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The Effects of 20-hydroxyecdysone in *Drosophila* Kc Cells on the Ecdysteroidosis

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Abstract : Drosophila Kc cells are ecdysone-responsive : hormone treatment leads rapidly to increased synthesis of several ecdysone-inducible polypeptides (EIPs) and to commitment to eventual proloferative arrest. Later the treated cells undergo morphological transformation, cease to proliferate and to grow. These responses have proven useful as models for studying ecdysone action and environmental endocrine disrupting actions. In this study, we used 20-HE to check out the Kc cells properties to the ecdysone and this properties will be applied to the environmental chemicals to find out the endocrine disrupting action in ecosystem. The cell counts of cultures harvested after 3 days' growth in the presence of 20-hydroxyecdysone. In Kc cell cultures, there were statistically significant different from control cells at 20HE 10^7-10^5. The morphological effects of all the hormones were similar, differing only in the dose level at which they were initiated.

Keywords : 20-HE, *Drosophila*, Kc cell, ecdysteroidosis

Introduction

In 1979, the first symposium on estrogens in the environment was held, "... to determine what an estrogen is and how it works, and what effect estrogenic substances might have on human health ... since many chemicals with diverse chemical structures, some of which are environmental contaminants, have been endowed with 'estrogenic' properties". This seemed a fairly straightforward goal at the time, however, 20 years later the attainment of that goal remains elusive[1].

One of the difficulties in the field of environmental endocrine research is semantic. What is an environmental estrogen? According to The American Heritage Dictionary of the English Language, ed 4 (2000), an estrogen is "any of several steroid hormones produced chiefly by the ovaries and resposible for promoting estrus and the development and maintenance of female secondary sex characteristics". The word, which first appeared in 1927, is comprised of the following components, "est(us)" (again, from the same dictionary, "estrus [is] the periodic state of sexual excitement in the female of most mammals, excluding humans, that immediately precedes ovulation and during which the female is most receptive to mating; heat") plus "o" (the combining form) and "-gen" ("producer; one that is produced."). Thus, estrogens is a word of recent origin with a functional definition, i.e., something that produces a period of heat in a female a signal[2].

To "induce estrus" is a behavioral and physiological process involving many organ systems and a commitment of time. As scientific knowledge of estrogen action has evolved, so has the functional definition. Over time, an estrogen has been defined in the scientific literature as a chemical capable of inducing vaginal cornification in an immature mouse; a chemical that increases uterine weight in an ovariectomized mouse; chemicals associated with proliferation of the uterine epithelium in castrate female mice; chemicals capable of stimulating an increased number of cells from estrogen target organs grown in tissue culture; chemicals that form ligands for the ER and displace radiolabelled estradiol from its binding; chemicals that regulate the expression of estrogen

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target genes; and, chemicals that transactivate ER-driven reporter genes in cells in culture[1,2].

The signaling molecule, estradiol, regulates reproduction in many invertebrates and all vertebrates. Of invertebrates, crustacean, mollusks (snail), and echinoderms (starfish) are reported to produce estradiol. The phylogenetic distribution of estradiol production in the animal kingdom suggests that estrogenically active chemicals may be evolutionarily conserved signals. It also suggests the possibility that all animals are sensitive to estrogens, whether endogenous or envionmental. In addition to the ligand signal, it appears that the signal recognition system is also widely distributed phylogenetically. ERs have been found in many vertebrate species. In those species in which it has been studied-including mammals[3].

Insect growth is marked by a series of discontinuous events called molts. Molting is a complicated process involving numerous biological systems and initiated by the steroid hormone ecdysone. Much of the molting process is not understood but disruption can be lethal and nonsteroidal ecdysteroid agonists are being developed for use as insecticides.

The use of Drosophila cell lines as research tools for the study of hormone action has become well established in recent years. Various lines have been used to investigate the action of 20-hydroxyecdysone (20HE) in some depth. Molting and metamorphosis in Drosophila are controlled by the hormones 20-hydroxyecdysone (20HE) and juvenile hormone. A number of genes that are directly induced by 20HE have been characterized. Drosophila Kc tissue cells offer several advantages for studying 20HE regulation of insect genes, since a homogenous population of cells responds rapidly and specifically to 20HE[2,3].

In this study, we used 20-HE to check out the Kc cells properties to the ecdysone and this properties will be applied to the environmental chemicals to find out the endocrine disrupting action in ecosystem.

Materials and Methods

Cell Cultures

Kc cells obtained from Dr. Lucy Cherbas (Indiana State University) were used for cell differentiation experiments. Cells were maintatined in HyQ CCM3 (Hyclone Co.) cell culture medium (Sigma). On day 0, 1×10^6 cells were placed in 98 well culture dishes in 200 μl of medium. Compounds were added in either 1 μl ethanol, and the cells were incubated at 25°C. Total cell number and the number of cells with ecdysone induced processes were determined 24 hours later.

20-hydroxyecdysone (20-HE) was purchased Sigma and stored and diluted in refrigerated temperature. The resuspended cells were centrifuged at 2500 rpm for 10 min. The cell pellet was resuspended in fresh medium and used for subcultures. Stock solutions of 20HE were made in reagent grade ethylalcohol and the concentration of ethanol was maintained at 0.1% in all the treatments as well as the controls. Serial dilutions of 20HE flowable formulation were prepared in the respective tissue culture media.

The morphological response was assayed as desscribed by L. Cherbas *et al.*[4].

Cell Countings

Cell growth and proliferations were analyzed by chemiluminescence methods (Cytolite). All Cytolite assays were carried out in white, 96 well View Plates (Packacrd Instrument Company), and the luminescence measured on a TopCount Microplate Scintillation and Luminescence counter (Packard Instrument Company) using 0.6 second count time in SPC (single photon counting) mode at 25, follwing a five minute precount delay. Kc cells were pre-calibrated to determine the optimum volume of Cytolite amplifier solution per well.

The cell growth and proliferations were evaluated with cytolite using chemiluminescent. The calibration procedure were as follows.

Kc cell were cultured in medium in 98 well culture plate. The control cells concentration were determined by an hemocytometer. The stock suspensions of 10^5 cell/ml were prepared in the same medium. Duplicate sereal dilutions (1:2) were prepared cell line starting from 10^5 cell/ml in a total volume of 200 μl in microplate. 25 μl/ well of activator solution was added. 100 μl/well of amplifier solution was added. Luminescence was measured on a Packard for 0.3 sec./well in SPC

(single photon counting) mode with temperature set at 25°C, following a five minute counting delay.

Results and Discussions

It was shown that Drosophila Kc cells respond to treatment with ecdysone by extending long processes and subsequently aggregating. This response has been used to characterize the nonsteroidal ecdysteroid agonist. Drosophila Kc cells treated with the ethanol vehicle alone are not significantly different from untreated cells. These cells have a round morphology with very few elaborating processes. Treatment for 24 hours with 20-hydroxyecdysone caused to flatten and to develop the long processes of cells described by others[4]. This morphology is also exhibited by cells treated with Ponasterone A, as would be expected for an ecdysteroid agonist[4,5].

Insects undergo periodic molting of their exoskeleton to accommodate growth during development and metamorphosis, which results not only in an increase in size but also in a change of form. The process of molting and metamorphosis are under endocrine control and several insect hormones including ecdysteroids and juvenile hormones play important regulatory roles in initiating and coordinating these processes. Therefore, it is not surprising that researchers in both academic and industrial laboratories have searched for new molecules that would serve as insecticidal agonists/antagonists of the two hormones. Although success in discovering insecticidal compounds with JH (juvenile hormone) activity came much earlier, it is only recently that agonists of 20 HE with insecticidal activity have been discovered[5].

Ecdysone is the central element of a steroid hormonal system operating in arthropods. Such a hormonal system is exemplified in the following general scheme, which is assumed to be valid for all steroid hormones including that of vertebrates. The hormonal system can be divided into several parts. One part is involved in the formation and secretion of the signal, (i.e. the hormones) ; another part is involved in distribution, conversion and inactivation (metabolism) ; and a third part is active in the perception of and the reaction to the signal. The role of the former parts of the hormonal system is the control of the steroid hormone titer. For that purpose they include several components : cells which synthesize and secrete the hormone immediately into the blood (gland cells) : possibly plasma proteins (carriers) able to bind the sterod hormones ; cells which take up the hormone, metabolize it and secrete the metabolites back into the circulatory system : and finally cells which are able to remove the hormone and its metabolites from the blood by excretion. The latter part of the hormonal system is represented only by the cells of the target organs, which take up the hormone from the circulatory system and react to the steroid with a specific respose. These parts of the hormonal system are linked by the hormone in the blood. Only the free steroid which is not bound to plasma proteins may be assumed to be the active hormone that links the different parts of the hormonal system[5,6].

The cell counts of cultures harvested after 3 days' growth in the presence of 20-hydroxyecdysone were shown in Table 1. In Kc cell cultures, there were statistically significant different from control cells at 20HE 10^{-7}-10^{-5}.

Table 1. The effects of 20-hydroxyecdysone on Kc cell growth (cells/ml)

group/hours	0	24	60
contol	4.3×10^6 (100)[a]	7.8×10^6 (181)	2.1×10^7 (488)
20-HE 10^{-7}	2.8×10^6 (100)	3.3×10^6 (118)	4.4×10^6 (157)
20-HE 10^{-6}	4.4×10^6 (100)	5.6×10^6 (127)	5.8×10^6 (132)
20-HE 10^{-5}	2.8×10^6 (100)	2.4×10^6 (86)	4.3×10^6 (154)

[a]% of cells have process more 3 times longer than that of the diameter.

Table 2. The effects of 20-hydroxyecdysone on Kc cell proliferations

group/hours	0	24	60
contol	2.5[a] (100)[b]	2.6 (104)	1.4 (56)
20-HE 10^{-7}	2.6 (100)	10.4 (400)	48.9 (1881)
20-HE 10^{-6}	2.3 (100)	17.7 (770)	25.2 (1096)
20-HE 10^{-5}	2.2 (100)	9.1 (414)	32.5 (1477)

[a]% of cells have process more 3 times longer than that of the diameter.

[b]% of elongated cells compared to that of the time 0.

In most higher dose levels, especially in 20HE 10^5, the cell count indicated fewer cells than the seeding level (3×10^6) after 24 hours, due to cell death and lysis.

The morphological effects of all the hormones were similar, as described above, differing only in the dose level at which they were initiated. The first markedly affected treatment for each hormone is shown in Table 2.

Ecdysteroid hormones play a crucial role in the control of growth and differentiation in insects. Several cultured Drosophila cell lines appear to be quite sensitive to physiological concentrations of 20HE and display within a few hours or days of treatment a characteristic array of morphological and metabolic modifications. This experimental model, although it is more delicate to handle than the heatshock system, is perhaps even more productive, since it combines the modulations of the best known genome of all higher organsims with the mechanism of action of a steroid hormone[4,6].

It has been reported that the ecdysone agonists bind to the same receptors as do 20HE and other ecdysteroids. The similarity of effects of the different hormone, in terms of morphology, reduction in cell numbers and other markers. Kc line cells are sensitive target for ecdysteroid hormones.

Arthropods are ecologically very important often comprising the basis of terrestrial, aquatic, and marine food chains. Estuaries are especially vulnerable to waste products from human activity which often have aheavy impact on the crustacean community. Sublethal effects that have been found in crustaceans after PAH exposure include changes in molting and growth, survival, behavior, sex ratio and biochemistry[7].

The Drosophila melanogaster Kc cell line is an appropriate medel by which to better understand the mechanism of steroid hormone action at the molecular level. These cells are sensitive to 20-hydroxyecdysone, the steroid moulting hormone. Several modifications are induced after hormone addition to cultured cells including morphology, enzyme activities, and gene expression. One of our interests in using this model is to experiment on hormonal regulations, under controlled in vitro conditions. Important morphological changes of Kc cells induced by 20HE (emission of long pseudopodia, motility) suggest participation of the cytoskeleton and particularly of its two major components : actin and tubulin. Tubulin is an heterodimer composed of one α and one β subunit and represents the main element of microtubules in eukariotic cells. In multicellular organisms, tubulins are encoded by amutigene family (four α and four β tubulin genes in Drosophila). The four β tubulin genes are located at 56D, 60C, 85D, and 97F[5-7].

Ecdysone is a late-comer within the steroid hormone family. Because it was isolated until 1954 and was only structurally identified in 1965, research on it lags about a quarter of a century behind that on the other classical steroid hormones. With respect to the chemical structures there are some characteristic features in which ecdysone and the ecdysteroids (compounds structurally related to ecdysone) differ from the other steroid hormones. These features are :

(a) the entire skeleton of cholesterol with 27 carbon atoms (or in a few cases with 28 carbon atoms), (b) an α,β-unsaturated keto-function located in ring-B, (c) an ususual bend in the steroid nucleus between rings A and B caused by the hydrogen at C-5 in th β-position and, (d) a number of hydroxyl groups in the steroid nucleus as well as in the side-chain. Due to these hydroxyls the ecdysteroids are the most complex and the most polar steroid hormones found in animals[5,9].

Ecdysteroids are the most wide-spread steroid hormones : they are found in more than 90% of the species in the animal kingdom, i.e. in arthropods which amount to about one million species. Increasing evidence suggests that further phyla of the lower animals, namely Annelida, Nematoda and Mollusca, might also use ecdysteroids to control certain vital processes in their life cycle. Ecdysteroids are thus physiologically old steroid hormones when compared with the steroid hormones of the 45,000 species of vertebrates[5,6,8].

Ecdysteroids control physiological processes involved in growth, differentiation and morphogenesis in arthropods. Three developmental periods in their life cycle have been shown to be under the control of ecdysteroids : embryonic developement ; post-embryonic development through several larval

stages (including a pupal stage in holometabolous insects) ; and the period of reproduction. In insect larvae ecdysteroids control the events which finally result in a moult. For this reason ecdysteroids are also called "moulting hormones". This is a broad term which refers to many compound that react physiologically like ecdysone. The chemical identity of these molecules is still a matter of discussion. The term "ecdysone" refers to a defined chemical compound and "ecdysteroids" as a generic term refers to a family of compounds with a common structure[9,10].

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