata	Amaranthaceae	Suma	Roots
Cyathula capitata	Amaranthaceae	Chuan niu hsi	Roots
Cyanotis vaga	Commelinaceae		Whole Plant
Polypodium lepidopteris	Polypodiaceae	Samambaia	Leaves

Table 1. Traditionally Used Ecdysteroid Containing Plants. Above is a summary of well known ecdysteroid containing plants used in traditional medicine. The scientific and common name is listed along with the plant family and plant organs used.

called Maral root, native to Eastern Europe, is used in traditional medicine to treat fatigue, anemia, and to increase working as well as learning capacity (Saratikov 1949). The initial identification was made by local hunters in the Altay Mountains who were fascinated by the behavior of Maral deer during mating season, which after eating the Leuzea root would regain their strength.

Pfaffia pniculata, also known as Brazilian ginseng, or Suma, has been used in South America for centuries in traditional medicine to treat diabetes, wounds, low energy, and as an aphrodisiac (Oliveira, 1986).

Cyanotis vaga or *C. arachnoides* has a long history of use, although not as a medicine. Extracts of Cyanotis are used for the synchronization of spinning in silkworm larvae (Guo 1989; Chandrakala et al. 1998).

Commercial Availability

Over 100 different ecdysteroid containing preparations can be found on the market containing either crude plant extracts or purified extracts with defined ecdysteroid content (Table 2). Most, which are sold over the internet, are marketed to athletes, specifically body builders. For a survey of products offered online, see Ecdybase at www.ecdybase.org. Although mainly suggested to increase muscle, ecdysteroids are also sold to treat diabetes, to increase energy, or as aphrodisiacs. Some are even advertised for animals, including horses and dogs. In addition, ecdysteroids are also present in some cosmetics designed to improve moisture in the skin (Phenomen and Hydrastar from C. Dior).

Name	Ecdysteroid Content	Source	Indication
Max Anabol	20 mg Turkesterone	*Ajuga*	Male Performance
Fuse	? of 20HE + Turkesterone	*Ajuga* + *Leuzea*	Anabolic
REAP	60 mg total ecdys.	*Ajuga* + *Leuzea*	Performance Enhancer
Prime One	?	*Leuzea*	Muscle Building
E-Bol	80 mg 20HE	*Leuzea*	Anabolic Performance Enhancer
Russian Secret	?	*Pfaffia*	Anabolic
Lepti-Trim	?	*Pfaffia*	Weight Loss
Hydra-Star	?	*Cyanotis*	Moisturizer
Phenomen-A	?	*Cyanotis*	Anti-Wrinkle
Excite	4 mg 20HE	*Polypodium*	Performance Enhancer

Table 2. Ecdysteroid Containing Dietary Supplements. A selection of products containing ecdysteroids is listed. The amount of ecdysteroids present, plant source, and the indicated uses are shown. ? denotes no information on ecdysteroid content available.

Ecdysteroid Containing Plants

Although there are many plants high in ecdysteroids, a few of which were previously mentioned, two species, *Ajuga turkestanica* and *Spinacia oleracea* were selected for further study, including characterization of ecdysteroid content, and confirmation of their therapeutic activity.

Ajuga turkestanica

Ajuga turkestanica, in the family Lamiaceae, is native to the mountainous area of Uzbekistan in Central Asia. It is a perennial herb that flowers in the spring, producing light purple inflorescences. The local inhabitants, who call it "sanabor" which means the charmer, use *A. turkestanica* traditionally as a tonic and as a liver protectant (Syrov et al. 2003). The genus Ajuga is traditionally used for its medicinal properties in many regions in the world (Hilaly et al. 2004). More recently, dietary supplements containing *A. turkestanica* are marketed as performance enhancers and as anabolics. This species specifically was selected for further study because it contains high levels (3 mg/g dried weight) of turkesterone (Fig. 2), a more uncommon ecdysteroid which is hydroxylated at the C11 position. There have been claims that this hydroxylation gives turkesterone more potent anabolic activity compared with other ecdysteroids (Syrov and Kurmukov 1976).

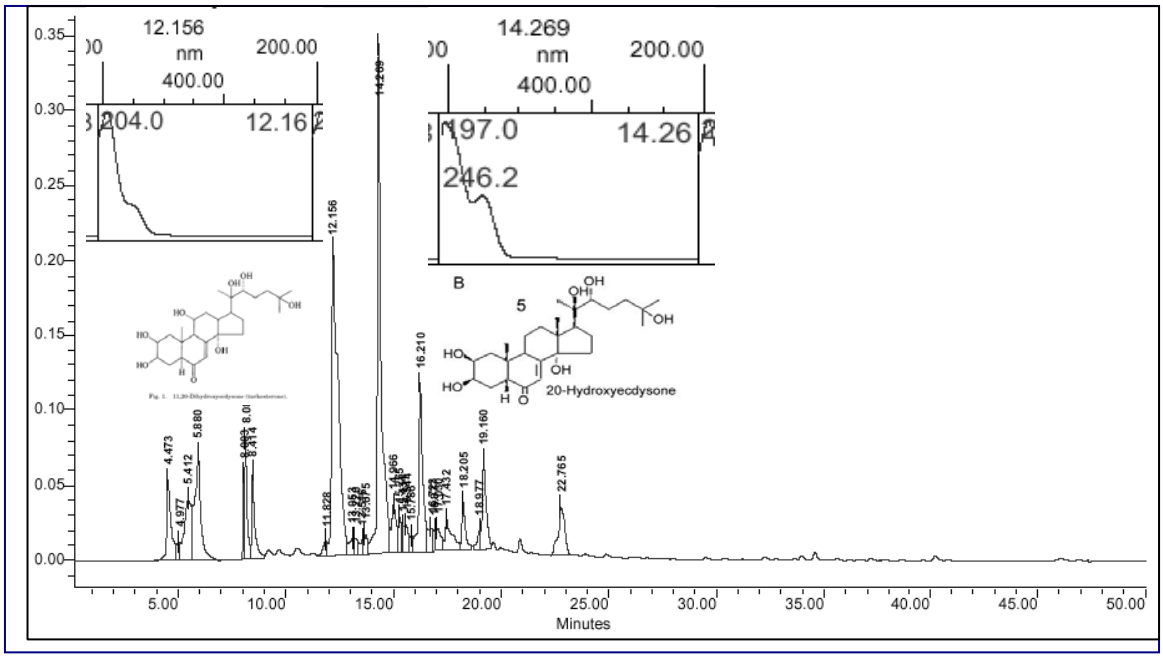


Fig. 2. LC-MS Chromatograph of Ethanolic *Ajuga turkestanica* Extract. Dried aerial portion of *A. turkestanica* was extracted in 95% ethanol and run on a HPLC Phenomenex Luna C-8 reverse phase column, size 150×2 mm, particle size 3 µM. The mobile phase consisted of 2 components: Solvent A (0.5% ACS grade acetic acid in double distilled deionized water, pH 3–3.5), and Solvent B (100% Acetonitrile). The mobile phase flow was adjusted at 0.25 ml/min with a gradient from 5% B to 95% B over 35 min. The chromatograph shows absorbance at 254 nm. The two largest peaks at 12.16 min and 14.26 min correspond with turkesterone and 20-hydroxyecdysone based on retention times. Above the 2 major peaks is their UV spectra recorded with a photodiode array detector. A maxima absorbance at around 245 nm is characteristic of the 7-en-6-one chromophore.

Spinach

Of the major food crops, *Spinacia oleracea* contains the highest amounts of ecdysteroids, about 0.01% fresh weight (Grebenok et al. 1994). Other related plants, including quinoa (Zhu et al. 2001) and asparagus (Dinan et al. 2001b), also contain significant levels as measured using HPLC and RIA. Since spinach has a long history of use as a food crop and is generally recognized as safe, it was selected as an ideal species to study the therapeutic activity of ecdysteroid enriched foods.

There are some data supporting the beneficial effects of spinach. A spinach enriched diet conferred a neuroprotective effect on aging rats, improving both their learning capacity and motor skills (Cartford et al. 2002), and of course, Popeye's use of spinach is well documented.

Mammalian studies

The first recorded study showing ecdysteroid activity in mammals was in 1961, when Burdette and Richards (1961) showed 20HE increased growth and proliferation in mammalian cells. Since then there have been various studies showing biological activity. For a review of the subject, see Lafont and Dinan (2003). Unfortunately, the majority of studies, performed in non-western countries, were published in primarily non-English journals, making them inaccessible to most western scientists. We will describe briefly the major effects of ecdysteroids known. These include anabolic, adaptogenic, hypoglycemic, and protectant properties (Table 2).

Effect	Species	Reference
Increase Protein Incorporation	Rat, Mouse, Sheep, Pig, Quail	Syrov & Kurmakov 1976; Todorov et al. 2000; Purser & Baker 1994; Kratky et al. 1997; Koudea et al. 1995
Adaptogenic	Rat	Syrov 1996
Anti Arrhythmic	Rabbit	Khushbaktova et al. 1987
Hepatoprotective	Rat	Syrov et al. 1992
Lower Blood Glucose	Rat, Mouse	Yoshida et al. 1991; Kosovskii et al. 1989
Lower Cholesterol	Rat	Catalan et al. 1985
Anti Inflammatory	Rat, Mouse	Takei et al. 1991; Kurmukov & Syrov 1988
Immune Stimulant	Mouse	Chiang et al. 1992
Spasmolytic	Rat	Babich et al. 1992
Wound Healing	Human	Meybeck and Bonte 1990

Table 2. Important Pharmacological Effects of Ecdysteroids. A summary of ecdysteroids' wide array of pharmacological effects and the species in which they were observed are listed. Note the increase in protein incorporation, also known as anabolic activity, and the multiple species in which it has been observed.

Anabolic Effects

One of the most interesting properties of ecdysteroids is their ability in animals to increase growth, protein content, and muscle mass, also know as anabolic activity. The majority of the supporting studies were performed by Syrov et al. in the former Soviet Union. The basic experimental design was to administer daily 0.5-50 mg/kg of 20HE or other ecdysteroid intraperitoneally for 10 days or longer to castrated rats (Syrov and Kurkamov 1976). Increased mass of specific muscles (levator ani and m. tibialis anterior) or liver as well as increase in total protein content were indications of anabolic activity. Chermnykh et al. (1988) observed ecdysteroids' anabolic effects in mice, administering 0.25 mg/kg of 20HE intraperitoneally for 7 days. Observed anabolic effects included 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 (Chermnykh et al. 1988). Increased growth and protein content was also observed in ecdysteroid-treated mouse liver and kidneys (Hikino et al. 1969).

In addition to rats and mice, ecdysteroids have been shown to increase growth in a wide variety of animals, including sheep, pigs, and quail. Intravenous ecdysone (0.02 g/kg) increased body and wool growth rate in sheep (Purser and Baker 1994). 0.4 mg/kg of 20HE orally administered daily increased body weight in pigs by 15% (Kratky et al. 1997). Dietary 20HE increased growth in Japanese quails after 30 days (Koudela et al. 1995).

Adaptogenic Effects

In addition to the anabolic effect, ecdysteroids are reported to produce a myriad of different pharmacological responses, including adaptogenic activity. The term adaptogen means something which increases the body's resistance to stress, counteracting fatigue and increasing energy (Brekhman and Dardymov, 1969). Ecdysteroids have been shown to have adaptogenic activity, increasing the rate of survival at elevated temperatures and after continuous swimming (Syrov and Kurmakov 1976). Administration of 5 mg/kg of 20HE or turkesterone significantly reduced the expression of stress reactions in rats after swimming for 5 h or immobilization for 16 h (Syrov 1996). Ecdysteroid treatment produced a decrease in gastrointestinal ulcers, and prevented adrenal gland hypertrophy and thymicolymphatic symptoms.

Hypoglycemic Effects

Ecdysteroids have been shown to produce hypoglycemic effects, suppressing hyperglycemia in several mouse and rat diabetic models (Tashmukhamedova et al. 1985; Kosovskii et al. 1989). Glucogan induced hyperglycemic mice were treated with 0.5 mg/kg 20HE intraperitoneally. Ecdysteroid treatment reduced blood glucose levels by 50% (Yoshida et al. 1971). Oral administration of 5 mg/kg 20HE or turkesterone for 15 days produced similar effects in alloxan induced diabetic rats, lowering blood glucose levels and restoring liver function (Syrov et al. 1992).

Protectant Effects

Ecdysteroids have a protective effect on various organs, including the liver, kidneys, and brain, protecting them from chemical induced toxicity. 5 mg/kg 20HE administered to rats with heliotrine induced toxic hepatitis stimulated bile secretion and improved the composition of bile, increasing the bile acid and bilirubin while decreasing the cholesterol content (Syrov et al. 1992). Oral administration of 5 mg/kg 20HE suppressed albuminuria and restored glomerular filtration in rats treated with a nephrotoxic mixture of uranyl acetate and glycerol (Syrov and Khushbatkova, 2001). Ecdysteroid treatment reduced glutamate induced cell death in rat cortex neurons (Aikake et al., 1996) and protected against diazepam induced memory loss in rats tested with the Morris water maze (Xu et al., 1999).

In addition, ecdysteroids have also been shown to lower cholesterol, aid in wound healing, and have spasmolytic (Babich et al. 1992), immune stimulating (Chiang et al. 1992), anti inflammatory (Takei et al. 1991; Kurmukov & Syrov 1988), and anti arrhythmic activity. Injection of 10 μg/kg ecdysone lowered hepatic cholesterol levels and reduced cholesterol biosynthesis in rats (Lupien et al. 1969). Phytoecdysteroid containing liposomes have been shown to stimulate wound healing in rats (Meybeck and Bonte 1990). 20HE treatment removed aconitine induced arrhythmia in rats (Khushbaktov et al. 1987). Ecdysteroids may produce more therapeutic activities, and more research is needed to verify the many claimed effects. However this is beyond the scope of this work which will deal more specifically with ecdysteroids' powerful anabolic activity.

Ecdysteroid Toxicity

Ecdysteroid toxicity in mammals is very low (Simon and Koolman, 1989). In mice, 20HE produced an LD_{50} of 6.4 g/kg when administered intraperitoneally and 9.2 g/kg when given orally (Oswaga et al. 1974). The extremely low level of toxicity is supported by studies where animals were fed Leuzea seeds containing more than 20 g/kg ecdysteroids with no apparent side effects (Koudela et al. 1995).

Anabolic - Androgenic Relationship

One of the most interesting aspects of ecdysteroids' anabolic activity is that it seems to lack the androgenic side effects common among anabolic compounds. Sergeev et al. (1991) investigated the thymolytic activity of ecdysteroids in mice, administering 0.5 mg/kg of either 20HE or the anabolic steroid, methandrostenolone, for 10 days. While metandrostenolone exhibited clear thymolytic effects, 20E was ineffective. In contrast to anabolic analogues of vertebrate steroid hormones, the anabolic actions of 20E were not associated with androgenic, antigonadotropic or thymolytic side effects (Syrov 1984; Sergeev et al., 1991).

Classical Anabolic Steroids

Anabolic androgenic steroids, structurally and functionally related to testosterone, often produce myotrophic effects in mammals (Shahidi 2001). Synthetic anabolic androgenic steroids exert their effect mainly through binding to the intracellular androgen receptor,

responsible for 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 (Shahidi 2001). Therefore, separating anabolic and androgenic effects is an important research target in human pharmacology.

Ecdysteroids Lack Androgenic Activity

Although they are also steroids, ecdysteroids are structurally very different from androgens and androgen analogues. Ecdysteroids lack the ketone group in the C3 position (Fig. 1). They contain multiple hydroxyl groups, making ecdysteroids much more polar and water soluble then the very lipophilic androgens, and they have a long carbon chain at C17, making them much bulkier than androgens. This reduces the likelihood that ecdysteroids would fit in the ligand binding pockets of the nuclear receptors designed for androgens. These major structural differences may be behind ecdysteroids' lack of androgenic, thymolytic, or antigonadotropic side effects.

Project Goals

Develop Plant Source of Ecdysteroids

Plants material harvested from the wild may introduce many variables due to genetic and environmental differences which may affect ecdysteroid content and activity. To reduce this variability, the selected plants should be cultivated under controlled conditions for further study. While spinach has a long history of cultivation and is easily grown, *A. turkestanica* is a wild herb which has never been cultivated. After being grown in a greenhouse under controlled conditions, the plants will be analyzed to identify the ecdysteroids present and quantify their content.

Elucidate Anabolic Mode of Action in Mammals

Since the documented effects of ecdysteroids are rather broad and not very specific, a single specific effect was selected for further verification and characterization in a controlled setting. In this manner a clear reproducible phenomenon could be established in the lab. This target would serve as an entrance into unraveling the mechanism of action of ecdysteroids.

The most well documented and one of the most interesting therapeutic properties of ecdysteroids is their anabolic activity. Although some animal studies have already documented this effect (Stopka et al. 1999; Todorov et al. 2000; Syrov 2000; Syrov and Kurmakov 1976), they were not published in widely known English journals and may have been performed under less than ideal conditions. To confirm the authenticity of all the prior research, an animal study was planned to verify the anabolic activity of ecdysteroids and ecdysteroid containing plants.

Cellular Model – Skeletal Muscle

The anabolic effect is more accurately defined as an increase in protein incorporation, which leads to increased protein content and mass. This can most easily be measured *in vitro* in a cell based system, removing many of the difficulties found in animal models where many unknown variables introduce sources of error. The cellular model to be used should be very responsive to ecdysteroids, producing an anabolic effect. Skeletal muscle, one of the main sites for anabolic activity, was chosen as the cell type for study. The ideal cellular model of skeletal muscle should closely resemble skeletal muscle found in the whole organism in humans. Obviously cells grown in culture are different than cells growing within an organism, but strong similarities in major physiological responses make cellular models a powerful tool for research.

The C_2C_{12} murine skeletal myoblast cell line, and L6, another murine skeletal myoblast cell line, were chosen. These cells were selected based on their ease in growth and

maintenance, and their robustness in use with cellular assays. Primary human skeletal myoblasts were also selected. The cell lines utilized were able to differentiate into myotubes, producing multinucleated fibers very similar to those found in the whole organism

Distinguishing Androgens and Ecdysteroids

Through development of a cellular model responsive to ecdysteroids, ecdysteroids' unique mode of action can be characterized and contrasted with that of androgens. The first step is to verify ecdysteroids' anabolic effect in a cellular model and then proceed to characterizing the pathways involved. This characterization will be attempted on various levels, beginning with the initial cellular activation, through the signaling cascade, and eventually leading to the physiological response.

Protein Metabolism

The first area to be studied is ecdysteroids effect on protein metabolism, including protein synthesis and degradation. Since anabolic activity is defined as increased protein synthesis and decreased degradation, this area will be focused on to characterize ecdysteroids anabolic activity.

Synthesis

Protein synthesis is defined as the formation of protein from amino acid building blocks. The rate of this process is heavily regulated and is effected by many factors including hormonal influence. Protein synthesis is an important therapeutic target as many diseases cause decreases in protein synthesis.

Degradation

Protein degradation, the breaking down of protein into peptides and amino acids is another vital aspect of protein metabolism. This process is also heavily regulated, with various degradation pathways involved.

Proteasome

A specific pathway of protein degradation, the proteasome, was also studied to determine if ecdysteroids alter proteasome activity. The proteasome is a multi protein complex that breaks down ubiquitin tagged proteins into smaller peptides (Rechsteiner and Mill 2005). Regulation of proteasome activity is an important means of controlling net protein synthesis. If degradation is reduced, net protein synthesis will increase, even if protein synthesis is unchanged, producing an anabolic effect. Inhibiting the proteasome may be a powerful means for treating many diseases which produce a catabolic state, many through increased protein degradation.

Receptors

The next area of study is receptor binding and activation. To distinguish ecdysteroids from androgens, ecdysteroids will be tested for binding to the androgen receptor, the nuclear receptor presumably responsible for androgen induced effects. Using bioinformatic techniques searching through the mammalian genome, potential receptors can be identified which may be activated by ecdysteroids based on their similarity in structure with the insect Ecdysone receptor. These receptors will be tested for ecdysteroid binding and activation.

Signaling Pathway

After characterizing the effect of ecdysteroids on protein metabolism, the signaling pathway leading to the effect will be studied. One of the most common methods for unraveling signaling pathways is through the use of inhibitors. Specific inhibitors of major signaling proteins can help to identify the pathways involved in producing the observed effect. Inhibitors of various key signaling molecules will be utilized to gain more insight into the pathway. The rapid response of immediate signalers as well as longer duration protein activation will be analyzed to better understand the mode of action of ecdysteroids in skeletal muscle.

In summary, the goals of this project are to identify and characterize beneficial plant sources of ecydysteroids, verify their anabolic activity, both *in vitro* and *in vivo*, and elucidate the molecular mode of action.

Plant Cultivation and Extraction

Ajuga

Ajuga turkestanica, with its rich ethnobotanical history (Syrov et al. 2003) and prior studies showing its potent anabolic activity, was selected as an ideal ecdysteroid containing plant for study. The initial analysis of *A. turkestanica* was performed using material collected from the field.

Collection

A. turkestanica was collected from the Surkhandarya region of Uzbekistan and voucher specimens cataloged in the Rutgers Herbarium. The dried aerial portion from flowering plants was weighed and 100 g extracted in 1 L of 95% ethanol.

Identification

The composition of plant extracts was initially identified using HPLC. 1 μg of plant extract wash injected onto a Phenomenex Luna C-8 reverse phase column, size 150×2 mm, particle size 3 μM. The mobile phase consisted of 2 components: Solvent A (0.5%

ACS grade acetic acid in double distilled deionized water, pH 3–3.5), and Solvent B (100% Acetonitrile). The mobile phase flow was adjusted at 0.25 ml/min with a gradient from 5% B to 95% B over 35 min. 20HE and turkesterone were identified as major components of the *A. turkestanica* extract using retention time and UV spectra (Fig. 2). The 7-en-6-on moiety of ecdysteroids produces a UV spectra with a peak around 245. Although the chromatogram supported the presence of ecdysteroids, accurate quantification was difficult as the ecdysteroids did not completely separate into distinct peaks from other compounds, producing multiple maximums in the UV spectra (Fig. 2 inset). Further purification was needed to more accurately quantify the ecdysteroids present in the extract.

Cultivation

We attempted to cultivate wild *A. turkestanica* under controlled conditions to better understand its ecdysteroid production and remove the variation produced in the field. Initially wild seeds were collected from Uzbekistan, in addition to dried plant material. We attempted to germinate approximately 300 seeds in the greenhouse, but no seedlings developed. It was not known if the lack of germination was due to an unknown requirement of the germination syndrome of *A. turkestanica,* or if the seeds collected were not viable due to poor collection or storage practices.

Many species require certain events to bring seeds out of dormancy and induce seed germination. The most common requirement is stratification, where seeds must undergo a cold period before vernalization, when warmer temperatures make the seed receptive to germination. Another common factor affecting germination is hormonal control. A classic example is gibberelic acid, which causes a chain of enzymatic events eventually leading to germination.

A second batch of approximately 500 seeds was collected from the field in Uzbekistan and brought to the laboratory at Rutgers. Gibberelic acid and vernalization treatments were performed in an attempt to aid in germination. *A.turkestanica* seeds were treated with 500, 750, or 1000 ppm gibberelic acid, but after 3 months showed no response. After a cold treatment of three months at 4° C, 3 seeds, less then 1%, germinated. The seedlings were transferred to pots and placed in growth chambers under standard conditions of 18 h / 6 h light/dark cycle, 750 mmol/m^2s, at a constant 22^{o}C, 20% relative humidity. The low germination rate and the subsequent lack of sufficient vegetative material led us to consider tissue culture as a potential means of propagation.

Cuttings of leaf material from mature plants were taken and sent to Phytocell (New York) for tissue culture. Proprietary culture techniques were utilized to culture *A. turkestanica* and produce plantlets. Plantlets were transferred to mist chambers for rooting. After four weeks, roots developed and plants were transferred to 4 inch pots. After 2 months, the plants flowered, producing *A. turkestanica*'s characteristic inflorescences (Fig. 3). All of the plants were propagated from one initial specimen and should be genetically identical.

They were all grown under similar conditions in the greenhouse, hopefully removing any major environmentally induced variation. The aerial portion was collected and dried for further study.

Plant Extraction

Although the initial *A. turkestanica* plant material was extracted only in ethanol, further purification was utilized to more clearly identify the ecdysteroids present. Based on previous phytoecdysteroid extraction protocols (Koudela et al. 1995), the ethanolic extract (1 g in 100 ml) was partitioned with butanol to remove the more polar compounds, and the butanolic phase (150 mg in 30 ml) was dried and used for further analysis.

Fig. 3. *Ajuga turkestanica* inflorescence.

Flowers of a 2 month old plant vegetatively propagated through tissue culture. The original material used for tissue culture was from a plant grown from seed collected from the Surkhandarya region in Uzbekistan.

Spinach

Introduction

Well known foods may contain not so well known therapeutic activities. While spinach is considered a healthy food, its anabolic properties are less appreciated. Spinach has been known to contain ecdysteroids for some time (Bathori et al. 1982), but the amounts present are considerably lower than in many of the other known ecdysteroid containing plants.

One of the benefits of studying spinach is that has been under cultivation for a long time, and is readily available from a variety of controlled sources. The ease of availability combined with its already widespread use as a food crop, make spinach a very good candidate for development of ecdysteroid enriched foods.

Extraction

1 kg of locally grown dried spinach powder (*Spinacia oleracea*) from ARC Greenhouses (Dayton NJ) was extracted in 10 L of 95% ethanol for 24 h. After the removal of ethanol, the extract (70 g) was resuspended in 7 L of water and partitioned with heptane (2 L) to remove the more apolar compounds. The organic phase was removed and the water

phase was partitioned with butanol (3 L) with the more polar compounds remaining in the water phase. The butanolic phase was dried (1.2 g) and used for testing.

Quantification

Ecdysteroid content of plant extracts were determined using positively charged Electro Spray Ionization Liquid Chromatograpy-Mass Spectometry (+ESI LC-MS) using HPLC conditions mentioned previously. A standard curve was generated using increasing amounts of purified turkesterone or 20HE. 20-hydroxyecdysone (99% purity verified by HPLC) was purchased from Scitech (Praha, Czech Rep.). Turkesterone (90% purity verified by HPLC) was a gift from the Tashkent Institute of Cardiology (Uzbekistan). 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 standards to quantify the amount of turkesterone and 20HE present.

Results

A standard curve was generated using known amounts of 20HE and turkesterone, and a linear relationship was established between peak area and amount of ecdysteroid from 250 ng to 5 μg (Fig. 4 inset).

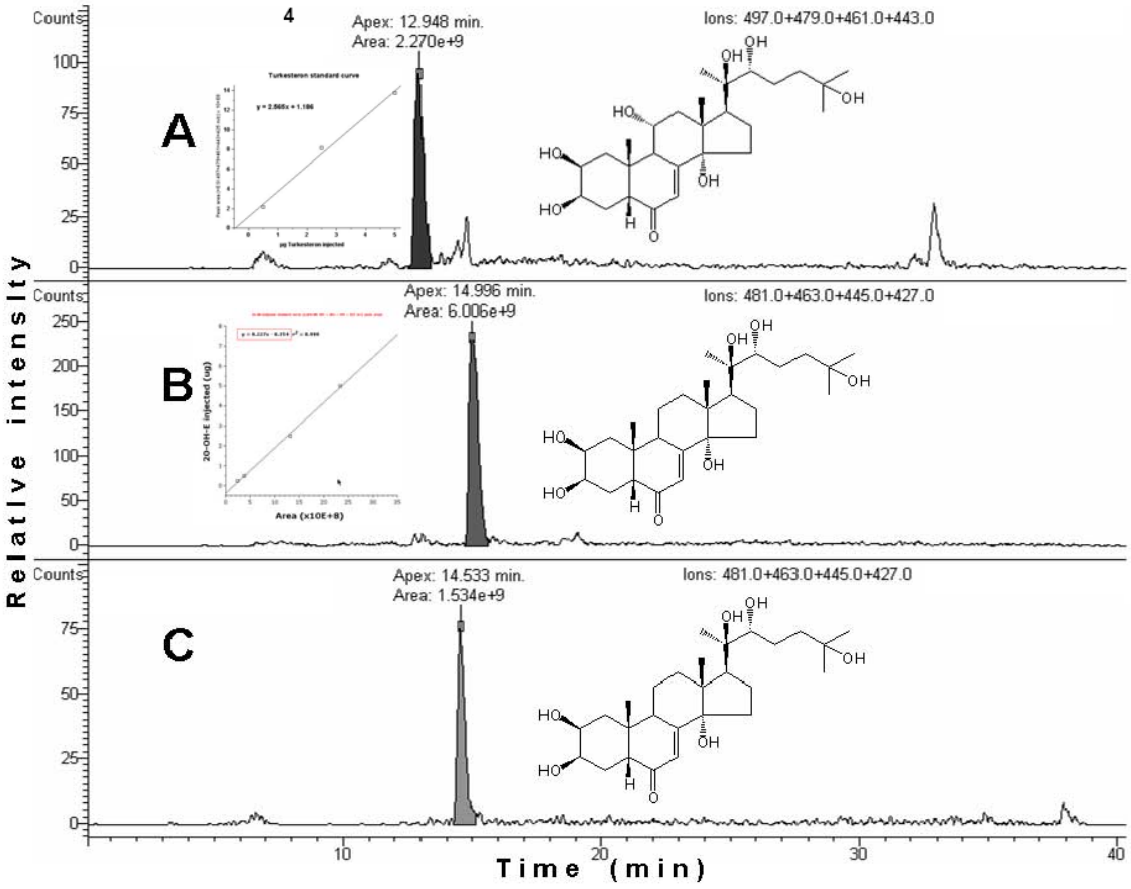


Fig. 4. Chromatograms of Ecdysteroid Containing Plant Extracts.

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. In the insets are calibration curves for (**A**) turkesterone and (**B**) 20HE using pure standards.

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% (wt/wt) turkesterone and 0.9% (wt/wt) 20HE (Fig. 4). Other ecdysteroids were also present, including 22-acetylcyasterone, but in smaller amounts, comprising less than 5% of the total ecdysteroid content.

The spinach extract contained 3.0% (wt/wt) 20HE. Both extracts contained other ecdysteroids, but at much lower levels, making quantification difficult (data not shown). Although the concentration of total ecdysteroids was similar in both plant extracts, the levels found in the original plants varied. The total ecdysteroid content 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 (Syrov et al. 2001; Soriano et al. 2004).

The difference in purification between the two species is due to the different extraction protocols. The extraction procedure for spinach, involving more steps then the *A. turkestanica* extraction, concentrated the ecdysteroid content 750-fold. The extraction of *A. turkestanica*, in contrast, only concentrated the ecdysteroid content about 6-fold. Although modifications to the extraction procedure including adding more steps with more expensive solvents, like heptane, can increase the purification of ecdysteroids, the extract is sufficiently concentrated (~3% total ecdysteroids) to be used both for identification of ecdysteroids present, and for use in *in vitro*, cellular, and animal studies.

Animal Studies

Introduction

In order to verify ecdysteroids' claimed anabolic activity, an animal study was designed based on the previous animal studies performed in the former Soviet Union. The study measured increases in body weight, muscle mass, protein incorporation, and physical strength (Syrov and Kurmukov 1976; Chermnykh et al. 1988). The main objective of the experiment was to verify the anabolic activity and the lack of androgenic activity of ecdysteroids. A comparable experiment was designed using purified 20-hydroxyecdysone, ecdysteroid containing spinach extract, and the anabolic androgen methandrostenolone as a positive control for anabolic activity. However, since measuring protein incorporation in vivo requires producing radioactive animals, this parameter was not performed. Instead, grip strength was used to measure anabolic activity, as increased muscle mass and protein content can translate into increased strength. Although not as straightforward of a gauge, grip strength has previously been utilized to access anabolic activity in both rats (Borst et al. 2007) and humans (Sih et al. 1997).

Methods

Male Sprague-Dawley rats, 7-8 weeks old (213-230 g) supplied by Harlan Inc. (Somerville, NJ), were subjected to one of four treatments: vehicle (water), 50 mg/kg 20HE (Scitech, Praha Czec Rep.), 1,000 mg/kg spinach extract (produced as previous mentioned), or 10 mg/kg methandrostenolone (Steraloids, Newport RI). Concentrations were determined based on previous experiments of Syrov et al. (1976). Spinach extract treatment contained comparable levels of ecdysteroids as the 20HE treatment. Ten animals per group were orally gavaged daily for 28 days. All animals were given standard rodent chow and filtered tap water *ad libitum*. Weekly body weight and food consumption was measured. On Day 28, grip strength on front limbs was assessed using a Wagner Force Five Digital Force Gage, Model FDV-5 (Greenwich, CT). After the rats' front paws grip the screen, the animals were quickly pulled by the base of the tail until the front paws release from the screen, and the required release force was recorded. Three trials on each animal were performed, and significance determined using the Student's *t* test ($p<0.05$). Brain, heart, liver, prostate, seminal vesicles, kidney, testes, and two skeletal muscles, Tibialis anterior and Levator ani, were harvested and weighed. Protein content of skeletal muscle was measured using the Bradford technique (Bradford 1976). Blood was collected and plasma used to calculate testosterone levels using ELISA (DRG International, Germany). Animal care followed the *Guide for the Care and Use of Laboratory Animals DHEW* of the NIH.

Results

To verify ecdysteroids' anabolic activity in animals, rats were daily gavaged with 20HE, spinach extract or methandrostenolone for 28 d, and the front limb grip strength determined at the end of the treatment period (Fig. 5). 20HE increased the grip strength by 18% compared to the control ($p<0.05$), while the spinach extract increased grip strength by 24% ($p<0.005$). Methandrostenolone increased grip strength by 21% ($p<0.01$). All animals gained weight equally and there was no difference in food intake between treatments (Data not shown).

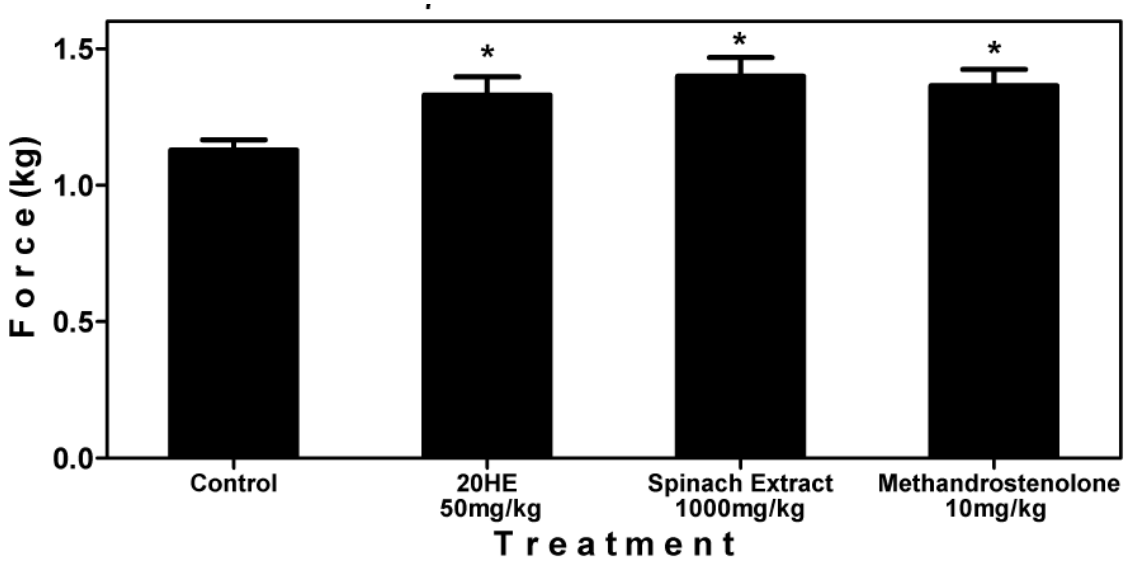


Fig. 5. Rat Front Limb Grip Strength After 28 Day Treatment with 20HE, Spinach extract, or Methandrostenolone. Adult male rats were gavaged daily with either 50 mg/kg 20HE, 1 g/kg spinach extract, 10 mg/kg methandrostenolone, or vehicle. On day 28, front limb grip strength was evaluated. * indicates $P<0.05$ compared with control (Student's t test).

While methandrostenolone significantly increased prostate weight by over 30%, neither 20HE nor spinach extract significantly increased prostate weight (Fig. 6). 20HE significantly increased brain mass by over 10% (Fig 7). Methandrostenolone increased brain mass by over 5%, while the increased brain mass due to spinach extract was not statistically significant (Fig. 7). There were no differences in all other organ weights measured, including heart, liver, seminal vesicles, kidney, testes, and two skeletal muscles, Tibialis anterior and Levator ani (data not shown). There was also no change in protein content of the skeletal muscle (data not shown).

Surprisingly, both 20HE and spinach treatments lowered plasma testosterone concentrations by over 40% compared to the control (Fig. 8).

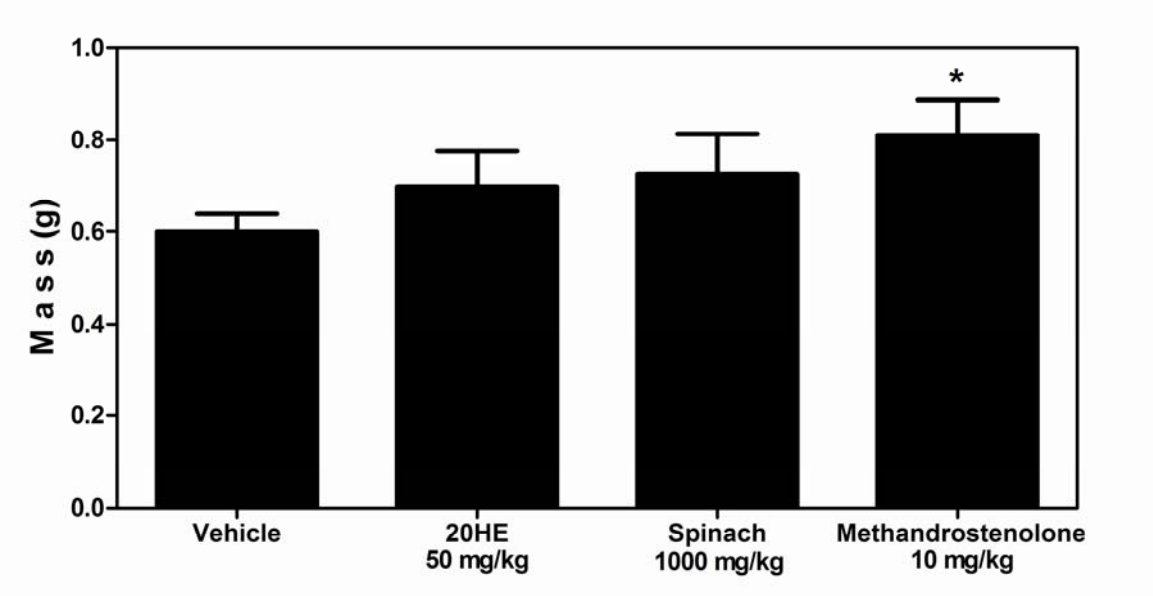


Fig 6. Prostate Mass of Rats Treated with 20HE, Spinach extract, or Methandrostenolone for 21 days. Adult male rats were gavaged daily with either 50 mg/kg 20HE, 1 g/kg spinach extract, 10 mg/kg methandrostenolone, or vehicle. On day 28, brains were harvested and weighed. * indicates $P<0.05$ compared with control (Student's t test).

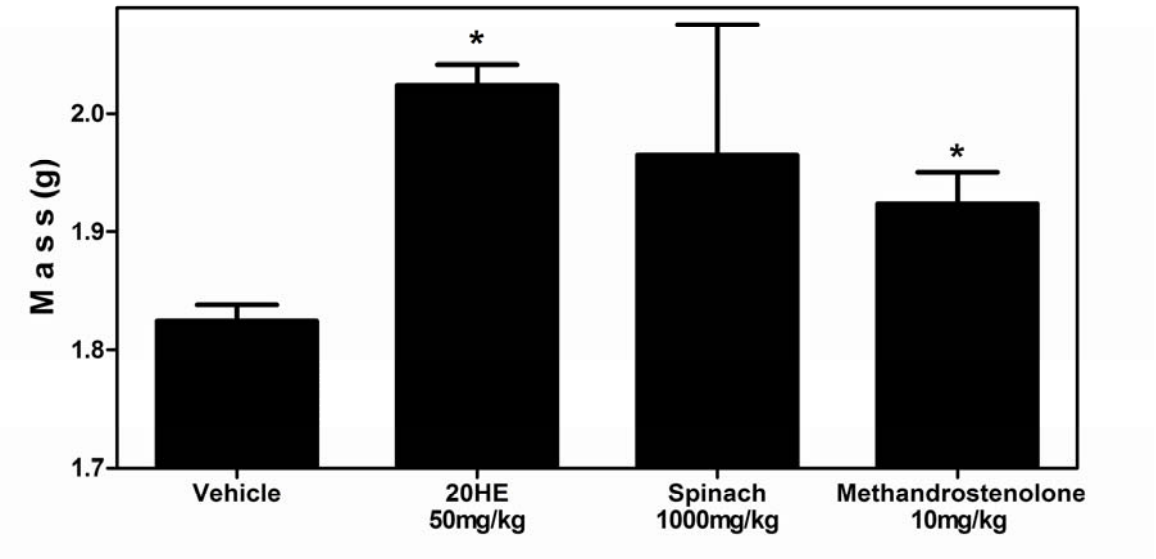


Fig 7. Brain Mass of Rats Treated with 20HE, Spinach Extract, or Methandrostenolone for 21 days. Adult male rats were gavaged daily with either 50 mg/kg 20HE, 1 g/kg spinach extract, 10 mg/kg methandrostenolone, or vehicle. On day 28, brains were harvested and weighed. * indicates $P<0.05$ compared with control (Student's t test).

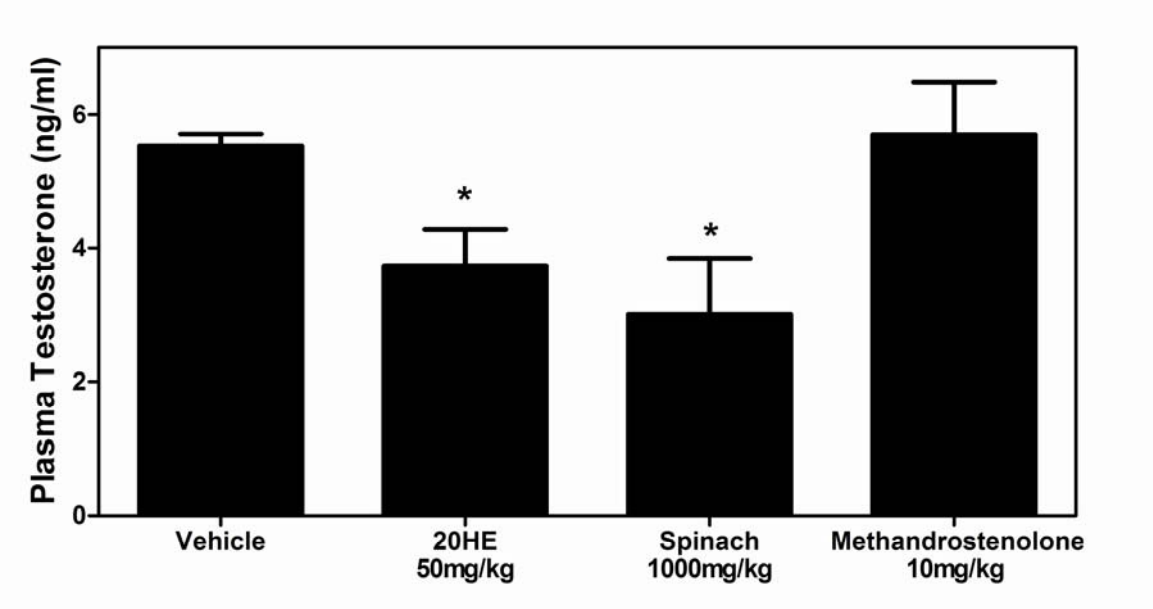


Fig. 8. Plasma Testosterone of Rats Treated with 20HE, Spinach extract, or Methandrostenolone for 21 days. Adult male rats were gavaged daily with either 50 mg/kg 20HE, 1 g/kg spinach extract, 10 mg/kg methandrostenolone, or vehicle. On day 28, blood was taken and plasma testosterone calculated using ELISA. * indicates $P<0.05$ compared with control (Student's t test).

Discussion

Our results confirmed the prior claims that ecdysteroids increase strength in animals, with both 20HE and spinach extract producing effects comparable with the anabolic androgen, methandrostenolone (Fig 5). Although prior studies showed increased physical endurance using a swimming test (Chermnykh et al. 1988), this is the first example of increased grip strength due to ecdysteroid or ecdysteroid containing plant extract treatment. A different factor than endurance, grip strength may be a clearer indicator of increased muscle and anabolic activity (Borst et al. 2007).

Other parameters of the anabolic effect were not as supportive. In contrast to previous studies which claimed increased body weight after 25 days (Stopka et al. 1999), we saw no change in body weight. There was also no increase in the mass of skeletal muscle or in protein content, unlike prior studies (Chermnykh et al. 1988). This discrepancy is puzzling and remains unclear. Ecdysteroids may produce changes in muscle fiber composition, increasing muscle strength without affecting total muscle mass or protein content. Further work is necessary to determine ecdysteroids specific effects on rat muscle.

The other objective was to test ecdysteroids' possible androgenic effects. Increased prostate weight is a common indicator of androgenic activity. While methandrostenolone treatment significantly increased the prostate weight by over 30%, neither ecdysteroid

treatment produced a significant increase (Fig. 6). This distinction further supports the claim that ecdysteroids do not possess androgenic activity.

An unexpected result was the increase in brain weight in 20HE and methandrostenolone treated animals (Fig. 7). Although ecdysteroids are reported to be neuroprotectants (Chaudhary et al., 1969; Catalan et al., 1984; Aikake et al., 1996; Xu et al., 1999) and aid in memory (Cartford et al. 2002), this is the first report of increases in brain mass. It is unclear if this effect is linked to ecdysteroids' neuroprotective or anabolic activity.

Another unexpected result was the decreased levels of plasma testosterone in ecdysteroid treated animals (Fig. 8). The significance of this finding is unclear. Although androgens can lower plasma testosterone levels by a central negative feedback mechanism, this is probably not the case with ecdysteroids, as methandrostenolone did not lower testosterone. Decreased testosterone can be caused by a variety of other factors including stress, toxicants, or hormonal disruption (Lephart et al. 1987). It is unclear if ecdysteroids directly modulate testosterone synthesis or secretion or if the decrease is an indirect result of a different effect.

Cellular Model

Introduction

Although the anabolic effect of ecdysteroids was verified in animals (Fig. 5), a cellular model of the anabolic effect was needed. Animal studies require more time and resources then cellular studies and involve many variables and uncertainties. It is also more difficult to analyze signaling pathways and rapid responses *in vivo*. For these reasons, a cellular assay for anabolic activity was developed.

As previously mentioned, skeletal muscle was chosen as a model tissue for analysis of ecdysteroids' anabolic activity. Comprising 40% of human body weight, skeletal muscle is one of the most metabolically active tissues in the body, providing precursors for glucose via gluconeogenesis, and serving as a repository of free amino acids and protein. Skeletal muscle protein is in a constant state of remodeling, maintaining the balance between synthesis and breakdown during all stages of life. The net synthesis of protein or protein accretion occurs when protein synthesis exceeds protein breakdown. Alternatively, when protein breakdown exceeds protein synthesis, such as during periods of injury or catabolic illness, a net loss of protein occurs. Factors affecting the homeostatic balance of muscle include hormonal balance (Rommel et al. 2001), nutritional status (Fryburg et al. 1990; Volpi et al. 2003), exercise (Rennie and Tipton 2000), aging (Volpi and Rasmussen 2000), and disease (Lim et al. 2003).

Anabolic hormones stimulate muscle growth in humans by increasing protein synthesis, by decreasing protein breakdown or both. Hormones such as testosterone, insulin, insulin-like growth factor-I (IGF-I) profoundly influence human skeletal muscle. They are important regulators of the remodeling process, responsible for modulating positive or negative muscle protein balance. The way a specific hormone exerts its anabolic action on muscle depends on many factors, many of which are not clearly understood. Ultimately, the increase in protein synthesis enables new contractile filaments to be added to the pre-existing muscle fiber, enabling the muscle to generate greater force, and produce the anabolic effect observed in animals (Fig. 5).

Cell Types

Three models of skeletal muscle were utilized for cellular studies. They were selected based on their ease of use, robustness, and similarity to skeletal muscle tissue.

C_2C_{12} Cell Line

C_2C_{12}, a mouse skeletal muscle cell line, can be grown indefinitely as undifferentiated myoblasts, and when needed can be differentiated into multinucleated myotubes. These cells behave in many ways like skeletal muscle fibers, contracting when stimulated and expressing characteristic muscle proteins like myogenin, myosin heavy chains (MyHC),

and the androgen receptor (Tannu et al. 2004). Because much work has already been performed using this cell line, the parameters of its physiology have been well characterized making it an ideal model for skeletal muscle.

L6 Cell Line

L6, a rat skeletal myoblast cell line, is also a very robust cellular model (Richler and Yaffe 1970). It can differentiate into multinucleated myotubes and behaves similar to C_2C_{12}, although it takes longer to differentiate. L6 differs slightly from C_2C_{12} in morphology, producing thinner longer myotubes. Using multiple species strengthens the claim that the pharmacological effects are not specific to one cell line or species, but rather reflects a general phenomenon in skeletal muscle.

Human Primary Myotubes

After studying the 20HE response in murine cell lines, human primary cells were used to verify the effect. Primary cells, although more difficult to maintain and reproduce consistent results, have benefits over cell lines for research. They differentiate into fibers more similar to human skeletal muscle, expressing a wider array of myo-proteins and responding to stimuli, such as hormones, which cell lines may become insensitive to. Primary myoblasts have better proliferation ability and better differentiation characteristics based on creatine phosphokinase (CPK), major histocompatibility complex (MHC), and multi-nucleated myotubule determination compared to murine

myoblast C_2C_{12} cells (Li et al. 2008). The response of human cells, as opposed to murine cells, is more likely to accurately reflect the clinical effect in humans, the ultimate intended use.

Methods

C_2C_{12} cells (ATCC, CRL-1772) between passages 3 and 10 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 Media (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 10 mM HEPES, 6 mM glutamine, 1 mM pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, New York). Cells were grown for 48 h in 5% CO_2 at 37 °C. Some of the initial experiments were performed on undifferentiated myoblasts. However, because the differentiated myotubes are more physiologically relevant, the majority of work was performed using myotubes. After cells reached 80% confluence, the media was replaced with differentiation media (DMEM containing 2% horse serum, 10 mM HEPES, 6 mM glutamine, 1 mM pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, NY). Media was replaced every 2 days, and after 5 days, the myoblasts had fused into multinucleated myotubes.

L6 cells (ATCC, CRL-1458) were cultured in a similar manner as C_2C_{12}, grown first as undifferentiated myoblasts in DMEM with 10% FBS, and then differentiated into myotubes by transferring the cells into differentiation media containing DMEM with 2% horse serum.

Primary human skeletal muscle cells from healthy lean adults (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 in 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 media was replaced with differentiation media (DMEM with 2% horse serum). Media was replaced every 3 d, and after 18 d, myoblasts fused into multinucleated myotubes.

Protein Metabolism

Introduction

The various skeletal muscle models were used to verify and characterize the anabolic activity of ecdysteroids. Although shown in animals (Fig. 5), it was not clear if the anabolic effect could be reproduced with isolated tissues or cells. Muscle may not be directly receptive to ecdysteroids, as there are no prior studies using isolated muscle. Ecdysteroids may require multiple tissues and systems, and the anabolic effect seen *in vivo* in muscle may only be the result of responses elsewhere in the body. Therefore, a cellular assay was developed to test the direct effect of ecdysteroids on protein metabolism in skeletal muscle.

Protein metabolism is a dynamic process with frequent shifts in rates of synthesis and degradation (Guttridge 2004). This process can be very complex with compartmentalization of different rates in different areas in the cells. Different classes of proteins may be synthesized and degraded at different rates.

Although very complex, a simpler look at net gain or loss can also be informative about the state of metabolism. Since there was no prior knowledge of ecdysteroids' anabolic effects on specific areas or types of proteins, it was difficult to look more closely at protein metabolism. For this reason, total protein synthesis was evaluated by measuring the incorporation of tritiated amino acids into protein.

Protein Synthesis

The first step was to confirm in fact that ecdysteroids increase total protein synthesis in skeletal muscle. Four different ecdysteroids were tested at concentrations from 10 nM to 10 μM and compared with the anabolic androgen methandrostenolone in the mouse cell line, C_2C_{12}. Ecdysteroids' effect on protein synthesis was then measured in rat myotubes and human primary skeletal muscle to assess ecdysteroids' anabolic activity in multiple species. A time course was generated of ecdysteroids' effects from 30 min to 24 h.

Finally, ecdysteroid containing plant extracts were tested to confirm that the anabolic effect exists within the complex mixture produced by plants.

Protein Degradation

After characterizing the effect of ecdysteroids on protein synthesis, focus was turned to the other main component of protein metabolism, protein degradation. Skeletal muscle atrophy, which can be caused by injury, illness related cachexia or sarcopenia, or hormones like glucocorticoids, is characterized by increases in protein degradation, including the ubiquitin proteasome pathway, eventually leading to decreased muscle mass and fiber size (Mitch and Goldberg 1996).

Many anabolic factors, like testosterone, insulin, and IGF-1, not only exert their effects through increased protein synthesis, but also lower protein degradation to increase net protein accumulation. In order to evaluate ecdysteroids' effects on protein degradation,

C_2C_{12} and L6 myotubes were prelabeled with tritiated amino acids, treated with ecdysteroids, and the ratio of radioactivity secreted into the media was measured to determine protein degradation. Positive and negative controls were used for comparison. The anabolic factor, IGF-1, known to both increase protein synthesis and decrease degradation (Fiorotto et al 2003) was used, as well as epomoxicin, a specific proteasome inhibitor. Dexamethasone, a glucocorticoid known to increase protein degradation was used as a negative control.

Proteasome

The ubiquitin proteasome pathway is a major degradation pathway comprised of the proteasome and other proteins involved in the tagging of proteins for degradation, called ubiquitination. The 26S proteasome is a multiprotein complex comprised of a single 20S core and capped by a 19S regulatory subunit that degrades misfolded and non assembled proteins (Baumeister et al. 1998).

Because the proteasome pathway is so important in maintaining protein homeostasis, altering its activity can have major effects on cellular physiology. Therefore, ecdysteroids effect on proteasome activity was studied in skeletal myotubes. IGF-1, and the proteasome inhibitor, epomoxicin, were used as positive controls for comparison.

Methods

Cell Treatment

For the ecdysteroid dose response, differentiated C_2C_{12} myotubes 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. 20HE, ponesterone, and polypodine B (99% purity verified by HPLC) was supplied by Scitech (Praha, Czech Rep.). Turkesterone (90% purity verified by HPLC) was a gift from the Tashkent Institute of Cardiology (Uzbekistan). Methandrostenolone (99% purity verified by HPLC) was supplied by Steraloids (Newport, RI). Compounds were added to serum-free media containing 5 μCi/ml [1,3,5 ^{3}H] - leucine. Cells were incubated for 4 h before protein measurement. Similar treatments were performed with L6 myotubes and human primary myotubes, although incubation for human primary myotubes was for 24 h due to lower rates of protein synthesis. For the 20HE time course study, C_2C_{12} cells were treated with 1 μM 20HE for 0.5 to 24 h in serum-free media containing 5 μCi/ml [^{3}H] leucine before protein measurement. For the plant extract study C_2C_{12} 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.

Determination of Protein Synthesis

Protein synthesis was determined by measuring the incorporation of the tritiated amino acid, leucine, based on Montgomery et al. (2002). 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 5ml of scintillation fluid (Ready Safe, Beckman Coulter, Fullerton CA). Decays per minute (DPM) were measured in a liquid scintillation counter (LS 6500, Beckman Coulter, Fullerton CA). 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 mg total protein. Each experiment was performed in triplicate. The results were expressed as mean ± SEM. Statistical significance was determined using the Student's t test ($p<0.05$).

Determination of Protein Degradation

Protein degradation was calculated based on (Tsujinaka et al. 1995) by measuring the release of tricholoroacetic acid (TCA) soluble radioactivity from protein labeled with [1,3,5 ^{3}H]-leucine. Tritiated leucine, dexamethasone, epomoxicin, IGF-1 and all other supplies were purchased from Sigma-Aldrich (St. Louis, MO). Both C_2C_{12} and L6 myotubes were used. After differentiation, cells were labeled with 1 μCi of [^{3}H]

leucine/ml for 24 h in DMEM. Cells were washed with PBS, and nonradioactive media containing 2 mM leucine was added. Cells were treated with 1 μM 20HE, 50ng/ml IGF-1, 10nM epomoxicin, or 25 μM dexamethasone for 24 h. After incubation, media was collected and TCA added to reach a final concentration of 10%. Samples were incubated at 4° C for 1 h and centrifuged for 5 min at 10000 rpm. Aliquots from the supernatant were transferred to scintillation tubes and TCA soluble radioactivity was determined in a scintillation counter. Cells were washed with ice cold PBS and dissolved in 0.5 M NaOH containing 0.1% Triton X-100 and aliquots were transferred to scintillation tubes. Radioactivity from the cells as well as from the TCA insoluble media were also measured in a scintillation counter. Protein degradation was calculated by dividing the TCA soluble radioactivity from the media by the total radioactivity, which included both the media and the cells, and expressed as % degradation.

Proteasome

Proteasome activity was measured based on Moravec et al. (2006). The Proteasome-Glo™ Cell-Based Assay (Promega, Madison WI) was conducted according to manufacturer's instructions. Differentiated C_2C_{12} myotubes cultured on 96 well plates were treated with 1 μM 20HE, 100 ng/ml IGF-1, 10 nM epomoxicin, or vehicle for 4 h. Treated cells were incubated with the Proteasome-Glo Cell-Based Assay Reagent for 10 min at room temperature. The chemotrypsin-like proteasome activity was detected as the relative light units (RLU) generated from the cleaved substrate in the reaction. Luminescence was measured with a Synergy™ HT Multi-Mode Microplate Reader

(Biotek, Winooski VT) and 4 wells per treatment were averaged and compared with the control.

Results

Protein Synthesis

All of the tested ecdysteroids increased protein synthesis in C_2C_{12} myotubes in a dose dependant manner after 4 h of treatment (Fig. 9). 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 these results were not statistically significant. Methandrostenolone, an anabolic steroid, had no significant effect on protein synthesis at up to 10 μM.

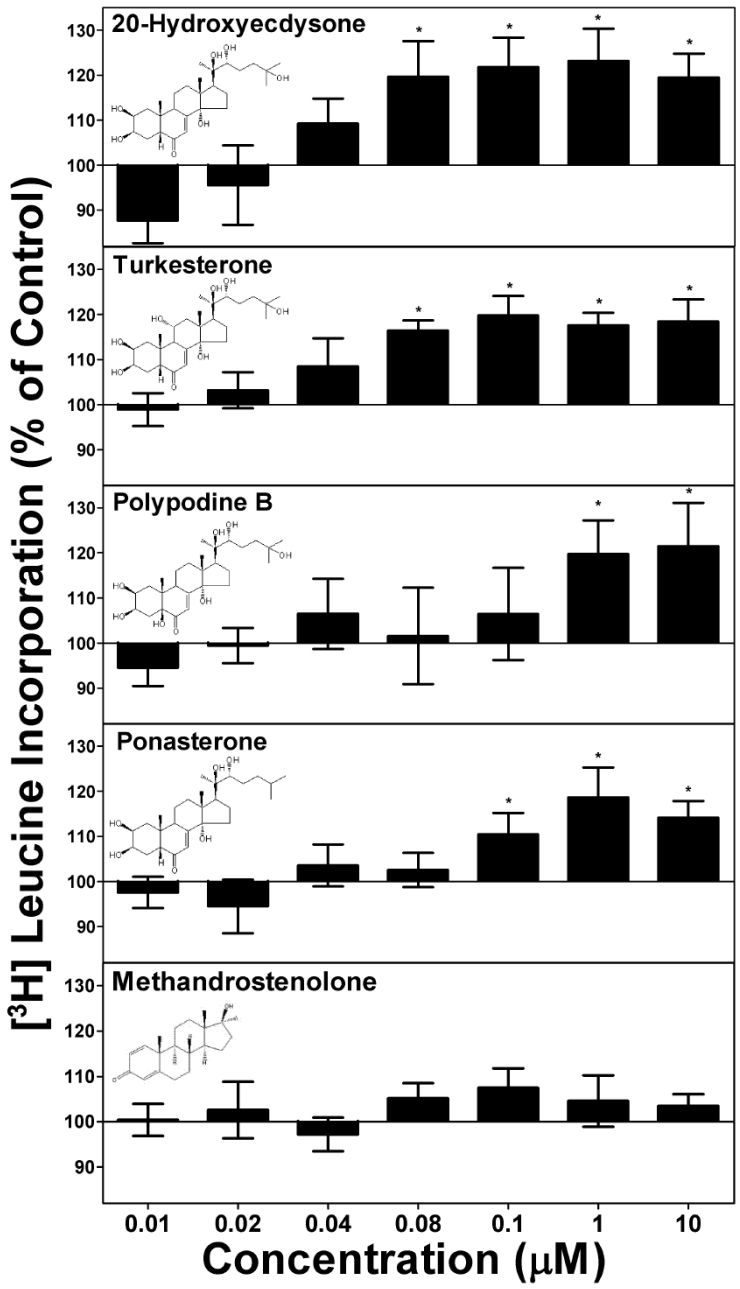


Fig. 9. [^{3}H] Leucine Incorporation in C_2C_{12} Myotubes Treated with Various Ecdysteroids or the Androgen, Methandrostenolone. Differentiated myotubes were treated for 4 h with increasing concentrations of 20HE, turkesterone, ponastersone, polypodine B, methandrostenolone, or vehicle. DPM were normalized by total protein. The data represent the mean values ± S.E.M. of four experiments, each done in triplicate.* indicates $P<0.05$ compared with control (Student's t test).

20HE treatment for 4 h also increased protein synthesis in L6 cells, a rat skeletal muscle cell line (Fig. 10). A significant increase was observed with 100 nM 20HE, increasing protein synthesis by over 15%. This increase was still observed at 1 μM.

In human skeletal myotubes, 20HE produced a similar dose dependant increase in protein synthesis after 24 h of treatment (Fig. 11). Treatment with 100 nM of 20HE increased protein synthesis by 120% of control. This increase was observed at up to 10 μM.

In the 24 h 20HE time course study, 20HE increased protein synthesis in C_2C_{12} myotubes by up to 120% of control (Fig. 12). This increase became statistically significant after 2 h, peaked at 8 h, and remained for the 24 h duration of the experiment.

Both A. turkestanica and spinach extracts stimulated protein synthesis in C_2C_{12} myotubes (Fig. 13). At the low concentration of 0.8 μg/ml, approximately 50 nM of 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 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.

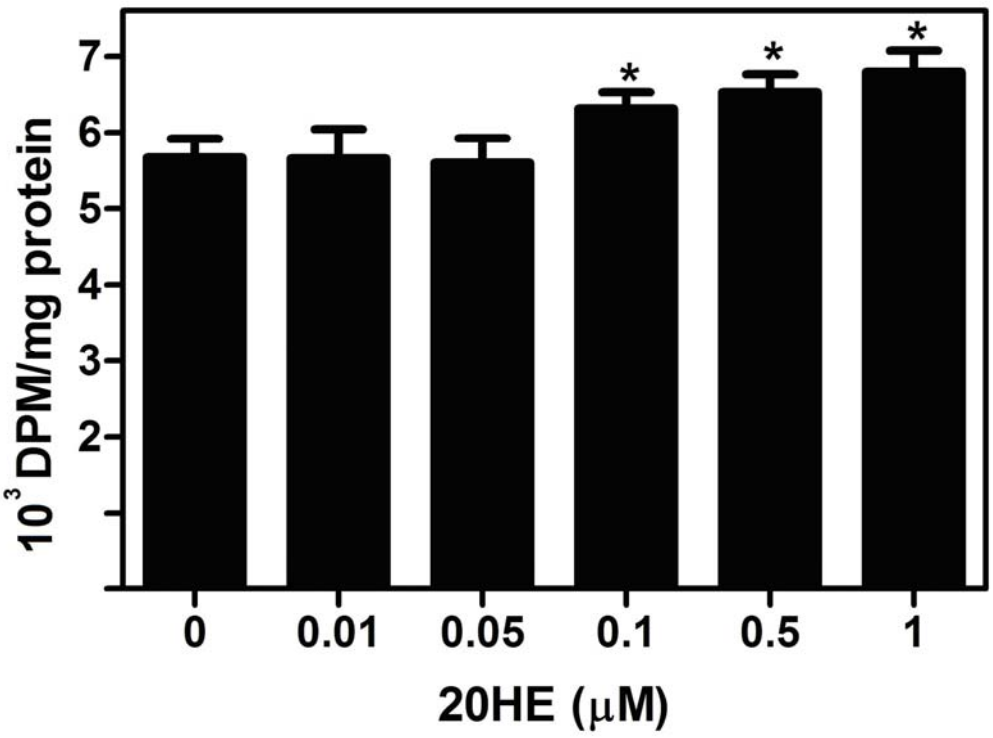


Fig. 10. [^{3}H] Leucine Incorporation in L6 Myotubes Treated with 20-hydroxyecdysone (20HE). Differentiated myotubes were treated for 4 h with increasing concentrations of 20HE or vehicle. DPM were normalized by total protein. The data represent the mean values ± S.E.M. of three experiments, each done in triplicate.* indicates $P<0.05$ compared with control (Student's t test)

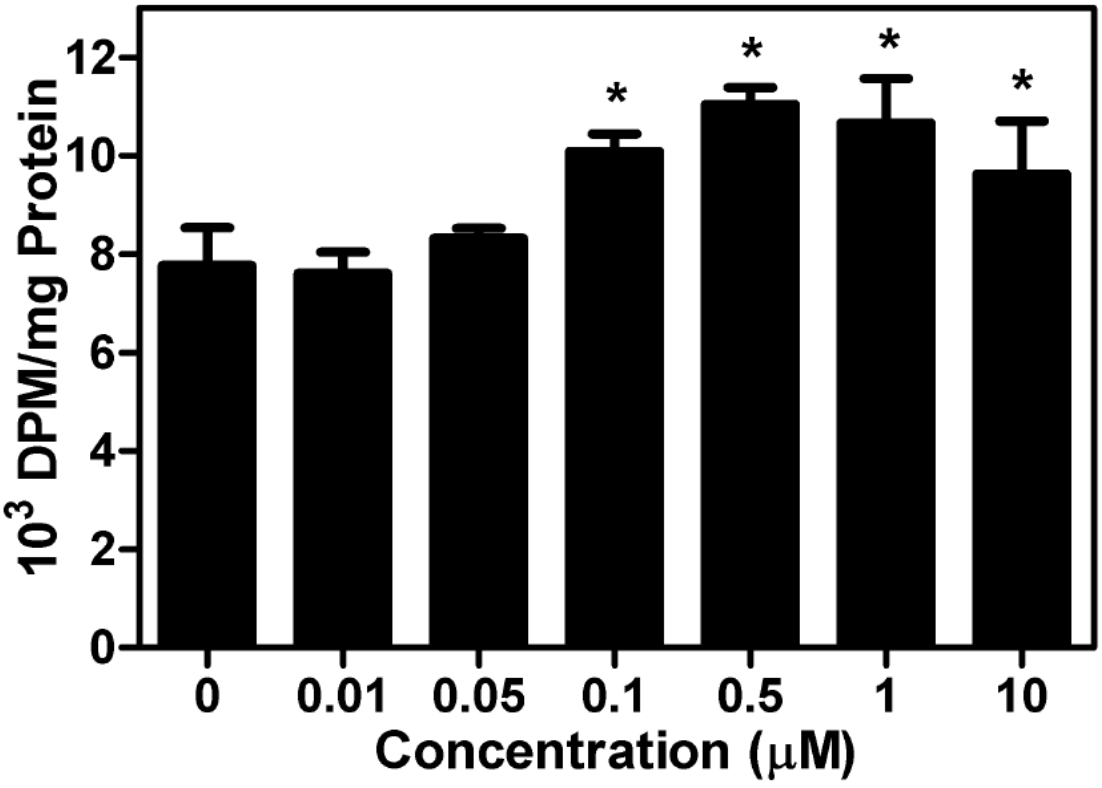


Fig. 11. Effect of Different Concentrations of 20-Hydroxyecdysone (20HE) on [^{3}H] Leucine Incorporation in Human Skeletal Muscle Cells.

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 ± S.E.M. of four experiments, each done in triplicate. * indicates $P<0.05$ compared with control (Student's t test).

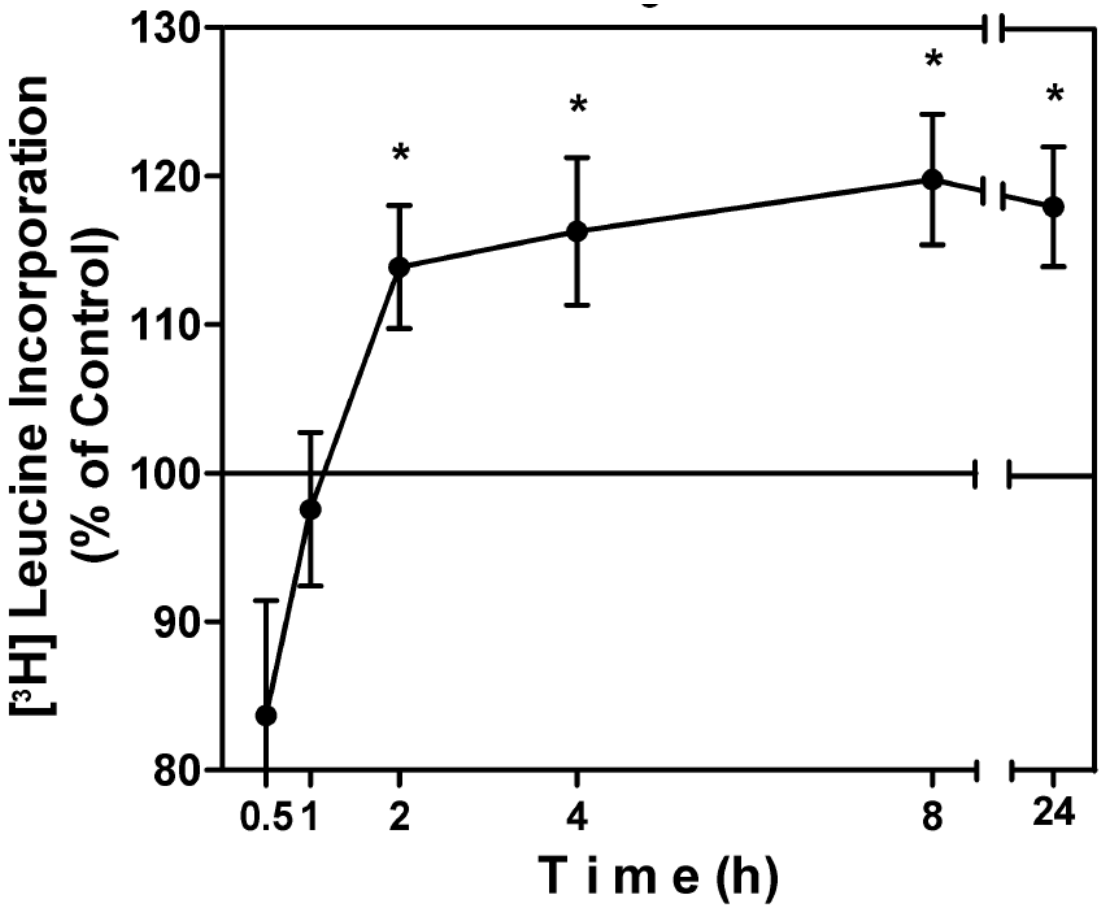


Fig. 12. Time Course of [^{3}H] Leucine Incorporation in C_2C_{12} Myotubes Treated with 20-Hydroxyecdysone (20HE).

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 ± S.E.M. of four experiments, each done in triplicate. * indicates $P<0.05$ compared with control (Student's t test).

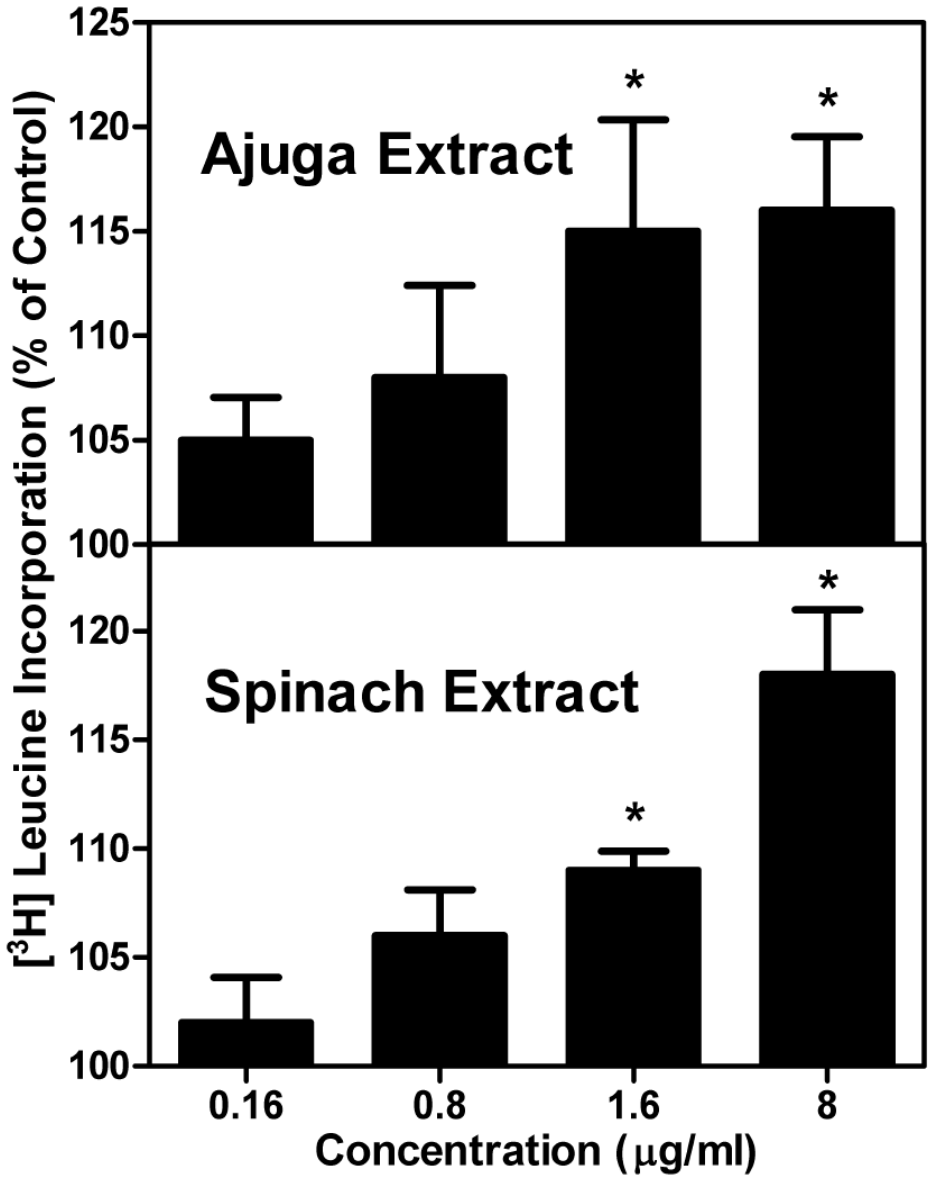


Fig. 13. [^{3}H] Leucine Incorporation in C_2C_{12} Myotubes Treated with Ecdysteroid Containing Plant Extracts. Differentiated myotubes were treated for 4 h with increasing concentrations of spinach extract, A. turkestanica extract, or vehicle. DPM were normalized by total protein. The data represent the mean values ± S.E.M. of four experiments, each done in triplicate. * indicates $P<0.05$ compared with control (Student's t test).

Protein Degradation

Ecdysteroids did not alter protein degradation in skeletal myotubes derived from either C_2C_{12} (Fig 14) or L6 cells (Data not shown). Both IGF-1 and the proteasome inhibitor epomoxicin decreased protein degradation compared to the control. However, dexamethasone, a glucocorticoid which increases protein degradation did not affect degradation in this study.

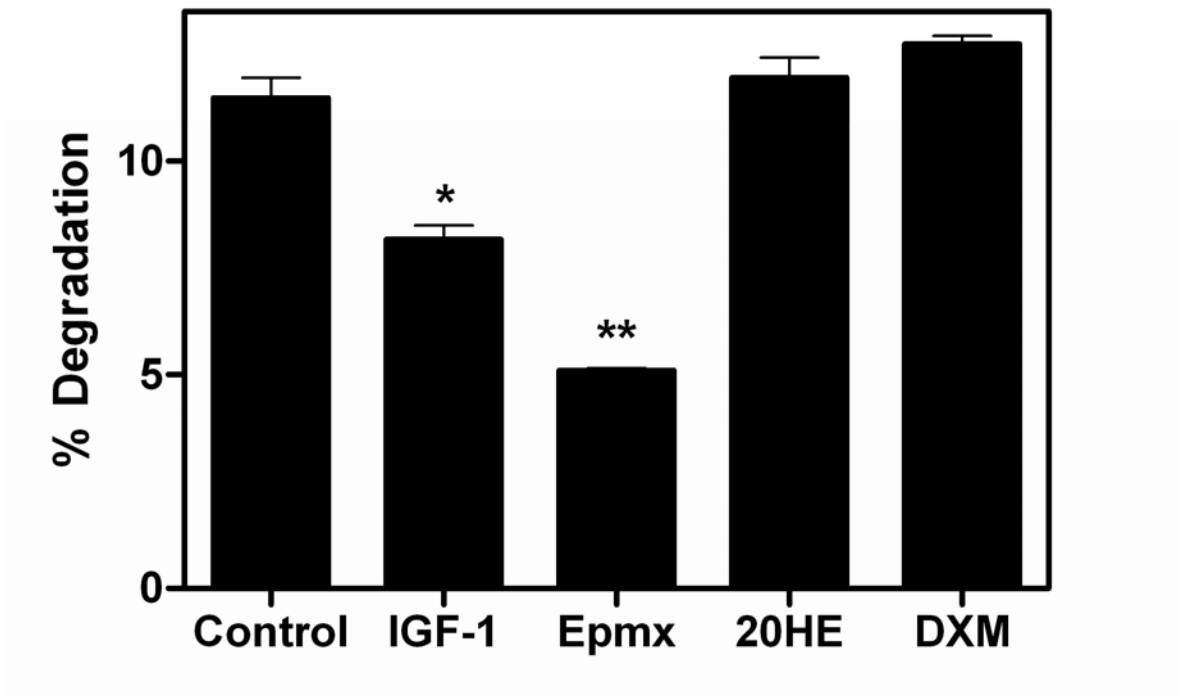


Fig. 14. Protein Degradation of C_2C_{12} Myotubes Treated for 24 h with 20-hydroxyecdysone (20HE), Insulin-like Growth Factor-1 (IGF-1), Epomoxicin (Epmx), or Dexamethasone (DXM). Differentiated myotubes were labeled with [^{3}H] leucine for 24 h prior to treatment. Cells were transferred to media containing cold leucine and 1μM 20HE, 50 ng/ml IGF-1, 10 nM Epmx, 25 μM DXM, or vehicle for 24 h. Protein degradation was calculated by dividing the TCA soluble radioactivity of the media by the total radioactivity, which included both the media and the cells and expressed as % degradation. The data represent the mean values ± S.E.M. of four experiments, each done in triplicate. * indicates $P<0.05$ compared with control (Student's *t* test).

Proteasome

1 μM 20HE significantly decreased proteasome activity after 4 h treatment in C_2C_{12} myotubes (Fig. 15). The 20% decrease in relative activity was comparable with the anabolic factor, IGF-1. The specific proteasome inhibitor, epomoxicin, reduced proteasome activity by 80%.

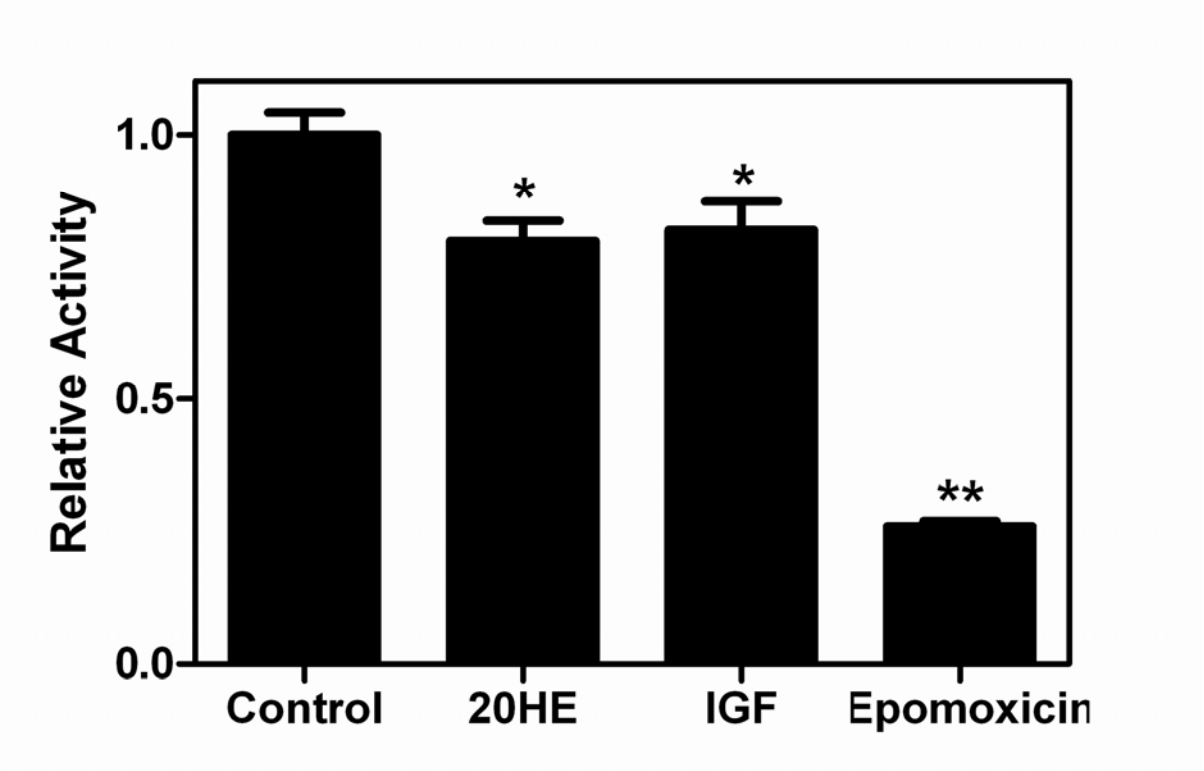


Fig. 15. Proteasome Activity in C_2C_{12} cells treated for 4 h. Differentiated myotubes were treated with 1 μM 20HE, 100 ng/ml IGF-1, 10 nM epomoxicin, or vehicle for 4 h. Cells were then incubated with proteasome activity reagent and fluorescence was measured. Data represents the average of 4 replicates and SEM. * indicates $p<0.05$ and ** $p<0.01$ compared to control (Student's *t* test).

Discussion

Protein Synthesis

Ecdysteroids used in this research increased protein synthesis in mouse, rat, and human skeletal muscle cells (Fig. 9, 10, and 11), in addition to the muscle strengthening effect observed *in vivo* (Fig. 5). 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 (Desler et al. 1996). This very clear distinction in cellular effects between ecdysteroids and anabolic steroids strongly supports the claim that ecdysteroids do not act in the same manner as androgenic anabolic steroids.

After confirming the overall anabolic effects of ecdysteroids, a closer look at different ecdysteroid analogues was taken in an attempt to develop a Structural Activity Relationship (SAR). Of the four different ecdysteroids tested, 20HE and turkesterone were the most anabolically active, significantly increasing protein synthesis at a concentration of only 80 nM (Fig. 9). The other ecdysteroids tested, ponesterone and polypodine B, produced slightly weaker anabolic effects, significantly increasing protein synthesis at concentrations of 100 nM and 1 μM respectively. Because the only structural differences between the tested ecdysteroids are the number and placement of hydroxyl groups, a clear SAR is difficult. The number of hydroxylations does not seem to be a factor, as turkesterone and polypodine B, which both possess 7 hydroxyl groups, differ in anabolic activity. The claim that the C11 hydroxylation confers increased anabolic

activity (Syrov et al. 2001) was not supported in this study, as the C11 containing ecdysteroid, turkesterone, produced increases in protein synthesis comparable to 20HE which lacks the C11 hydroxyl. The increased anabolic activity of turkesterone previously reported in animals may be due to a specific *in vivo* effect such as increased bioavailabilty which may not affect activity measured in cell culture. Addition of a C5 hydroxyl or loss of the C25 hydroxyl appears to weaken the anabolic activity. Ultimately, because the range of activity is narrow and the number of ecdysteroids tested small, a strong SAR was difficult to produce.

The anabolic effect of 20HE became significant after 2 h and was maintained for at least 24 h (Fig. 12). The effect became significant at 100 nM of 20HE, comparable to the affinity of an insect ecdysone receptor (Elmogy et al. 2004).

Similarly to pure compounds, extracts from plants known to contain ecdysteroids (i.e., *A. turkestanica* and spinach) also enhanced protein anabolism in muscle cells (Fig. 13). Levels of ecdysteroids present in the extracts were sufficient to explain the observed effects and correlated with the effective concentrations of pure compounds. However the results seem to question the supposed advantage of *A. turkestanica* over other ecdysteroid containing plants as a potential source of ecdysteroids. Although *A. turkestanica* extract produced anabolic effects, the activity was comparable to the other ecdysteroid containing plant extract from spinach (Fig. 13), which lacks turkesterone. The equivalent activity of turkesterone and 20HE, as demonstrated both from the plant extracts as well as the pure compounds (Fig. 11), does not support prior claims of *A. turkestanica*'s

increased anabolic activity. However, *A. turkestanica* may indeed be more potent due to other *in vivo* factors not addressed in the cellular model.

Similarly to methandrostenolone, 20HE and spinach extract normalized for 20HE dose were associated with significant increases in muscle strength in rats (Fig. 5). These results support previous findings that ecdysteroids increase muscle strength *in vivo* (Chermnykh et al. 1988), and indicate that extracts from both tested plants may stimulate muscle growth and strength. Although no change in total muscle mass was observed *in vivo*, the increase in strength may be due to changes in muscle fiber composition which may not increase overall mass.

Protein Degradation

The effects of ecdysteroids on protein degradation remain uncertain. Although neither C_2C_{12} nor L6 showed any change in degradation after treatment with 20HE, this does not necessarily mean ecdysteroids have no effect. The glucocorticoid dexamethasone also produced no effect on protein degradation in our study, in contrast to previous studies clearly showing its effect in the same cell lines (Desler et al. 1996). This discrepancy may be a result of the cells' acquired insensitivity to glucocorticoids. This insensitivity may alter the cells' regulation of protein degradation, making these cells a poor model for protein degradation. Although no clear results regarding ecdysteroids' effect on total protein degradation were obtained, looking at a specific pathway of degradation may be more enlightening. Therefore, attention was turned to the ubiquitin proteasome pathway.

Proteasome

Although ecdysteroids did not produce any change in total degradation, a statistically significant change in proteasome activity was observed. The importance of this finding remains to be determined, and may be dependant on the specific proteins whose degradation ecdysteroids may decrease. This requires further study focusing on metabolism of specific proteins, beyond the scope of this project.

Although their effects on protein degradation in skeletal muscle were not clearly characterized, ecdysteroids clearly alter protein metabolism, specifically synthesis. Once the anabolic effect of an ecdysteroid-induced increase in protein synthesis was verified, we moved on to characterize the signaling pathway involved. The initial step in many responses to stimuli, especially steroids, is the interaction or activation of a receptor. A receptor may be involved in mediating the anabolic effect of ecdysteroids in mammals. Identifying this potential receptor can be difficult, as very little work has previously been done suggesting any candidates.

Androgen and Glucocorticoid Receptor

Introduction

Because ecdysteroids produce anabolic effects similar to androgens, it has been conjectured that ecdysteroids bind to the androgen receptor. Therefore, the first step in receptor identification was to test the binding of ecdysteroids to the known androgen binding receptors.

Although ecdysteroids produce anabolic effects in animals similar to androgens, they seem to be pharmacologically very different, as ecdysteroids are reported not to produce the androgenic side effects associated with androgen analogues (Syrov and Kurmukov 1976). Even though both ecdysteroids and androgens contain the same steroid backbone, there are major structural differences which physiologically distinguish the two classes of compounds. As previously mentioned, these primarily include the 7-en-6-one moiety, the C17 alkyl side chain, and the multiple hydroxyl groups. These chemical differences may restrict ecdysteroids from activating the nuclear androgen receptor, and explain the difference in pharmacological responses.

In order to test this possible distinction, binding assays using the androgen receptor were used to compare 20HE with methandrostenolone, an androgen analogue used therapeutically as an anabolic agent.

Another nuclear receptor which is activated by androgens is the glucocorticoid receptor (Mayer and Rosen 1975). Because some of the anabolic effects of androgens may be mediated by the gluccorticoid receptor, binding assays using the glucocorticoid receptor were performed comparing 20HE and methandrostenolone.

Methods

Androgen Receptor Binding

Androgen receptor binding assays were performed by MDS Pharma Services, Taiwan, (study #1019130). The assay to test the androgen receptor binding was performed as described by Chang and Liao (1987). 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/ml 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 in order to quantify displacement of the labeled ligand. Each treatment was repeated four times, and the results were averaged.

Glucocorticoid Receptor Binding

Glucocorticoid receptor binding assays were also performed by MDS Pharma Services, Taiwan, (study #1063350), based on Cidlowski and Cidlowski (1981). Briefly, human glucocorticoid receptors purified from transfected HeLa S3 cells was combined with 6

nM [^{3}H] dexamethasone in a buffer of RPMI-1640, 10 mM HEPES, pH 7.2, for 2 h at 25 C. 100 μM of 20HE, methandrostenolone, or the vehicle (1% DMSO) were added for 20 h at 4 °C. DPM of the incubation buffer was measured in order to quantify displacement of the labeled ligand. Each treatment was repeated four times, and the results were averaged.

Results

In the rat androgen nuclear receptor binding assay, methandrostenolone produced specific binding to the androgen receptor with an IC_{50} of 24 nM, producing a binding curve similar to the endogenous ligand, testosterone (Fig. 16). However, 20HE showed no significant binding from concentrations of 1 μM up to 100 μM (Table 2). In the glucocorticoid receptor binding assay, 20HE displayed no significant binding at 100 μM (Table 3). Methandrostenolone produced 43% specific binding with the glucocorticoid receptor (data not shown).

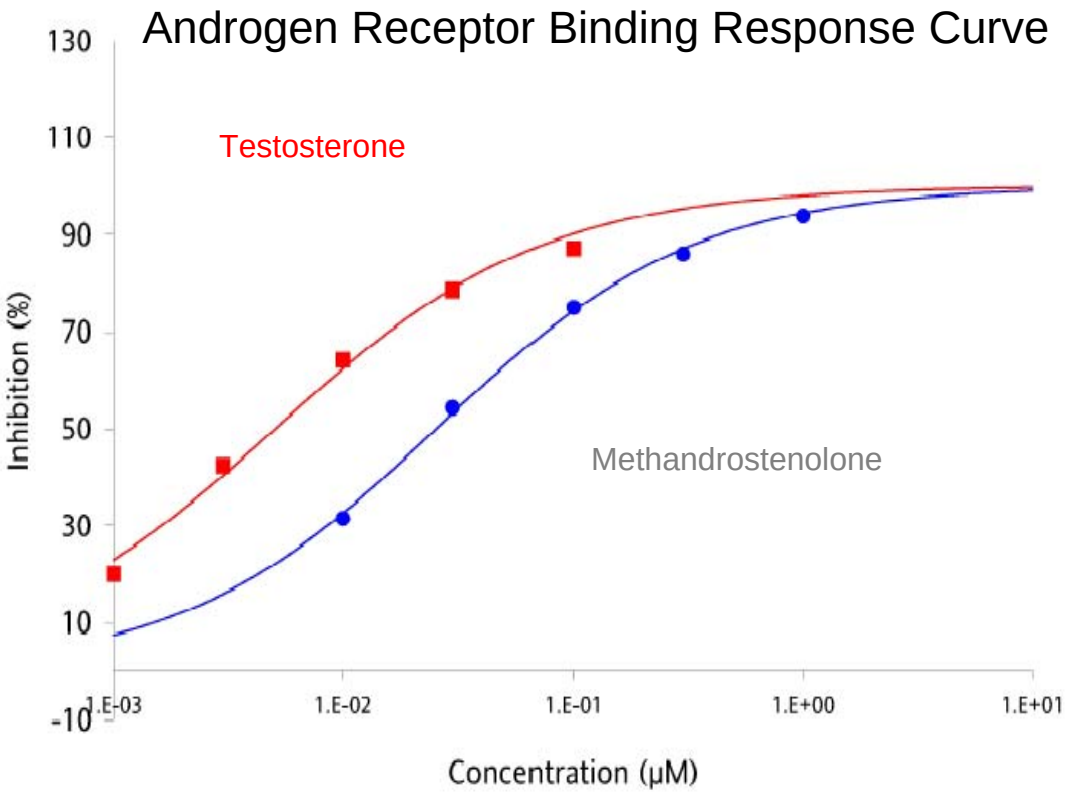


Fig. 16. Androgen Receptor binding of Testosterone or Methandrostenolone.

Binding assay of the rat Androgen Receptor using testosterone or the androgen analogue, methandrostenolone were performed measuring the displacement of the androgen [^{3}H] mibolerone. Each point was performed in triplicate. Note the similar response curve.

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

Table 3. Androgen Receptor Binding of 20-Hydroxyecdysone (20HE) and Methandrostenolone. Binding assays of the rat Androgen Receptor using 20-hydroxyecdysone or the androgen analogue, methandrostenolone, were performed measuring the displacement of the labeled androgen [^{3}H] mibolerone. Each point was performed in triplicate.

Discussion

The complete lack of specific binding of 20HE to the androgen receptor seems to reject the possibility that ecdysteroids activate the androgen receptor, and strongly supports the claim that ecdysteroids do not have androgenic activity.

In contrast with the claims that they may activate glucocorticoid receptors, ecdysteroids displayed no specific binding to the glucocorticoid receptor. Methandrostenolone showed significant binding, supporting previous evidence that androgens may activate the glucocorticoid receptor.

Ecdysteroids' anabolic activity, if not mediated through the androgen receptor, must work through a different pathway. A bioinformatic search for potential ecdysone activated receptors in mammals was undertaken in the hope of identifying the initial response that eventually leads to increased protein synthesis.

Nuclear Ecdysone Receptor Homologues

Introduction

Initially we theorized that putative ecdysteroid receptors (EcR) exist in mammals which function similarly to those found in insects. Since mammals seem to lack the EcR, other potential mammalian receptors for ecdysteroids were identified. The selection process was driven by protein-protein BLAST searches of the human, rat, and mouse proteome using the protein sequence of the *Drosophila melanogaster* nuclear ecdysone receptor.

After potential mammalian ecdysone receptors were identified, they were tested for binding with 20HE. 20HE activity was compared to the anabolic androgen methandrostenolone in an attempt to distinguish their binding activities. Binding studies were performed to see if 20HE would bind to any of the selected nuclear receptors. The more promising candidates, LXR and FXR, were subjected to additional activation assays.

Methods

BLAST

The first approach taken to identify potential receptors involved searching for putative receptors similar in structure to the insect EcR. The ecdysone receptor CG1765-PA,

isoform A (NP_724456.1) was used for searching the mouse, rat, and human proteome. Analysis of the complete amino acid sequence using protein-protein BLAST algorithms were performed and homologous proteins analyzed (Altschul et al. 1990).

Binding Studies

Thyroid Receptor

Thyroid receptor binding assays were performed by MDS Pharma Services, Taiwan, (study # 1063350) as described by Inoue et al. (1983). Briefly, thyroid receptor extracted from Sprague Dawley rat liver was combined with 0.03 nM [^{125}I] triiodothyronine in a buffer of 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 10% Glycerol, 2 mM EDTA, and 5 mM DTT. 100μM of 20HE, methandrostenolone, or the vehicle (1% DMSO) were added for 18 h at 4 °C. DPM of the incubation buffer was measured in order to quantify displacement of the labeled ligand. Each treatment was repeated four times and the results were averaged.

Vitamin D_3 Receptor

Vitamin D_3 receptor binding assays were also performed by MDS Pharma Services, Taiwan, (study # 1063350) based on Ross et al. (1991) and Sone et al. (1990). Briefly, recombinant human vitamin D_3 receptor from insect Sf9 cells was combined with 0.3 nM [^{3}H] 1α, 25-dihydroxyvitamin D_3 in a buffer of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 300 mM KCl, and 0.01% Tween 20. 100 μM of 20HE, methandrostenolone, or the vehicle (1% DMSO) were added for 20 h at 4 °C. DPM of the incubation buffer

was measured in order to quantify displacement of the labeled ligand. Each treatment was repeated four times and the results were averaged.

Activation Studies

FRET

FRET assays were performed by Phenex AG (Germany). Activation of selected nuclear receptors was tested using FRET, based on Glickman et al. (2002). 20HE or methandrostenolone were added to an assay buffer of 50 mM Tris, pH 7.4, 50 mM KCl, 1 mM DTT, and 0.1% BSA containing 1 nM labeled anti-glutathione-S-transferase (GST) antibody, the ligand binding domain of either LXR or FXR affinity tagged with GST, 2 nM allophycocyanin labeled streptavidin, and biotin labeled with either the Nuclear Receptor CoActivator 3, NCoA3, for LXR or SRC1 for FXR. Concentrations from 1 nM to 100 μM were tested in triplicate in an assay volume of 25 μl and fluorescence was measured.

Gal4 Transactivation Assay

Gal4 assays were also performed by Phenex AG (Germany). HEK293 cells were transiently transfected with the following plasmids:

1) pFR-Luc, containing a promoter with five tandem repeats of the yeast GAL4 binding sites that control expression of the *Photinus pyralis* luciferase gene.
2) pRL-CMV, containing the gene for *Renilla reniformis* (American Firefly) luciferase, driven by a constitutive promoter, for normalization against differences in transfection efficiency and cell growth.
3) pCMV-BD, to fuse the DNA-binding domain of the yeast protein GAL4.

Cells were treated with 20HE or methandrostenolone in medium containing a final concentration of 8.6% charcoal-dextran treated FBS (Hyclone), and lysed after 16h. Firefly and Renilla luciferase activities were measured sequentially in the same cell extract using a Dual-Light-Luciferase-Assay system. An eleven point dose response from 1 nM to 100 μM was performed in triplicate on 96 well plates. Significance was determined as greater then 10% increase in activity compared to control.

Results

BLAST

Liver X Receptors

The most similar receptors discovered were the liver X receptors. The mouse LXRα (NP_038867) produced a homology score of p = 3e-81 with 37% amino acid identity and 52% similarity with the insect EcR. LXRβ showed similar homology.

Farnesoid X Receptors

Another receptor identified was the farnesoid X receptor. The rat FXR (NP_033134) produced a homology score of p = 9e-59 with 36% amino acid identity and 52% similarity with the insect EcR.

Thyroid and Vitamin D_3 Receptors

Other potential receptors identified were the thyroid receptor and the vitamin D_3 receptor. The human thyroid hormone receptor α (NP_003241) produced a homology score of p = 2e-42 with 30% amino acid identity and 48% similarity with the insect EcR. The human 1,25-dihydroxyvitamin D_3 receptor (NP_000367) produced a homology score of p = 9e-23 with 43% amino acid identity and 63% similarity with the insect EcR.

Binding and Activation Studies

20HE, even up to 100 μM concentration, showed no significant binding or activation to any of the tested nuclear receptors (Table 3).

Discussion

Homologous Receptors

LXRs are in the same heterodimer nuclear receptor family as the EcR and share homology with the EcR receptor. They are involved in lipid and glucose homeostasis (Lafitte et al. 2003), but recently have been shown to play an important role in many physiological processes (Rader 2007).

Receptor	Assay	
	Binding	Activation
Androgen	-	NT
Glucocorticoid	-	NT
Thyroid	-	NT
Vitamin D	-	NT
Liver X α	NT	-
Liver X β	NT	-
Farnesoid X α	NT	-
Farnesoid X β	NT	-
β_2 Adrenergic	-	-

Table 4. Receptor binding and activation by 20HE. 8 nuclear receptors and a G-Protein Coupled Receptor were tested for activity with 100 μM 20HE. Binding was analyzed using displacement assays with labeled ligands. Activation was tested using FRET for LXRs and FXRs and a functional assay for cAMP for the β_2 Adrenergic receptor. All assays were performed in triplicate and significance established as 10% change from control. **-** means no significant displacement or activation. **NT** stands for not tested.

The FXRs are also in the same family of heterodimer nuclear receptors and share homology with the EcR. FXRs historically were considered bile acid receptors involved in lipid homestasis, but recent studies suggest a more pleiotropic role, including regulation of energy expenditure and the vasculature (Scotti et al. 2007). Although the hypothetical connection between FXR activation and increased protein synthesis is unclear, it is certainly an interesting possibility.

The thyroid and vitamin D receptors also belong in the same heterodimer nuclear receptor family as the insect EcR, so it is not surprising that they share homologous sequences. However the relevance of either thyroid receptor or vitamin D receptor activation with ecdysteroids' anabolic effect is unclear. Nevertheless, because of their similarity, these receptors were included in subsequent receptor binding studies.

The four identified receptors all share strong sequence homology with the insect EcR. This homology was also found when comparing only the ligand binding domain (data not shown). Although amino acid sequence similarity does not necessarily imply similar function, it is a reasonable, yet often incorrect, assumption. This assumption was partially supported by studies showing that oxysterols, which are structurally similar to ecdysteroids, bind to similar nuclear receptors (Schroepfer 2000). In fact, a steroid was found to bind and activate the FXR (Wang et al. 2006). For these reasons, the selected receptors were tested for activity with ecdysteroids.

Binding and Activation

All of the previously mentioned receptors tested in binding assays showed no significant binding of 20HE at concentrations of 100 μM (Table 3). This supports previous reports that 20HE showed no activity with another similar nuclear receptor, the pregnane X receptor (PXR) (Ekins et al. 2008).

In many cases ligands can activate receptors without displacing the main agonist (May et al. 2007). Therefore, lack of displacement from the previously mentioned binding studies does not necessarily mean there is no binding. A more accurate test of potential agonists is receptor activation studies. For this reason, the two most homologous receptors, LXR and FXR were subjected to Fluorescence Resonance Energy Transfer (FRET) and Gal4 transactivation assays to test ecdysteroid activity.

100 μM 20HE did not activate the LXRs or FXRs in either the FRET or Gal 4 assays (Table 3). With the exception of the androgen and glucocorticoid receptors, methandrostenolone also did not display significant binding or activation of any of the other receptors tested (data not shown).

The lack of binding of 20HE to all of the candidate receptors refutes the theory that a mammalian nuclear receptor, homologous to the insect Ecdysone receptor is responsible for ecdysteroids anabolic activity.

A different target was needed to identify the hypothetical mammalian ecdysone receptor. Although searches using the nuclear EcR were ultimately unsuccessful, perhaps searches using a distinct non-nuclear ecdysone receptor would produce more positive results.

Non-nuclear Ecdysone Receptor

Introduction

Fortuitously, a distinct non nulcear ecdysone receptor was identified in insects. The **d**opamine **e**cdysone **r**eceptor, DopEcR characterized in *Drosophila malangaster*, is a membrane bound GPCR which is activated by dopamine and 20HE (Srivastava et al. 2005). Unlike the nuclear EcR which requires time to produce a genomic response through gene transcription, the GPCR ecdysone receptor may be responsible for many of the rapid non-genomic responses to ecdysteroids. Therefore, it is possible that a mammalian GPCR similar to the insect DopEcR may be the receptor eliciting the anabolic activity of ecdysteroids. Initially, the DopEcR sequence was used to search for homologous receptors in mammals to be tested for activation by ecdysteroids.

Methods

BLAST

The protein sequence of *Drosophila melangaster* dopamine/ecdysteroid receptor, CG18314-PA, isoform A (NP_647897.2) was used for searching the mouse, rat, and human proteome. Analysis of the complete amino acid sequence using protein-protein BLAST algorithms was performed and homologous proteins were analyzed (Altschul et al. 1990).

Binding and Activation Studies

β_2 Andrenergic receptor binding assays were performed by Cerep (France), **(**Study # 13041), based on Smith and Teitler (1999). Briefly, recombinant human β_2 Andrenergic receptors expressed in insect Sf9 cells were combined with 0.2 nM [^{3}H] alprenolol in a buffer of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 300 mM KCl, and 0.01% Tween 20. 100 μM of 20HE, methandrostenolone, or the vehicle (1% DMSO) were added for 60 min at 22 °C. DPM of the incubation buffer was measured in order to quantify displacement of the labeled ligand. Each treatment was repeated four times and the results were averaged. The specific ligand binding to the receptor is defined as the difference between the total binding and the nonspecific binding determined in the presence of an excess of unlabelled ligand.

Functional activation of β_2 adrenergic receptor was tested using a cellular assay for cAMP production based on Baker (2004). CHO cells expressing human recombinant β_2 receptors were treated with 100 μM 20HE for 30 min at 22 °C. cAMP production was detected using homogeneous time resolved fluorescence (HTRF).

An assay for a potential antagonist effect was performed by co-treatment with the agonist 30 nM isopreterenol and 100 μM 20HE to determine if 20HE reduces the isoproterenol induced increase in cAMP.

Results

The human β_2 adrenergic receptor (NP_000015) was identified as possessing the greatest homology with the insect DopEcR, producing a homology score of $p = 3e^{-22}$ with 27% amino acid identity and 45% similarity.

20HE did not display any specific binding or functional activation of the β_2 adrenergic receptor at 100 μM (Table 3).

Discussion

The β_2 adrenergic receptor, a GPCR, is a very promising candidate for mediating ecdysteroids' anabolic activity. Agonists like clenbuterol have been shown to induce skeletal muscle hypertrophy and block atrophy (Hinkle et al. 2002), producing responses similar to ecdysteroids.

Based on the BLAST results and the known physiological responses, the β_2 adrenergic receptor was selected for binding studies with 20HE. Since displacement assays are not very conclusive, functional activation assays using cAMP were also performed.

The lack of binding or activation of the β_2 adrenergic receptor by 20HE seems to reject the hypothesis that ecdysteroids' anabolic activity is mediated through the β_2 adrenergic receptor. Perhaps a different GPCR may mediate the ecdysteroid response. There are over 1000 different GPCRs and most are uncharacterized (Rompler et al. 2007). It is quite possible that one of these GPCRs is an ecdysone receptor. However, since identifying the orphan GPCR is a rather daunting task, other strategies were utilized to elucidate ecdysteroids' mode of action.

GPCR Signaling

Introduction

Since direct analysis of a hypothetical ecdysone GPCR was not feasible, indirect studies of signaling pathways characteristic of GPCRs were performed. Presuming the existence of a mammalian ecdysone GPCR, assays were designed to analyze the hypothetical signaling pathway, looking at rapid signaling molecules, intermediary proteins, and the effect of specific inhibitors on longer term effects.

Like the name implies, GPCRs are coupled to G-proteins. G proteins are heterotrimeric guanine nucleotide-binding regulatory proteins, comprised of α, β, and γ subunits (Marinissen and Gutkind 2001). They transmit external signals from membrane bound GPCRs to intracellular effectors. There are four subfamilies of G proteins classified according to their α subunits: Gs, Gi, Gq, and G12. Agonists binding to GPCRs induce a conformational change of the transmembrane-spanning segments, which leads to the exchange of GDP for GTP bound to the α subunit of the G protein (Kobilka, 2007). The resulting G protein subunits regulate a wide range of effector molecules, including ion channels, phospholipase C, GPCR kinases, and PI3K (Bommakanti et al. 2000). Downstream, these signaling molecules produce a range of important cellular responses.

In order to begin to elucidate the hypothetical GPCR ecdysone receptor pathway, some of the first steps in G protein signaling were analyzed. Inhibition of G protein activation, the

phospholipase C pathway, and calcium flux were studied and their role in ecdysteroid-induced anabolic activity in skeletal muscle was characterized.

Methods

PTX

Differentiated C_2C_{12} myotubes were serum starved overnight and pretreated with 1 μg/ml PTX (Sigma Aldrich, St Louis MO) or vehicle for 1 h, prior to treatment with 1 μM 20HE, 50 ng/ml IGF-1, or vehicle for an additional 4 h. Protein incorporation was measured as mentioned previously.

PLC

PLC and IP_3R inhibition wase studied using the PLC inhibitor, 1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione (U-73122), and the IP_3R inhibitor, 2-aminoethoxydiphenyl borate (2-APB) purchased from Calbiochem (San Diego CA). C_2C_{12} myotubes were serum starved overnight and pretreated with either 10 μM U-73122, 10 μM 2-APB, or vehicle for 1 h before treatment with 1 μM 20HE, 50 ng/ml IGF-1, or vehicle for an additional 4 h. Protein incorporation was measured as mentioned previously.

Calcium

Ca^{2+} Flux

Measurements of intracellular calcium from C_2C_{12} skeletal myotubes was determined using Fluo-4 NW based on Gee et al. (2000) and according to manufacturer's instructions (Invitrogen, Carlsbad CA). C_2C_{12} myotubes plated on 96 well plates were loaded with the calcium fluophore, Fluo4-AM for 1 h prior to treatment. Cells were washed with either Ca^{2+} containing media (1 mM NaCl, 5 mM KCl, 2.6 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mm HEPES-Na, 5.6 mM glucose) or Ca^{2+} free medium (1 mM NaCl, 5 mM KCl, 1.0 mM EGTA, 3.6 mM $MgCl_2$, 10 mM HEPES-Na, 5.6 mM glucose). For inhibitor studies, cells were pretreated with either 1 μg/ml PTX, 3 mM EGTA, 10 μM U-73122, both 3 mM EGTA and 10 μM U-73122, 1 μM 2-APB, or vehicle for 1 h prior to treatment with either 1 μM 20HE or vehicle. Baseline fluorescence was monitored prior to treatment. Cells were treated with 1 μl of 1 μM 20HE, dissolved in assay buffer, and fluorescence was measured using a Synergy™ HT Multi-Mode Microplate Reader. Data were collected from three wells per treatment every five seconds for 180 seconds, and analyzed using Gen5 Data Analysis software. The changes in fluorescent intensity over baseline ($\Delta F/F_0$) were averaged and plotted as a function of time.

Effect of Ca^{2+} on Protein Synthesis

Removal of free Ca^{2+} was performed based on Dancui et al. (2003). For removal of free extra- or intracellular Ca^{2+}, differentiated C_2C_{12} myotubes were transferred to media supplemented with either 3 mM EGTA and/or 25 μM BAPTA-AM for 30 min prior to treatment with 1 μM 20HE, 50 ng/ml IGF-1, or vehicle for 4 h. Protein incorporation was measured as mentioned previously.

Results

PTX

Pretreatment with 1 μg/ml PTX, a G protein inhibitor, for 1 h completely abolished the 20HE-induced increase in protein synthesis in C_2C_{12} myotubes (Fig. 17). In contrast, the IGF-1 induced increase in protein synthesis was unchanged after PTX pretreatment.

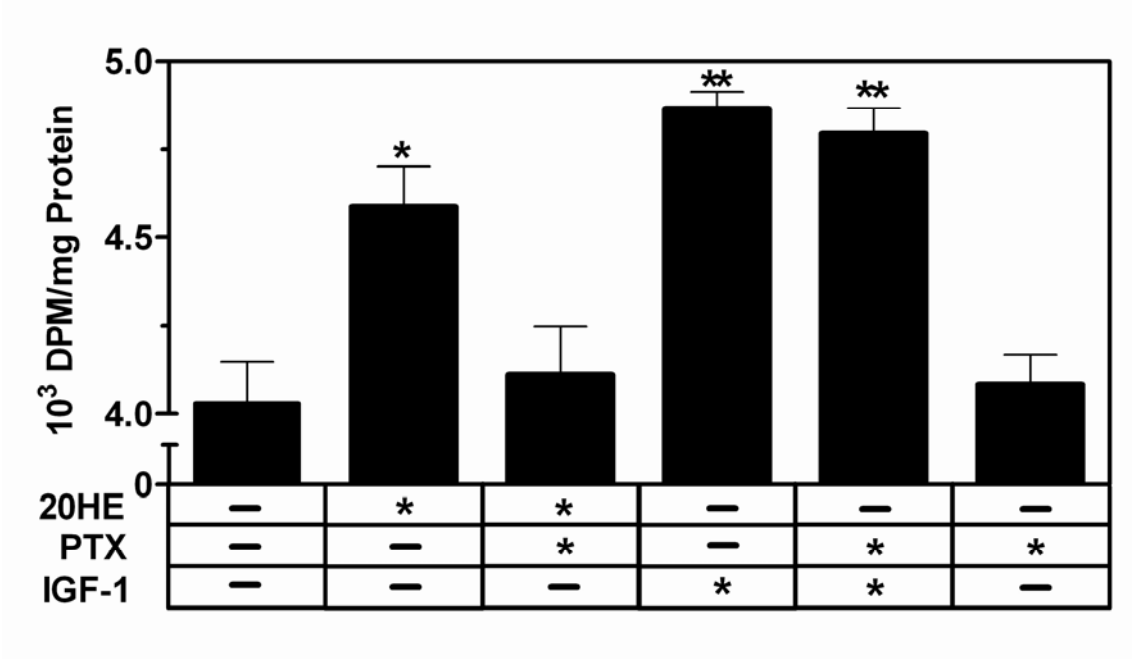


Fig. 17. [^{3}H] Leucine Incorporation in C_2C_{12} Myotubes Pretreated with G protein inhibitor, PTX.

Differentiated myotubes were pretreated with the G protein inhibitor, 1 μg/ml PTX, or vehicle for 1 h before treatment with either 1 μM 20HE, 50 ng/ml IGF-1, or vehicle for an additional 4 h. DPM were normalized by total protein. The data represent the mean values ± S.E.M. of three experiments. * indicates $P < 0.05$ and ** indicates $P < 0.01$ compared with control (Student's t test).

PLC

Pretreatment with either 10 μM U-73122 or 10 μM 2-APB for 1 h reduced the 20HE-induced increase in protein synthesis, but the effect was not additive when both inhibitors were combined (Fig. 18). Pretreatment with U-73122 significantly reduced the IGF-1 induced increase in protein synthesis while 2-APB caused only a slight decrease. Treatment with only U-73122 and/or 2-APB did not alter protein synthesis (data not shown).

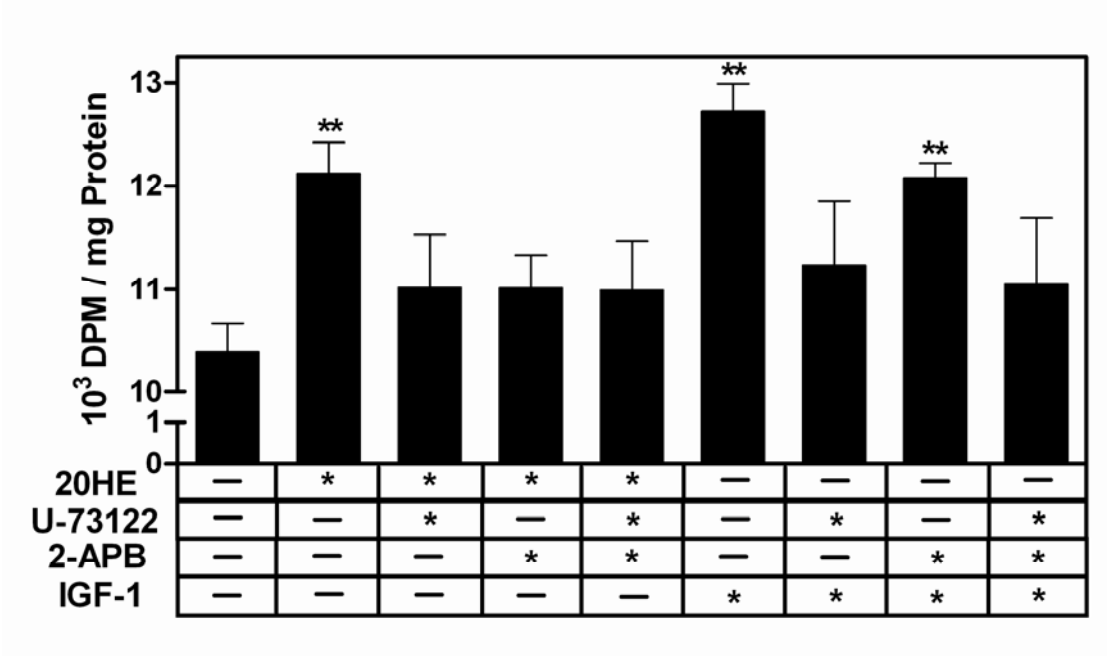


Fig. 18. [^{3}H] Leucine Incorporation in C_2C_{12} Myotubes Pretreated with Phospholipase C (PLC) or Inositol 3 Phosphate Receptor (IP_3R) Inhibitors. Differentiated myotubes were pretreated with either the PLC inhibitor, 10 μM U-73122, and/or the IP_3R inhibitor, 10 μM 2-APB, or vehicle for 30 min 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 ± S.E.M. of three experiments. ** indicates $P < 0.01$ compared with control (Student's t test).

Calcium

Ca^{2+} Flux

Treatment with 1 μM 20HE increased intracellular Ca^{2+} in C_2C_{12} myotubes within 10 sec (Fig. 19A). Intracellular Ca^{2+} peaked 35 sec after treatment, and began to decline after 70 sec. The decrease was gradual with some elevation of intracellular Ca^{2+} still observed after 180 sec. This effect was completely abolished when cells were pretreated with 1 μg/ml PTX, 1 h prior to 20HE treatment (Fig. 19B).

Ca^{2+} free media containing the extracellular Ca^{2+} chelator, 3 mM EGTA, slightly reduced and modified the 20HE response and decreased its duration (Fig. 19C).

Pretreatment with the Phospholipase C (PLC) inhibitor, 10 μM U-73122, for 1 h prior to 1 μM 20HE treatment also reduced the intensity and duration of the intracellular Ca^{2+} flux as compared to the 20HE treatment alone (Fig. 19D). The IP_3R inhibitor 2-APB produced similar results (data not shown). Both EGTA and U-73122 produced a more rapid oscillating decline in Ca^{2+}, returning close to control levels in less than 120 s (Fig. 19C and D). When the PLC inhibitor, U-73122, was added to EGTA containing media, the 20HE-induced Ca^{2+} flux was almost completely abolished (Fig. 19E).

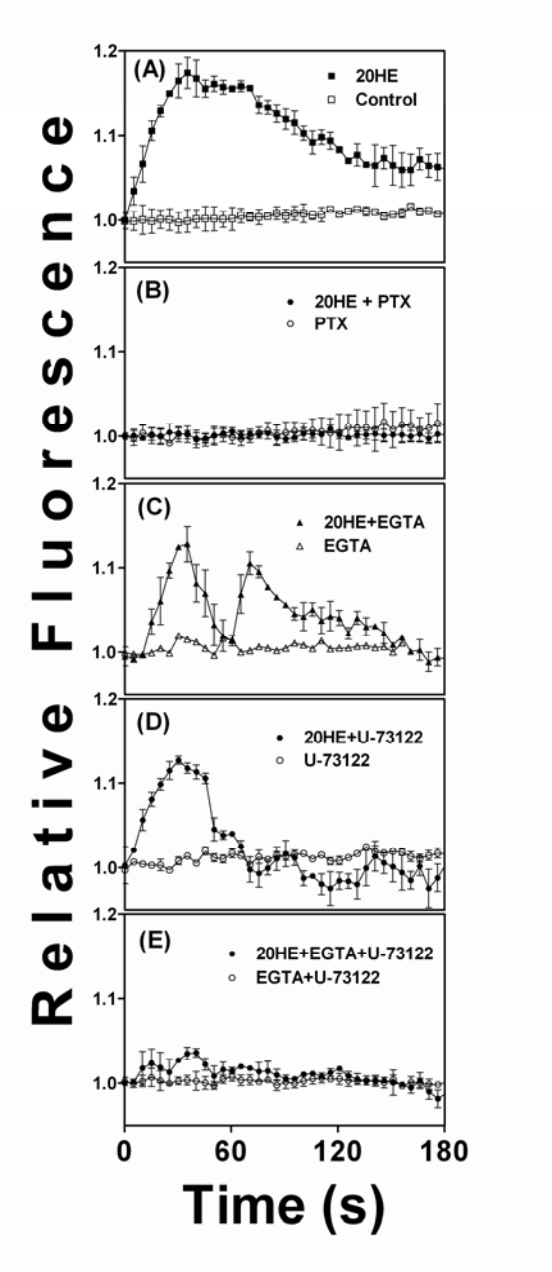


Fig 19. Intracellular Calcium in C_2C_{12} myotubes treated with 20-Hydroxyecdysone (20HE) and various inhibitors.

Differentiated myotubes were pretreated with either (**A**) vehicle, (**B**) 1 μg/ml PTX, (**C**) 3 mM EGTA, (**D**) 10 μM U-73122, (**E**) both 3 mM EGTA and 10 μM U-73122, for 1 h prior to treatment with either 1 μM 20HE (●) or vehicle (○) . Relative fluorescence was measured for 180 seconds after treatment. The data represent the mean values ± S.E.M. of three experiments.

Effect of Ca^{2+} on Protein Synthesis

Removal of extracellular Ca^{2+} abrogated the 20HE-induced increase in protein synthesis in C_2C_{12} myotubes (Fig. 20). In media containing 3 mM EGTA to chelate free extracellular Ca^{2+}, the 20HE-induced increase was only 8%, half of the 20HE-induced increase when using Ca^{2+} containing media. Pretreatment with 25 μM BAPTA-AM for 1 h prior to 20HE treatment similarly increased the protein synthesis by only 8%. When cells were pretreated with both EGTA and BAPTA-AM together for 1 h prior to treatment, the 20HE-induced increase was completely abolished. The IGF-1 induced increase in protein synthesis was also inhibited by both EGTA and BAPTA-AM. While IGF-l still increased protein synthesis in cells pretreated with EGTA, IGF-1 produced no increase in cells pretreated with BAPTA-AM. Treatment with only EGTA and/or BAPTA-AM did not alter protein synthesis (data not shown).

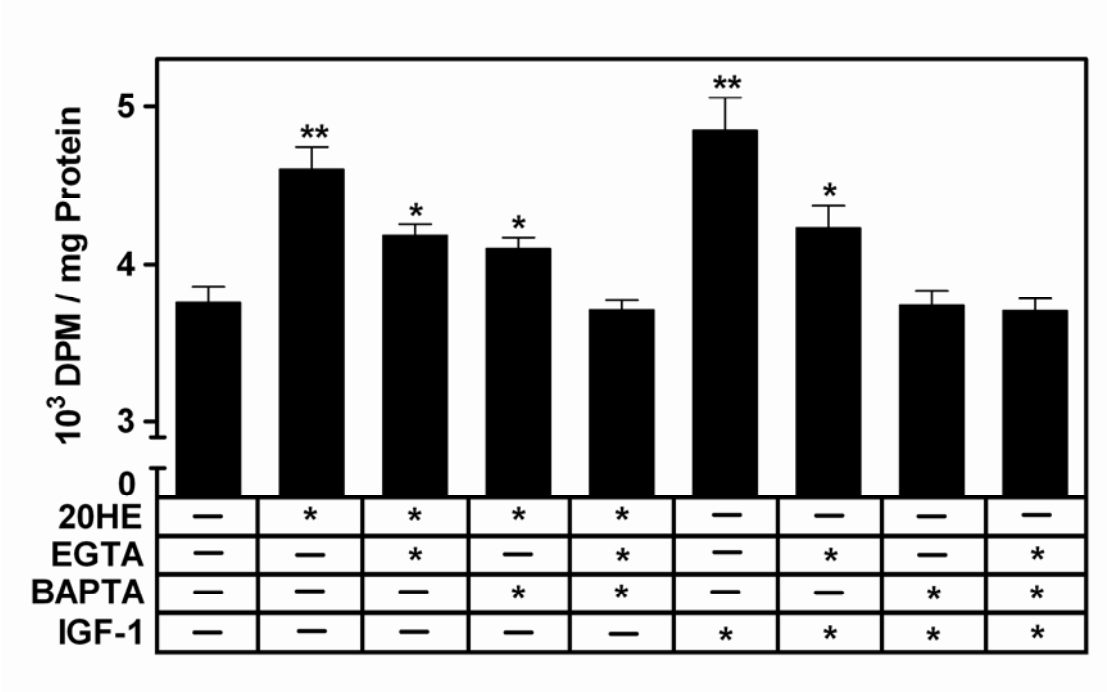


Fig. 20. [^{3}H] Leucine Incorporation in C_2C_{12} Myotubes Pretreated with the Calcium Chelators, EGTA and/or BAPTA-AM.

Differentiated myotubes were pretreated with either 1 mM EGTA or 20 μM BAPTA-AM, or both, or vehicle for 1 h 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 ± S.E.M. of three experiments. * indicates $P < 0.05$ and ** indicates $P < 0.01$ compared with control (Student's t test).

Discussion

PTX

Pertussis toxin (PTX), a protein-based AB5-type exotoxin produced by the bacterium *Bordetella pertussis* (Burns 1988), was used to block G protein signaling. PTX inhibits the activation of G_α proteins, thereby preventing interaction with the GPCRs. The effect of blocking GPCR signaling on ecdysteroids' anabolic activity was tested.

PTX treatment completely blocked the ecdysteroid-induced increase in protein synthesis (Fig. 17), suggesting that ecdysteroids act through a G protein mediated response. In contrast, IGF-1, known to activate the receptor tyrosine kinase IGF-1 receptor, was not blocked by PTX, supporting previous claims of a non G protein mediated signal (Rommel et al. 2001).

G protein activation can lead to a variety of signaling pathways including Akt/mTOR/p70S6K, Raf/MEK/ERK, and PKC/PKD, all of which are involved in cell growth and protein synthesis (Rozengurt 2007). The G protein induction of these pathways is usually through the activation of phospholipases which leads to the production of lipid derived second messengers, Ca^{2+} flux, and eventually protein activation.

PLC Pathway

The results of the PTX inhibitor study suggest that ecdysteroids' anabolic effects are, at least in part, mediated through the PLC-IP_3R pathway. Claims of PLC activation by ecdysteroids have previously been made based on rapid changes in phosphoinositide levels in rat brain and heart after ecdysteroid treatment (Kotsyuruba et al. 1999). It is quite possible PLC may also be involved in ecdysteroid action in skeletal muscle. To test the possible activation of PLC and the downstream IP_3R by ecdysteroids, specific inhibitors of PLC and IP_3R were utilized in an attempt to block the ecdysteroid induced increase in protein synthesis in C_2C_{12} myotubes.

The membrane bound enzyme, Phospholipase C (PLC), is an important effector stimulated by G protein activation. Many GPCR agonists activate the $G_{\alpha q}$ subunit, causing the exchange of bound GDP for GTP (Rhee 2001). This GTP-$G_{\alpha q}$ complex activates PLC. It is also possible the $G_{\alpha i}$ as well as the $\beta\gamma$ subunits of G proteins can also stimulate PLC (Rozengurt 2007). Once activated, PLC causes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), producing the two second messengers: inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to IP_3 receptors (IP_3R) in the endoplasmic reticulum (ER), or the sarcoplasmic reticulum (SR) found in skeletal muscle, and triggers the mobilization of Ca^{2+} from ER or SR stores, leading to a rapid increase in intracellular Ca^{2+} (Berridge et al. 2000). These secondary signalers lead

to cascades of protein phosphorylation, which eventually produce a range of important cellular responses, including protein synthesis.

Blocking either PLC or IP_3R produced similar decreases in the 20HE-induced increase in protein synthesis (Fig. 18). There was also no additive effect when both inhibitors were combined. This supports the current understanding that IP_3R and PLC lie along the same pathway (Rozengurt 2007).

One of the main downstream signals of the PLC/IP_3P pathway is intracellular Ca^{2+}. The previously mentioned activation of IP_3R causes the release of Ca^{2+} from the SR into the cytoplasm. Because increased Ca^{2+} flux can activate a range of important pathways, many of which lead to increased protein synthesis, ecdysteroids' effect on intracellular Ca^{2+} was studied in skeletal myotubes.

Calcium

Ca^{2+} flux is a key signaler in many GPCR mediated pathways. Although increased intracellular Ca^{2+} can be generated through the PLC/IP3R induced release of SR Ca^{2+} stores, this is not the only means. Various ion channels can be opened, allowing entry of extracellular Ca^{2+}. Therefore, in addition to studying ecdysteroids' effects on total Ca^{2+} flux, the specific effects on intra and extracellular pools of Ca^{2+} is also important in understanding the signaling pathway. This was studied by using the extracellular calcium

chelator, EGTA. Specific inhibitors of G protein signaling and PLC were utilized to better understand the relationship of Ca^{2+} with the upstream signalers.

The 20HE-induced rapid increase in Ca^{2+} flux shown in C_2C_{12} myotubes (Fig 19A) supports previous claims that ecdysteroids produce rapid non-genomic responses (Tamaschko 1999). The complete abolishment of 20HE-induced Ca^{2+} flux after treatment with the G protein inhibitor, PTX (Fig 19B), suggests a GPCR mediated pathway.

Since the Ca^{2+} flux can be the result of intra- or extracellular pools, the calcium chelator, EGTA was used to remove free calcium from the media. This did reduce and delay the 20HE-induced increase but did not completely remove it (Fig. 19C), suggesting extracellular Ca^{2+} pools are involved, but are not the only cause of the 20HE effect.

Our data show that the inhibition of PLC with U-73122 or the IP_3R using 2-APB shortened the duration of the 20HE-induced Ca^{2+} increase but did not completely abolish it (Fig. 19D and data not shown), further supporting that the PLC/IP_3R pathway is not the sole producer of 20HE-induced Ca^{2+} flux. However, PTX treatment completely blocked the 20HE-induced Ca^{2+} flux (Fig. 19B), which suggests that both the intracellular as well as the extracellular pools are transferred via a G protein mediated pathway. GPCR activated Ca^{2+} channels, which have been described in LNCaP prostate cancer cells (Sun et al. 2006), may also be activated by 20HE, allowing extracellular Ca^{2+} to flow into the cell.

Although this is the first documented case of 20HE-induced Ca^{2+} flux, this is not the first example of a steroid increasing intracellular Ca^{2+}. Steroids can rapidly alter Ca^{2+} levels both through influx and intracellular stores. This effect is steroid and cell type specific. In Serotoli cells, testosterone induced Ca^{2+} intake was due to PLC mediated closure of K^{+}-ATP channels (Loss et al. 2004). In T cells, while estradiol produced rapid Ca^{2+} flux from both extracellular and intracellular pools (Benten et al. 1998), testosterone induced only extracellular Ca^{2+} flux (Benten et al. 1997). Testosterone enhanced Ca^{2+} transport by opening T-type Ca^{2+} channels in rabbit kidney (Couchourel et al. 2004). Chronic testosterone treatment increased the current size and single channel activity of L-type Ca^{2+} channels in rat cardiomyocytes (Er et al. 2007). However, acute testosterone decreased activity of T-type Ca^{2+} channels (Michels et al. 2006). Estrada et al. (2006) described androgen induced Ca^{2+} oscillations in neurite cells which were blocked by the G protein inhibitor, PTX. This oscillation also required both intra- and extracellular Ca^{2+}. In human granulosa luteinizing cells, androstenedione increased Ca^{2+} through voltage-dependent Ca^{2+} channels in the plasma membrane, and phospholipase C activation via a pertussis toxin-sensitive G protein (Machelon et al. 1998).

The role of Ca^{2+} in steroid signaling is still unclear and may depend on many factors, including cell type, duration, and frequency of signal, as well as Ca^{2+} source. The role of the observed 20HE-induced flux in relation to the overall anabolic effect needs to be understood.

In order to evaluate the relevance of ecdysteroid induced Ca^{2+} flux, ecdysteroids' effect on protein synthesis was observed in the absence of intracellular or extracellular Ca^{2+} pools. The extracellular chelator, EGTA and intracellular chelator, BAPTA-AM were used to remove free Ca^{2+}.

Removal of free Ca^{2+} reduced the 20HE-inducedincrease in protein synthesis in skeletal myotubes (Fig. 20), suggesting Ca^{2+} is involved in the ecdysteroid pathway producing anabolic activity. Increased intracellular Ca^{2+} which is involved in GPCR signaling may come from either extracellular Ca^{2+} or internal stores (Berridge et al. 2000), either of which may be involved in ecdysteroids' anabolic activity. The removal of only intra- or extracellular Ca^{2+} alone reduced but did not completely remove the 20HE effect, implying both sources of Ca^{2+} are required for ecdysteroids anabolic activity.

Ca^{2+}, an important player in GPCR signaling, is involved in many downstream cellular functions. To narrow in on the specific Ca^{2+} mediated mechanism producing increased protein synthesis, potential signalers downstream in the GPCR pathway were selected.

Akt

Introduction

One plausible candidate for a downstream signaler in ecdysteroid-induced anabolic activity is Akt, which when activated, has been shown to increase protein synthesis (Rommel et al. 2001). Akt, a serine/threonine protein kinase, is involved in many important cellular processes including cell growth, survival, and glucose metabolism (Whiteman et al. 2002). The central role of Akt in so many different processes may explain ecdysteroids' wide range of effects.

There is some evidence that G proteins can activate Akt either directly or through intermediary signalers (Rozengurt 2007). Akt is activated by GPCR agonists including isoproternol, carbachol, and thrombin, and the pleckstrin homology domain of Akt has been shown to bind IP_3 and G proteins (Bommakanti et al. 2000). Because of its possible activation by GPCRs, and its central role in many processes affected by ecdysteroids, ecdysteroids were tested for activation of Akt in a cellular model of skeletal muscle.

The intial phase involved characterizing the effect of the inhibition of the Akt pathway on ecdysteroid's anabolic activity. If ecdysteroids' effect is Akt mediated, inhibition of the Akt pathway should block the increase in protein synthesis. After inhibition was verified, the effect of ecdysteroids on Akt activation was studied. Specific inhibitors as well as manipulation of available calcium were utilized to elucidate the specific pathways

involved in ecdysteroid-induced Akt activation. Potential pathways downstream of Akt were also studied in an attempt to further elucidate the mode of action of ecdysteroid's anabolic activity in skeletal muscle.

Methods

PI3K Inhibition

Differentiated C_2C_{12} myotubes were pretreated with a PI3K inhibitor, 10 μM LY294002 (LY) (Sigma Aldrich, St Louis MO) or vehicle, for 1 h prior to treatment with 1 μM 20HE, 100 ng/ml IGF-1, or vehicle. Protein synthesis after 4 h incubation was calculated as described previously.

Akt Activation

Cell Treatment

For the 20HE dose response, differentiated C_2C_{12} myotubes were washed with serum free DMEM and treated for 2 h with increasing concentrations of 20HE or vehicle. For the 20HE time course study, differentiated C_2C_{12} myotubes were treated with 1 μM 20HE for 5 min to 24 h in serum free media.

Western Blotting

After treatment, cells were lysed using 250 μl of cold lysis buffer per well. Lysis buffer consisted of 20 mM Tris, pH 7.4, 30 mM NaCl, 3.5 mM EDTA, 1 mM EGTA, 1.0% Triton X-100, 2.5 mM sodium pyrophosphate, 4 mM sodium fluoride, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride and Protease Inhibitor Cocktail (Sigma Aldrich, St Louis MO) consisting of 2.0 μg/ml leupeptin and 2.0 μg/ml aprotinin. Two wells per treatment were pooled and the whole cell lysates were sonicated for 15 seconds, boiled for 5 min, and centrifuged at 16,000 *g* for 5 min at 4° C. Protein content of the supernatant was quantified using a Nanodrop 1000 spectrophotometer. 40 μg of protein were separated using 8% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Amersham). Membranes were blocked in Tris Buffered Saline (TBS) containing 5% nonfat dry milk and 0.01% Tween at room temperature for 1 h. Membranes were then incubated in 5% BSA in TBS containing phospho-Akt (Ser^{473}) antibody (1:1000) or total Akt antibody (1:1000) (Cell Signaling) overnight at 4° C. Membranes were then rinsed and probed with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2000) (Cell Signaling) in 5% nonfat dry milk in TBS. After incubation with secondary antibodies, phosphorylated or total Akt protein was detected by enhanced chemiluminescence (Amersham). Band intensity was quantified by densitometry of immunoblots using NIH ImageJ, version 1.38x, and phosporalyted Akt levels normalized to total Akt.

Upstream Inhibition of Akt

Differentiated C_2C_{12} myotubes were pretreated with 10 μM LY, 1 μg/ml PTX, or vehicle for 1 h prior to treatment with 1 μM 20HE, 50 ng/ml IGF-1, or vehicle for 2 h. For removal of free extra- or intracellular Ca^{2+}, media was supplemented with either 3 mM EGTA or 25 μM BAPTA-AM for 30 min prior to treatment. Western blotting was performed as described earlier.

Downstream of Akt

Differentiated C_2C_{12} myotubes were serum starved overnight and pretreated with the mTOR inhibitor, 10 μM rapamycin (EMD, Madison WI), or vehicle for 30 min before treatment with 1 μM 20HE, 50 ng/ml IGF-1, or vehicle for 4 h. Protein incorporation was calculated as described previously

Results

PI3K Inhibition

When C_2C_{12} myotubes were pretreated with 10 μM of the PI3K inhibitor, LY294002, the effect of 20HE on protein synthesis was significantly reduced (Fig. 21). The PI3K inhibitor similarly reduced IGF-1 stimulated protein synthesis.

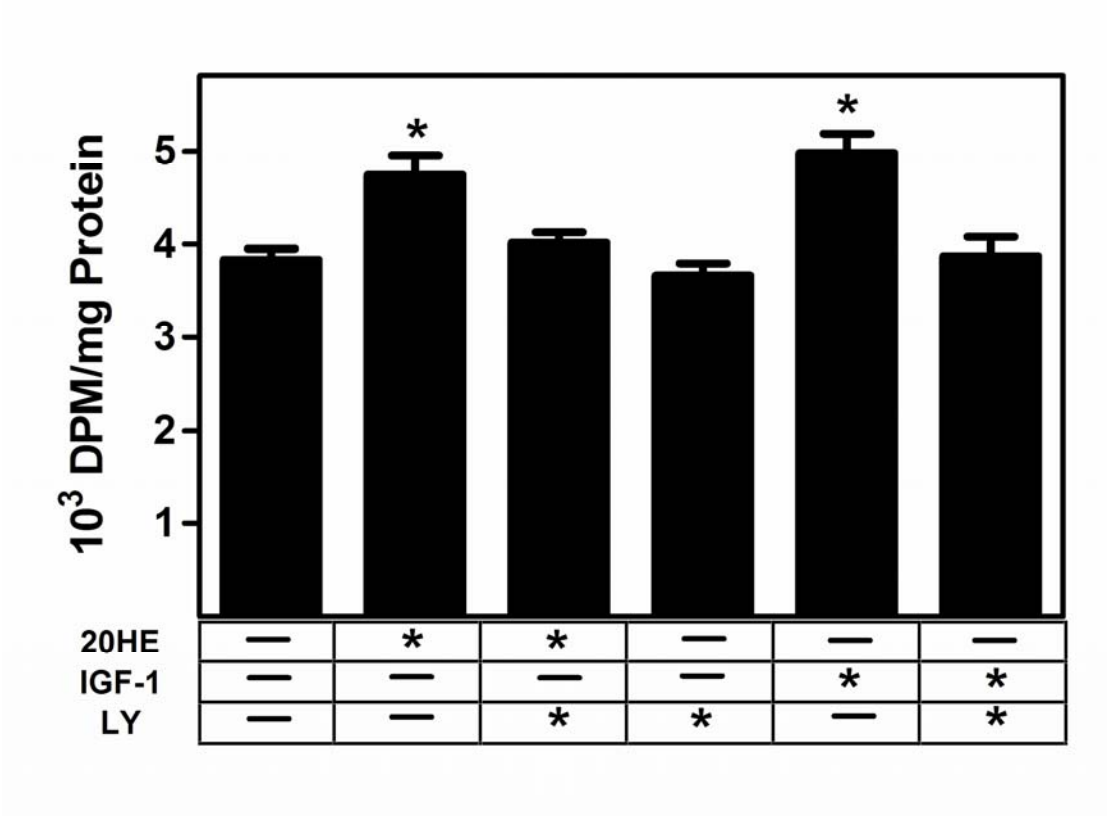


Fig. 21. [^{3}H] Leucine Incorporation in C_2C_{12} Myotubes Pretreated with PI3K Inhibitor.

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 ± S.E.M. of four experiments, each done in triplicate. * indicates $P<0.05$ compared with control (Student's t test).

Akt Activation

20HE treatment activated Akt in C_2C_{12} skeletal myotubes (Fig. 22). 100 nM 20HE significantly increased phosphorylation of Ser^{473} in Akt by more than threefold. Akt activation increased with increasing concentrations of 20HE up to almost fivefold with 10 μM. The time course of Akt activation after treatment with 1 μM 20HE was biphasic (Fig. 23A), with a small peak after 45 min, and a larger peak after 4 h (Fig. 23B). The increase in pAkt in C_2C_{12} was significant even 24 h after treatment.

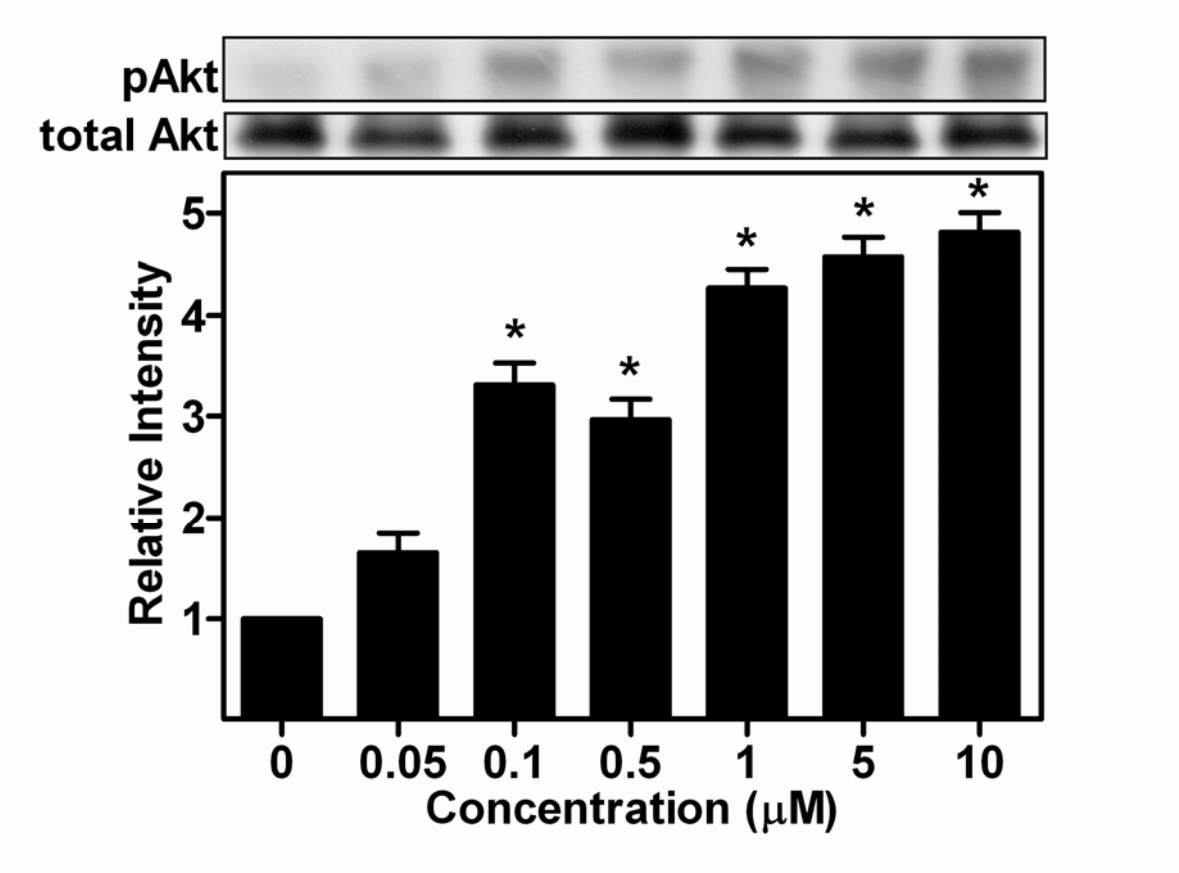


Fig. 22. Effect of Different Concentrations of 20-Hydroxyecdysone (20HE) on phosphorylation of Akt in C_2C_{12} Myotubes.

Differentiated myotubes were treated for 2 h with increasing concentrations of 20HE or vehicle. Cell lysates were subjected to Western Blotting and probed with either anti-pAkt or anti-Akt. The data shown represent the mean values of phosphorylated Akt normalized to total Akt ± S.E.M. of three experiments. * indicates $p < 0.05$ compared with control (Student's t test)

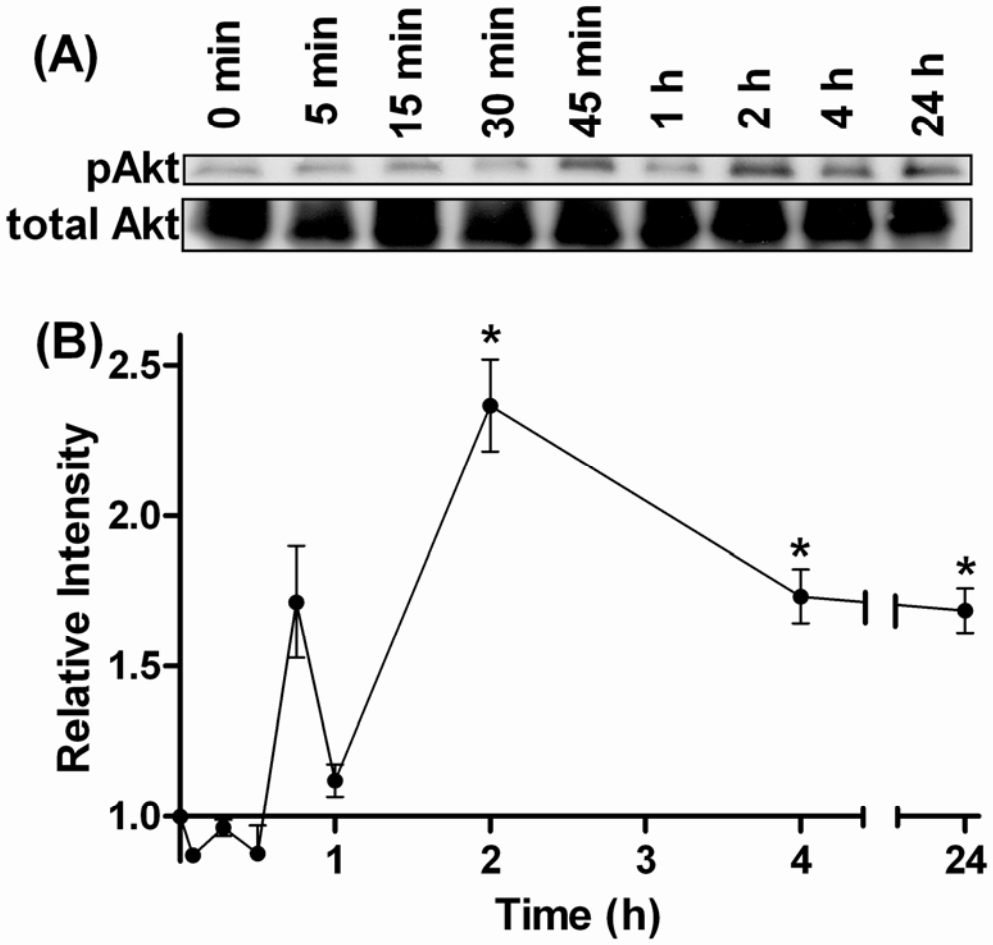


Fig. 23. Time Course of Akt phosphorylation in C_2C_{12} Myotubes Treated with 20-Hydroxyecdysone (20HE).

Differentiated myotubes were treated with 1 μM 20HE for between 5 min and 24 h. (A) Cell lysates were subjected to Western Blotting and probed with either anti-pAkt or anti-Akt. (B) The data shown represent the mean values of phosphorylated Akt normalized to total Akt ± S.E.M. of three experiments. * indicates $p < 0.05$ compared with control (Student's t test).

Upstream Inhibition of Akt

Pretreatment with either 10 μM LY or 1 μg/ml PTX for 1 h inhibited the 20HE-induced Akt activation in C_2C_{12} myotubes (Fig. 24). In contrast, activation of Akt by 50 ng/ml IGF-1 was inhibited by LY but not PTX.

Removal of available Ca^{2+} also inhibited the 20HE-induced activation of Akt. Treatment with either the extracellular chelator, 3 mM EGTA, or the intracellular chelator, 25 μM BAPTA-AM, 1 h prior to treatment reduced the 20HE-induced Akt phosphorylation (Fig. 25). Pretreatment with both EGTA and BAPTA-AM together completely abolished the 20HE-induced phosphorylation of Akt. IGF-1 induced activation of Akt was not affected by pretreatment with EGTA, BAPTA-AM, or both.

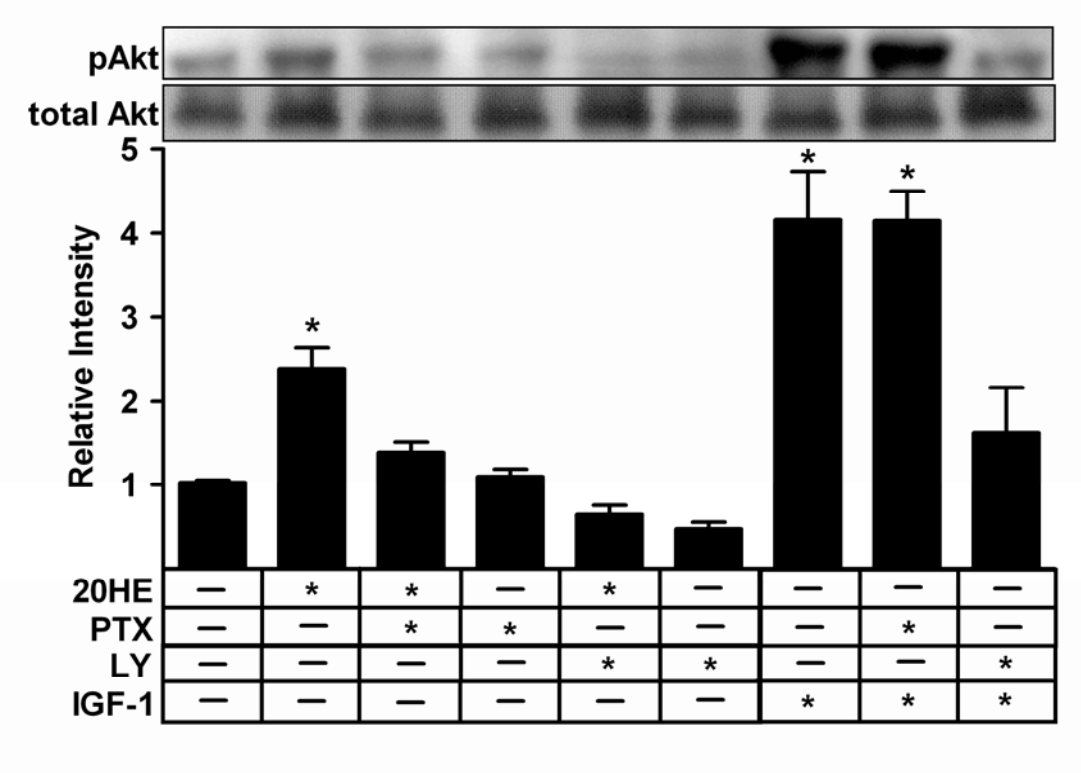


Fig. 24. G-protein and PI3K Inhibitors on Akt phophorylation in C_2C_{12} myotubes treated with 20-Hydroxyecdysone (20HE).

Differentiated myotubes were pretreated with either 1 μg/ml PTX, 10 mM LY, or vehicle 1 h prior to treatment with either 1 μM 20HE or 100 ng/ml IGF-1 for 2 h. Cell lysates were subjected to Western Blotting and probed with either anti-pAkt or anti-Akt. The data shown represent the mean values of phosphorylated Akt normalized to total Akt ± S.E.M. of three experiments. * indicates $p < 0.05$ compared with control (Student's t test).

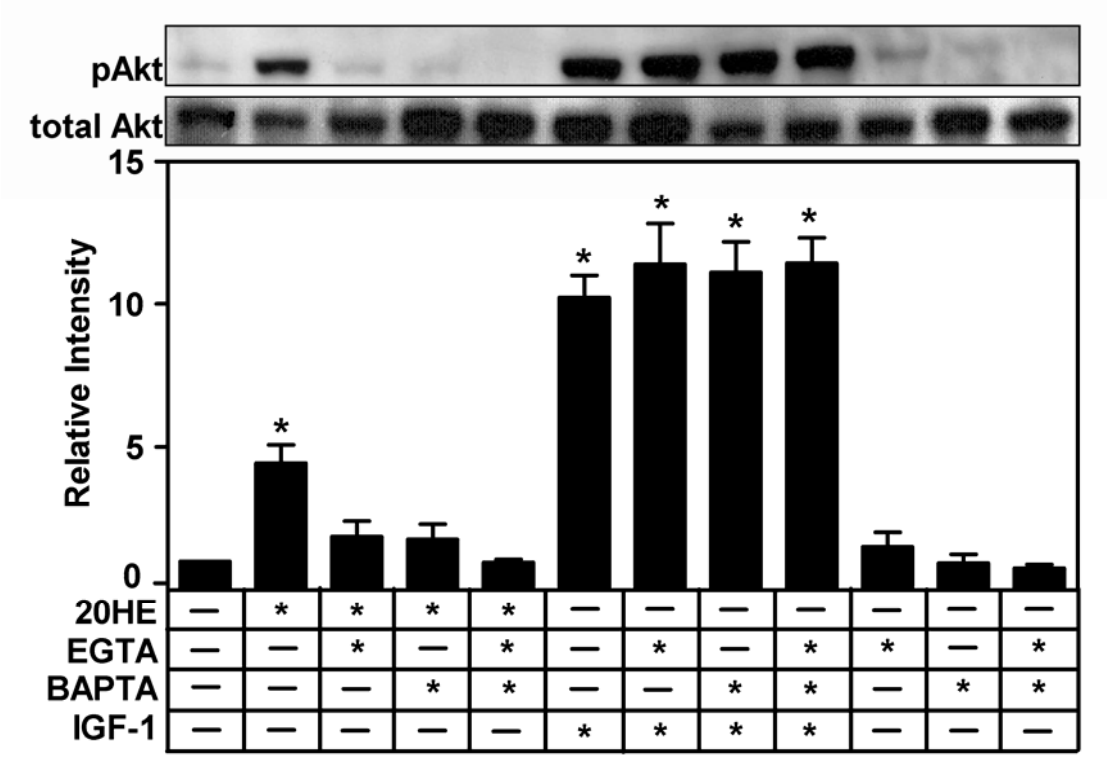
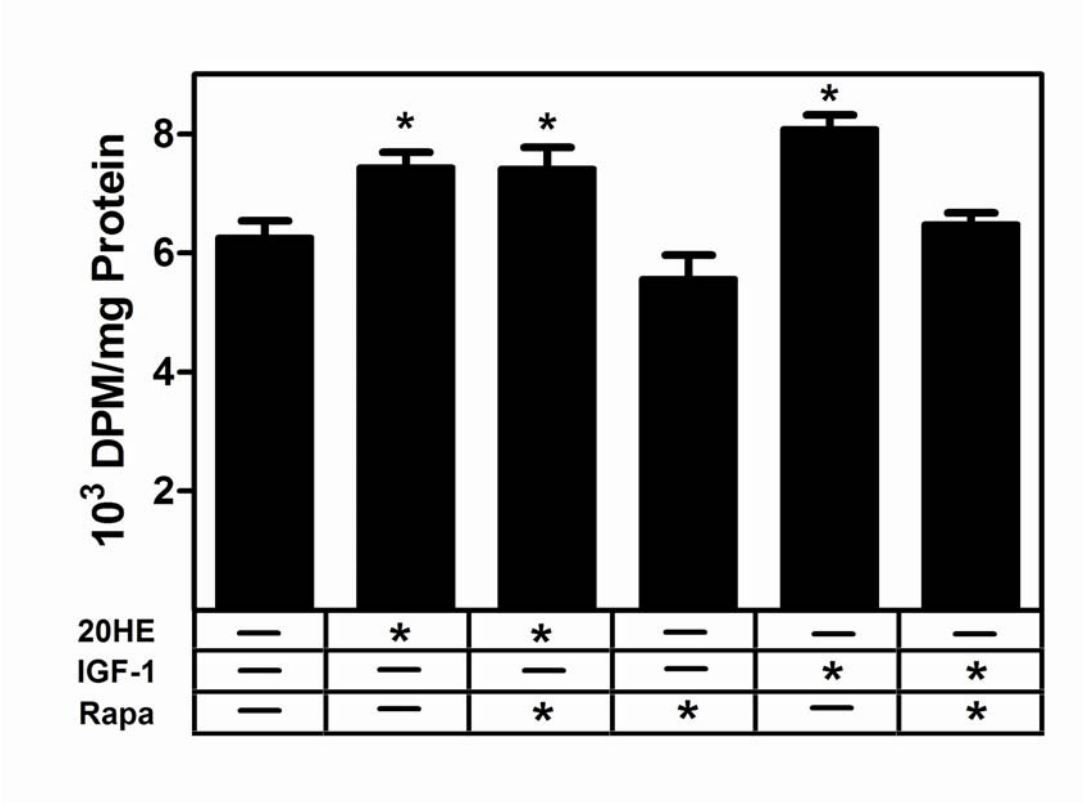


Fig. 25. Extra- and Intracellular Calcium Chelators on 20-Hydroxyedysone (20HE) induced Akt phosphorylation in C_2C_{12} myotubes.

Differentiated myotubes were pretreated with either 3 mM EGTA, 25 μM BAPTA-AM, both, or the vehicle, for 1 h prior to treatment with 1 μM 20HE or 100 ng/ml IGF-1 for 2 h. Cell lysates were subjected to Western Blotting and probed with either anti-pAkt or anti-Akt. The data shown represent the mean values of phosphorylated Akt normalized to total Akt ± S.E.M. of three experiments. * indicates $p < 0.05$ compared with control (Student's t test).

Downstream of Akt

Pretreatment with 10 μM rapamycin did not affect the 20HE stimulated increase in protein synthesis in C_2C_{12} myotubes (Fig. 26). In contrast, the IGF-1 induced increase was inhibited by rapamycin.



Fig, 26. [^{3}H] Leucine Incorporation in C_2C_{12} Myotubes Pretreated with mTOR Inhibitor.

Differentiated myotubes were pretreated with either 10 μM rapamycin 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 ± S.E.M. of four experiments, each done in triplicate. * indicates $P<0.05$ compared with control (Student's t test).

Discussion

PI3K Inhibition

The first step in elucidating Akt's potential role in the ecdysteroid induced increase in protein synthesis was the use of a specific inhibitor. LY-294002 (LY), a PI3K inhibitor, blocks one of the main signals directly upstream of Akt activation. PI3K generated phospholipids cause translocation of Akt to the plasma membrane, where it is phosphorylated by phosphoinositide dependant kinase (PDK1) (Whiteman et al. 2002). LY inhibits PI3K activation and blocks subsequent phosphorylation of Akt.

Pretreated with the PI3K inhibitor LY removed the 20HE-induced increase in protein synthesis in skeletal muscle cells (Fig. 25). The LY inhibition of the ecdysteroid-induced increase in protein synthesis suggests Akt may be involved in the effect. The PI3K/Akt pathway has been shown to increase protein synthesis in skeletal muscle (Hellyer et al. 2006), so it quite possible that Akt may mediate the anabolic effect in C_2C_{12} cells. However, although use of inhibitors is informative, it is indirect. Therefore, in order to further analyze Akt's role in the ecdysteroid effect, direct activation of Akt was studied.

Akt Activation

As previously mentioned, the PI3K pathway causes the translocation of Akt to the plasma membrane where it is phosphorylated by PDK1, and subsequently produces a phosphorylated signal cascade leading to various cellular responses, including increased protein synthesis (Rommel et al. 2001). Since phosphorylation of Akt leads to its downstream effects, activation of Akt by ecdysteroids was studied by measuring the 20HE-induced phosphorylation of Akt in skeletal muscle cells. A range of concentrations was tested, and a time course produced, in an attempt to characterize the response.

20HE activated Akt in C_2C_{12} skeletal myotubes in a dose (Fig. 22) and time dependant manner (Fig. 23). The effective 20HE concentration which produces Akt activation is comparable with the concentration required to increase protein synthesis (Fig. 9), suggesting Akt activation may be involved in protein synthesis.

Although 20HE-induced Akt phosphorylation has been described previously in mouse pro-B lymphocyte cells (Constantino et al. 2001) and RKO colon carcinoma cells (Ohme et al. 2006), this is the first example in skeletal muscle. Previous studies involved long treatment periods of 48 h, suggesting a non specific response. Our study shows a more rapid response, with activation after 45 min, although this is still a much slower response than other activators, like IGF-1, which phosphorylate AKT within 10 min. The delay in Akt activation is intriguing. It may be due to an indirect effect which only activates Akt

downstream of other signalers. In order to better understand the specific activator of Akt triggered by ecdysteroids, the potential signalers upstream in the Akt pathway were studied.

Upstream Inhibition of Akt

Akt can be activated by different upstream signals, including PI3K and G proteins. In order to elucidate the pathway leading to ecdysteroid induced Akt activation, specific inhibitors were used to block different segments of the potential pathway, beginning with PI3K, immediately upstream of Akt. Continuing upstream, PTX was utilized to study the effect of G proteins on 20HE-induced Akt activation. Finally, calcium chelators were used to look at the role of Ca^{2+} in 20HE-induced Akt activation. IGF-1, a known activator of Akt, was used for comparison.

The LY inhibition of 20HE-induced Akt activation (Fig. 24) suggests that ecdysteroids utilize the PI3K pathway to activate Akt. This is not so surprising, as PI3K is the main activator of Akt.

More surprising is the PTX inhibition of 20HE-induced Akt activation (Fig. 24). Although not the main route, it has been reported that the G-proteins can also activate the PI3K/Akt pathway (Crespo et al 1994). In yeast, Gαq interacts directly with PI3K subunits (Slessareva et al. 2006). Activated protease-activated receptor 2 (PAR-2), a GPCR, increases PI3K activity through a Gαq/Ca^{2+}-dependent pathway involving PYK2

and a Src-family kinase (Wang and DeFea 2006). This supports the hypothesis that an ecdysteroid GPCR may be responsible for 20HE-induced Akt activation and increased protein synthesis.

Ca^{2+} has been shown to be an important regulator of Akt activation (Danciua et al. 2003). Inhibiting PLC or chelating intracellular Ca^{2+} abolished the EGF induced activation of Akt in mammary carcinoma cells (Deb et al. 2004). In this study, we further support the role of Ca^{2+} in Akt activation, showing that removal of free Ca^{2+} reduces the 20HE-induced Akt phosphorylation (Fig. 25).

Although some of the key players involved in ecdysteroid-stimulated Akt activation may have potentially been identified, the link between Akt activation and increased protein synthesis was still not known.

Downstream of Akt

Downstream of Akt lie a few major pathways which regulate growth and protein synthesis, including mTOR and GSK3.

mTOR activation increases protein translation by two means. mTOR activates $p70^{s6k,}$, a positive regulator of translation, and inhibits 4E-BP1, an inhibitor of eIF-4E, a protein initiation factor (Nave et al. 1999). Through these two pathways mTOR causes increases in translation, thereby increasing protein synthesis, making mTOR a good candidate for mediating the 20HE-induced increase in protein synthesis. To test this hypothesis, the effect of a specific inhibitor of mTOR activation on the 20HE stimulated increase in protein synthesis was studied in skeletal muscle cells.

Surprisingly, mTOR inhibition did not affect the ecdysteroid anabolic activity in skeletal muscle cells (Fig. 26). Although closely linked to Akt, and an important regulator of protein synthesis, mTOR appears not to be directly involved in the ecdysteroid effect.

There are other downstream pathways of Akt which may potentially mediate the ecdysteroid effect. One candidate is Glycogen synthase kinase 3β (GSK3β). Activation of Akt causes the inactivation of GSK3β (Cross et al. 1995). GSK3β inactivation has been shown to induce hypertrophy in skeletal muscle cells (Rommel et al. 2001). Further study is needed to confirm if GSK3β is in fact involved in the ecdysteroid effect.

Proposed Pathway

To summarize, we have shown that 20HE treatment activates Akt, and that blocking Akt activation also blocks increased protein synthesis, supporting the key role of Akt in the 20HE-induced increase in protein synthesis in skeletal muscle. Although the connection between Akt and G protein signaling is not completely clear, there is evidence that Akt can be activated through GPCR pathways, specifically $G_{\alpha q}$ (Wang and Defea 2006). Removal of either extracellular or intracellular Ca^{2+} reduced the 20HE effect on protein synthesis (Fig. 20), suggesting that Ca^{2+} flux is a necessary signaler in the 20HE-induced response leading to increased protein synthesis.

However, although the induced Ca^{2+} flux is a rapid response, the 20HE increase in protein synthesis is not observed until after 2 h (Fig. 10). A possible reason for the delay may involve the downstream activation of Akt. Although many Akt activators, like IGF-1, phosphorylate Akt within 5 min, 20HE-induced activation of Akt is much slower, only appearing after 45 min and then after 2 h.

The link between Akt activation and protein synthesis also remains to be elucidated. Although mTOR is a major signaler in Akt activated protein synthesis, our findings do not support their role in ecdysteroid-induced anabolic activity (Fig. 26). Other candidates, including GS3K, should be analyzed for involvement in ecdysteroids activity.

Although the mode of action of 20HE in skeletal muscle is still largely a mystery, the current evidence suggests the existence of a 20HE-activated membrane bound GPCR, similar to the one found in insects. High affinity membrane binding sites in rat lymphocytes, erythrocytes, and hepatocytes have already been described using 20HE bound to magnetic nanoparticles (Mykhaylyk et al. 2001). This putative 20HE GPCR may activate the PLC-IP3 pathway as well as open Ca^{2+} channels, leading to G-α protein-dependant activation of PI3K/Akt and increased protein synthesis (Fig. 27). Although there is some evidence supporting this pathway, more work, including identifaction of the putative receptor, is needed to confirm the hypothesis.

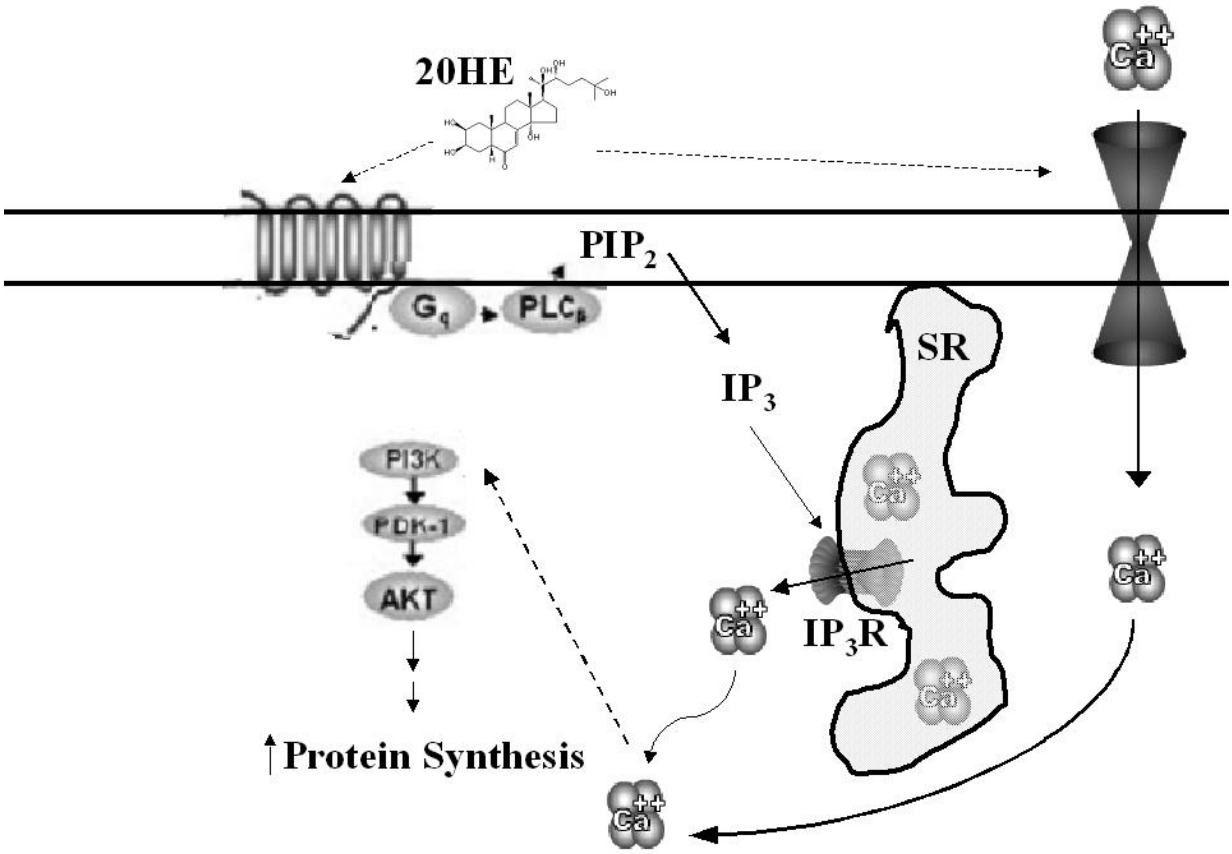


Fig. 27. Schematic representation of the proposed mode of action of 20HE in skeletal muscle.

The proposed pathway of 20HE includes activation of a putative membrane bound GPCR. This leads to PLC activation producing IP_3. IP_3 activates IP_3R in the SR releasing intracellular stores of Ca^{2+} into the cytoplasm. 20HE may also elicit the opening of extracellular Ca^{2+} channels. The total Ca^{2+} flux leads to phosphorylation of Akt which leads to increased protein synthesis. Hypothetical signaling is described with dashed arrows.

Conclusion

Plants are a rich source of therapeutic ecdysteroids. In this study we hoped to characterize two potentially beneficial ecdysteroid containing plants. Although *A. turkestanica* was cultivated, and its ecdysteroid content quantified, its therapeutic benefits over other ecdysteroid containing plants remains questionable. Extracts from *A. turkestanica* produced anabolic effects in skeletal muscle cells, but these effects were not any greater than effects produced from other ecdysteroid sources. Difficulty in cultivation combined with lack of evidence for the increased potency claims of turkesterone did not clearly support *A. turkestanica* as a superior source of ecdysteroids. Further research is needed to clearly refute or confirm prior claims of *A. turkestanica*'s increased potency, which may be a result of specific *in vivo* effects.

In contrast spinach, a major food crop, has clear potential as an ecdysteroid enriched food. Extracts from spinach, which is easily cultivated, produced anabolic activity both *in vivo* and *in vitro*. Although acute studies with concentrated spinach produced anabolic effects, further work is needed to evaluate the long term therapeutic properties of spinach enriched diets. The characterization of ecdysteroids' anabolic activity requires a better understanding of the ecdysteroid pathway.

While some of the ecdysteroid pathway has been uncovered, more remains a mystery. Since the initial discovery of anabolic activity in 1961, phytoecdysteroid research has made some progress, but there is still much more work to be done. Considering that there

has been over 40 years of research and over 30 papers published, there is still very little known.

This study focused on phytoecdysteroids' anabolic activity, only one of their many therapeutic properties. Although many of the claims need to be verified, the evidence for some of the effects continues to build. We have generated further support for ecdysteroids' anabolic activity, showing effects both *in vitro* and *in vivo*, and hopefully more research confirming its activity will add to our knowledge. Furthermore, we have begun to uncover the cellular mode of action of ecdysteroids' anabolic activity. Our work suggests the existence of an ecdysone GPCR, which may mediate ecdysteroids' anabolic activity through activation of the PLC-IP3 pathway, increased Ca^{2+} flux, and downstream activation of the PI3K/Akt pathway. This chain of events may eventually lead to increased protein synthesis

It is tempting to suggest that many of the pleiotropic effects of ecdysteroids are mediated through the same mechanism, the putative mammalian ecdysone GPCR and signaling pathway implicated with the anabolic effects. This suggestion seems more plausible considering the important signalling role Akt plays in many cellular processes, including cell growth, survival, and metabolism. However it is also possible that the wide array of effects may be the result of completely different mechanisms, all produced by one class of compounds. Either way, there is much more to discover.

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