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ORIGINAL ARTICLE

Effect of concomitant progesterone administration or the effect of removal of estrogen capsule on changes caused by long-term estrogen treatment in pituitary VIP immunoreactivities

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Abstract In the anterior pituitary besides the classical tropic hormones, peptides of a small molecular weight are also synthesized. One of them is the vasoactive intestinal polypeptide (VIP). VIP immunoreactivity is readily detected in human and monkey pituitaries; however, in the rat VIP immunoreactive cells were observed in about 50% of intact rats. In estrogen treated rats VIP immunoreactive cells were observed in the anterior pituitary of all animals. In this work, we have examined the effect of long-term sexual steroid treatments on the VIP immunoreactivity of the anterior pituitary using diethylstilbestrol (DES) or progesterone (P) filled capsules. The effectiveness of steroid treatments was tested by the measurement of plasma prolactin (PRL) level and by the appearance of prolactinoma. DES enhanced the plasma PRL level and 5 months later it induced prolactinomas, the concomitant P treatment prevented both the elevation of plasma PRL level and the formation of prolactinomas. These results indicated that there was enough steroid in the capsules. There was a positive correlation between the duration of DES influence and the number of VIP immunoreactive cells. Two months after the implantation of DES there was a considerable number of VIP cells in the anterior pituitary, and 5 months after implantation the number of VIP cells was greatly increased so as to form a VIP-oma. Concomitant implantation of P prevented the formation of VIP-oma. Two months after the implantation, the DES capsule was removed. Already 2 months after removal the number of VIP cells approximated to the control level. It has been concluded that P can prevent the undesired effect of DES not only on the PRL, but on the VIP immunoreactivity as well.

Keywords Anterior pituitary · Immunohistochemistry · Prolactin · Radioimmunoassay

Abbreviations

DES	Diethylstilbestrol
E	Estrogen
E2	Estradiol
ECAP	Empty capsule
ER	Estrogen receptor
FSCs	Folliculostellate cells
FSH	Follicle stimulating hormone
ir	Immunoreactive
LH	Luteinizing hormone
P	Progesterone
PR	Progesterone receptor
PRL	Prolactin
VIP	Vasoactive intestinal polypeptide

Introduction

The pituitary tropic hormone secretion is regulated by the hypothalamic releasing and inhibiting hormones and by the peripheral sexual steroids, estrogen (E), and progesterone (P), via a feed-back mechanism. It was demonstrated two decades ago that diethylstilbestrol (DES) induced the appearance of prolactin (PRL) secreting tumors in the pituitary gland [1].

Estrogen and progesterone receptors (ERs and PRs) were identified in various pituitary hormone secreting cells

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and in various hypothalamic and extrahypothalamic structures. Both ER and PR have two isoforms. ERα was identified by Koike and his co-workers [2], ERβ was cloned by Kuiper and his co-workers [3]. ERα is localized in the largest number in lactotropes, and in decreasing number in somatotropes, thyrotropes, and gonadotropes. The number of ERβ was much lower and it was identified in somatotropes, lactotropes, and gonadotropes [4]. ERs are present in the nucleus, rough endoplasmic reticulum, secretory granules, and in the free cytosol. The intracellular localization of these receptors is dependent on the stage of estrous cycle. In another work ERβ was shown in follicle stimulating hormone (FSH) secreting cells [5]. The two isoforms of PR are named A and B which were demonstrated in the anterior lobe with the use of RT-PCR technique [6] and immunohistochemistry [7]. The predominant form is the A-isoform (PRA). In rat PR immunostaining was seen in the nuclei of gonadotropes. ERs and PRs were also throughly mapped in the central nervous system including hypothalamic structures [8–11].

In the anterior pituitary not only classical tropic hormones but many other molecules are synthesized including neurotransmitters, neuropeptides, and growth factors, among them vasoactive intestinal polypeptide (VIP) [12]. In intact rats the number of VIP cells is very limited. In other species, such as monkey and human, VIP immunoreactive (ir) cells are commonly present [13]. It was also found that VIP colocalized with all classical hormone immunoreactivities in human pituitaries [14]. However, in rat pituitaries colocalization was only found between VIP and PRL immunoreactivities. In our previous work, it was also observed that E enhanced the number of VIP cells [15]. Hemolytic plaque assay has shown that VIP has a stimulatory role on the PRL release and this effect is carried out in an autocrine manner [16].

In clinical practice sexual steroids are very frequently given to patients. For women these steroids are given as contraceptive treatment. It is well known that administration of E or the combination of E and P prevents the luteinizing hormone (LH) surge and the ovulation. Hormone contraception was suggested about 70 years ago [17–19]. Sexual steroids are also applied to synchronize the ovarian cyclicity, to maintain the endometrium during pregnancy [20, 21]. E is even recently used as replacement therapy after surgical removal of ovaries or in menopause as well [22–24]. In men E is used to treat prostatic cancer having ERs [25].

On the basis of the data available in the literature we hypothesized that P could prevent the undesired effect of E on the enhanced VIP secretion in the anterior pituitary, and after the removal of E influence (removal of E containing capsule) the changed VIP immunoreactivity could be normalized. In this work, we investigated the effect of a long-term E treatment on the VIP immunoreactivity and how P implanted with E influenced the effect of E on the VIP immunoreactivity in the anterior pituitary. It was not investigated beforehand either in humans and rats but it is well known that there is an interaction between VIP and PRL secretion. We have also examined whether removal of E capsule 2 months after implantation could reverse the E induced changes one or 2 months following the removal. The effectiveness of steroid treatments was tested by the measurement of the weight of anterior pituitaries and the plasma PRL level and by the appearance of prolactinoma in the anterior lobe. For E treatment we have applied DES which is commonly used for animal experiments.

Results

Effect of treatments on the weight of anterior pituitaries

DES treatment enhanced the weight of anterior pituitaries. In the case of 5 month-survival it was much higher than in the case of 2 month-survival. The effect of DES was significantly attenuated by a concomitant P treatment. Implantation of P alone or an empty capsule (ECAP) did not influence the weight of the anterior lobes. After removal of DES capsule the weight of the anterior pituitaries decreased to the aged-matched control level. Figure 1 shows the weight of anterior lobes in the various groups.

Effect of treatments on VIP immunoreactivity in the anterior pituitaries

The occurrence of VIP ir cells is not consistent in control rats. If they are present, their number is very limited (Fig. 2a). DES implantation induced and enhanced the number of VIP ir cells. Their number was considerably higher 2 months after implantation (Fig. 2b) and their numbers were extremely enhanced in the case of 5 month-survival. In the pituitary of these animals VIP-omas (VIP cells arranged in groups resembling adenomas) were formed (Fig. 2c). Implantation of P with DES prevented the formation of VIP-omas; however, VIP cells were always observed in the animals of this group, but their number was low (Fig. 2d). Implantation of P alone or ECAP did not influence the VIP immunostaining (not shown). When the DES capsule was removed after 2 months the number of VIP ir cells started to decrease in a month after removal (Fig. 2e), but remained a little higher than in intact rats even 2 months after removal (Fig. 2f).

Counting all VIP ir cells in three mid-level horizontal sections of three anterior pituitaries of the various experimental groups supported our morphological observations (Fig. 3).

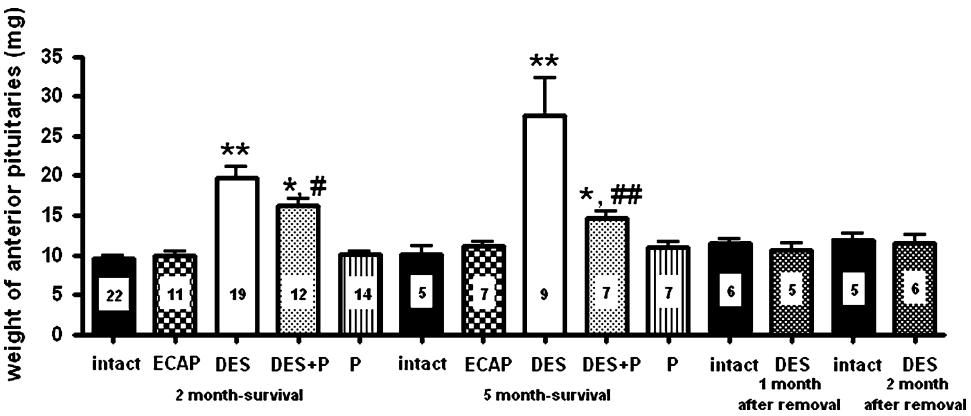

Fig. 1 Diagrams showing the weight of anterior pituitaries in various experimental groups. The implantation of DES capsule significantly enhanced the weight of anterior pituitaries compared to age-matched controls. The elevation was more pronounced in 5 months than 2 months later. Concomitant implantation of P containing capsule attenuated the weight gain in the case of both 2 and 5 month-survival. ECAP and P alone did not influence the weight of anterior pituitaries. The removal of DES capsule 2 months later reversed the elevation of the weight of the anterior pituitaries which returned to the age-matched control levels. The *numbers* in the columns indicate the number of animals. * $P < 0.01$ intact versus DES + P; ** $P < 0.001$ intact versus DES treatment; # $P < 0.05$ DES versus DES + P (2 month-survival)

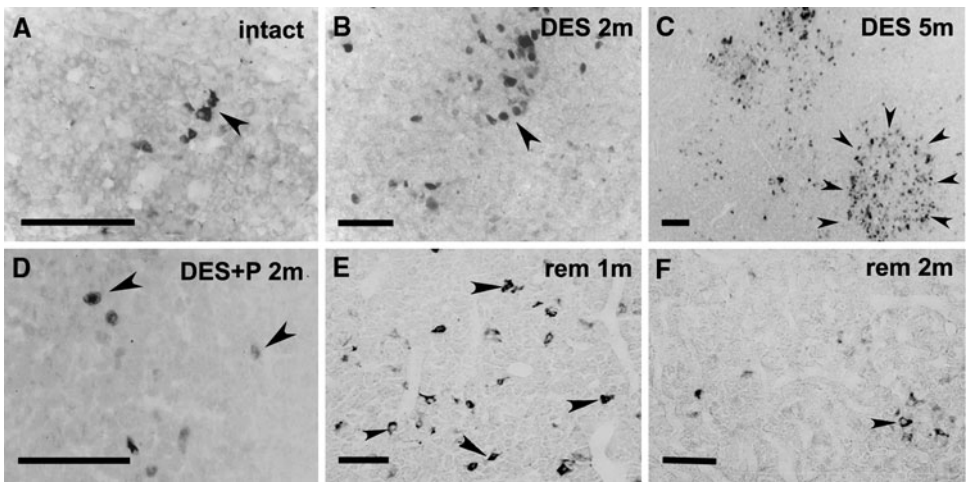

Fig. 2 Microphotographs showing VIP immunoreactivity in the anterior pituitary of the animals of various experimental groups. ABC technique. Antigen–antibody complex was visualized by nickel intensified DAB reaction. **a** In intact rats the number of VIP cells are limited. **b** Two-month DES influence enhanced the number of VIP immunoreactive cells. They were arranged in groups. **c** Five-month DES influence extremely enhanced the number of VIP cells. They formed well defined rounded or ovoid groups resembling VIP-oma. **d** P implantation concomitant with DES prevented the enhancement of VIP immunoreactive cells. The number of VIP cells was similar to that of intact rats. **e** and **f** Removal of DES capsule 2 months after its implantation gradually restored the histological image of VIP immunoreactivity in the anterior pituitary. Two months after removal the number of VIP cells was similar to that of intact rats. *Arrowheads* show VIP immunoreactive cells. *DES 2m* two months after DES capsule implantation, *DES 5m* five months after DES capsule implantation, *DES+P 2m* two months after DES + P capsule implantation, *rem 1m* one month after the removal of DES capsule, *rem 2m* two months after the removal of DES capsule. Scale 100 μm

Effect of treatments on PRL immunoreactivity in the anterior pituitary

In intact male rats the PRL cells are small and cup-shaped (Fig. 4a). DES enhanced the number and the size of PRL cells. In these rats the cells were large, ovoid, or rounded, and their diameter was nearly double than that of intact rats already after 2 month-steroid influence (Fig. 4b). P alone did not influence the appearance of PRL cells. The concomitant DES + P treatment after 2 month-survival moderately enhanced the size of the cells compared to control rats and they lost their cup-shape (Fig. 4c). Five months after implantation of DES prolactinomas developed. Figure 4d shows a well developed prolactinoma. P prevented

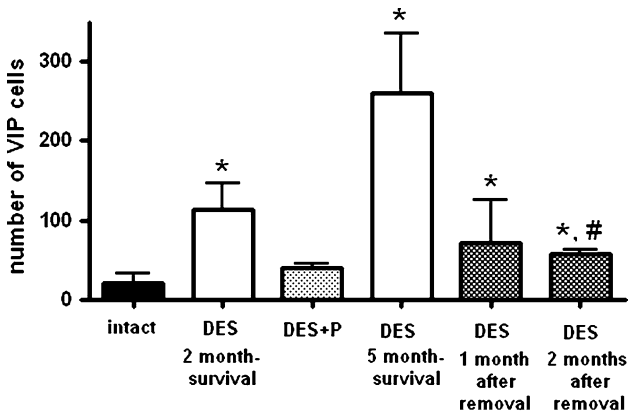

Fig. 3 Quantitative analysis of the number of VIP immunoreactive (ir) cells in the anterior pituitaries of various experimental groups. VIP cells were counted in three mid-level horizontal sections of the anterior pituitary gland of each animal. Three PFA-perfused male rats were included in the various experimental groups. DES treatment significantly enhanced the number of VIP ir cells. This elevation was more pronounced in the case of 5 month-survival than in 2 month-survival. When P was implanted with DES the number of VIP ir cells was near the control level. Removal of DES significantly decreased the number of VIP ir cells; however, their number remained a little above the control level. * $P < 0.05$ intact versus DES and DES (1 month after removal, 2 months after removal); # $P < 0.05$ DES (2 months after removal) versus DES (2 month-survival)

the prolactinoma-forming effect of DES. The immunostaining was similar to control rats. P alone and ECAP did not affect the PRL immunostaining (not shown).

Effect of treatments on the plasma PRL levels

As it was expected DES enhanced the basal plasma PRL levels in the animals. Two months after implantation P blunted the effect of DES and 5 months after implantation P completely reversed the effect of DES. P alone and implantation of ECAP had no effect on the PRL levels. Removal of DES capsule restored the basal plasma PRL levels compared to those of their own age-matched controls (Fig. 5).

Discussion

PRL immunohistochemistry in the anterior pituitary and plasma PRL levels clearly showed that steroid treatments were effective in our animals. Two months after implantation, DES induced hypertrophy of PRL cells, in the case of 5 month-survival prolactinomas developed indicating that there was enough DES in the capsule to continuously influence the hypertrophic process of the PRL cells. It is also true for P, that is there was enough hormone in the capsules even for 5 months, because in the animals having both DES and P capsules the PRL level remained low, it did not differ significantly from the control level.

As we supposed P significantly attenuated the undesired effect of DES on the enhancement of the number of VIP ir cells, and the effect of DES was reversible because after the removal of the DES influence the number of VIP ir cells decreased. These two statements are new and not published beforehand.

What may be the role of the enhanced VIP cell number upon E treatment? The enhanced VIP synthesis in the anterior pituitary may contribute to the enhanced PRL synthesis and release. It was demonstrated three decades ago that E enhanced the VIP content of the anterior pituitary measured by radioimmunoassay (RIA) [26]. Antiserum against VIP has been shown to inhibit pituitary PRL production as measured by both RIA of PRL in the media [27] and by counts of PRL ir cells in a pituitary cell culture [28]. Hemolytic plaque assay has shown that VIP stimulates the PRL release in an autocrine manner [16]. Our previous results, that demonstrated colocalization between VIP and PRL immunoreactivities in E-treated rats at light and electron microscopic levels [15, 29], also support the hypothesis of the autocrine mechanism.

A relationship between VIP and the dopaminergic system has been suggested by the finding that dopamine agonist bromocriptine reduces the pituitary VIP content in E-treated rats [30]. Moreover, an in vitro study has been performed [31] showing that DA reduces the release and transcript expression of VIP in pituitary cell cultures. The same research group 7 years later carried out a more complex experiment. In Fischer 344 rats DES was implanted under the skin. Twenty-five days later, the rats were treated with three different doses of a VIP receptor antagonist or the vehicle for 5 days. DES treatment resulted in a marked increase in serum PRL, pituitary PRL content, PRL mRNA expression, pituitary weight, and pituitary proliferating cell nuclear antigen. DES treatment also increased pituitary VIP content and VIP mRNA levels, but not in the hypothalamus and cerebral cortex. Simultaneously, DES treatment decreased the pituitary TGF-β1 content and increased the pituitary content of vascular endothelial growth factor. VIP receptor antagonist partially reverted the effect of DES on serum PRL and pituitary PRL, proliferating cell nuclear antigen, TGF-β1, and vascular endothelial growth factor contents, as well as on pituitary weight, in a dose-dependent manner. These data suggest that pituitary VIP mediates the effect of E on lactotrope hyperplasia, pituitary TGF-β1, and angiogenesis [32]. It has been also shown that E in vitro enhances and bromocriptine reduces not only expression and release of VIP in anterior pituitary cell culture, but the endothelial growth factor as well [33].

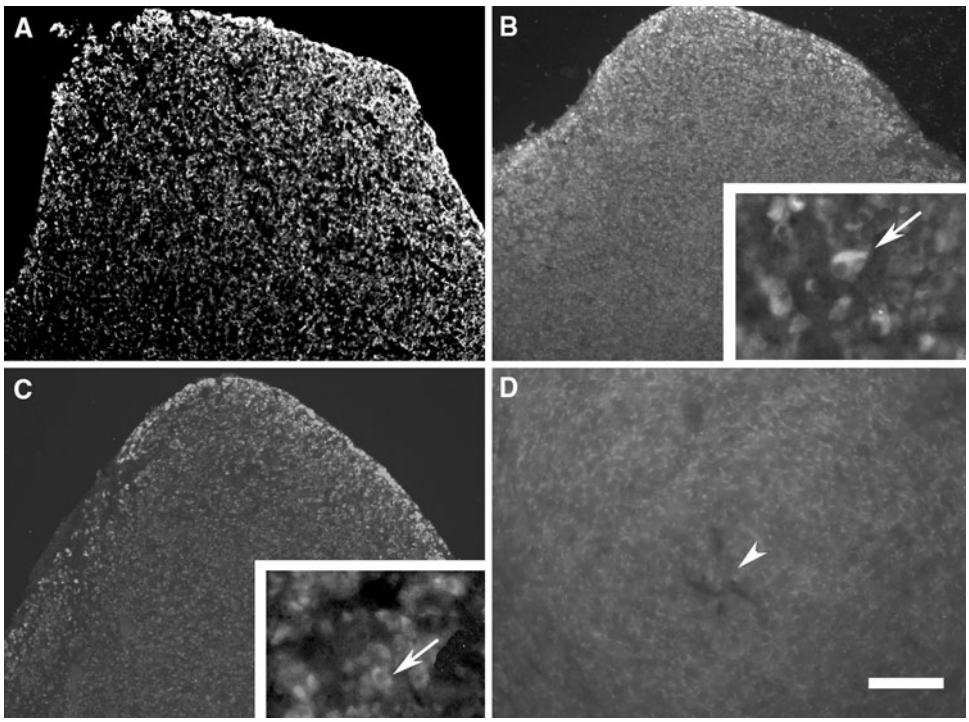

Fig. 4 Microphotographs showing PRL immunoreactivity in the anterior pituitary of animals of various experimental groups. Indirect immunofluorescence technique. **a** In intact rats the PRL cells are small and cup-shaped and evenly distributed in the anterior pituitary. **b** In DES implanted animals with 2 month-survival the PRL cells are rounded, hypertrophised and they are closely packed in the anterior lobe. *Arrow* in the insert shows large hypertrophised cell. **c** When P was implanted together with DES it moderated the rate of hypertrophy. It is indicated by the smaller size of PRL cells in the insert compared to that of the DES animal (*arrow*). **d** The size of pituitary is extremely enhanced in the animals treated with DES for 5 months. The photo shows a prolactinoma. In its middle there is a necrotized region (*arrowheads*). The cells are large and closely packed. Scale 200 μm in (**a–d**), and 50 μm in the *inserts*

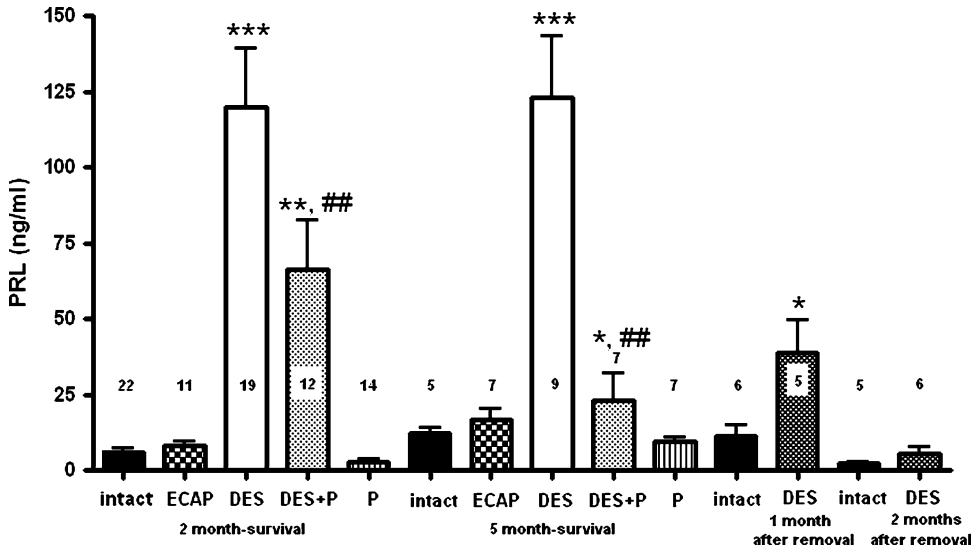

Fig. 5 Diagrams showing the effect of sexual steroid treatments on the basal PRL levels in various experimental groups. DES extremely enhanced the PRL level even 2 months after implantation. P blunted the effect of DES with 2 month-survival, and this effect was more pronounced in the animals with 5 month-survival. ECAP and P alone did not significantly influence the basal PRL levels. When DES capsule was removed 2 months after implantation the basal PRL levels gradually decreased and returned to control level. The *numbers* above or in the columns indicate the number of animals. * $P < 0.05$ DES (1 month after removal) or DES + P versus their own control; ** $P < 0.001$ DES + P versus its own control; *** $P < 0.0001$ DES versus their own control; ## $P < 0.001$ DES + P versus DES (with the same survival)

We have previously demonstrated that the DES treatment changed the distribution of S-100 immunoreactive folliculostellate cells (FSCs) and GH cells as well. Inside the prolactinomas there were only a few scattered FSCs and GH cells, not densely and evenly distributed as in normal pituitary tissue. However, the prolactinomas were surrounded by a densely packed FSCs and GH cells. FSCs formed a demarcation line around the prolactinomas. When DES was implanted with P the changes, characteristic for DES treatment, could not develop. Concomitant P influence prevented the morphological changes in the anterior pituitary. The distribution of FSCs was very similar to that of controls [34].

The question arises how E induces and P prevents the appearance of VIP ir cells. In Calderon and his coworkers' experiment [35] E induced the nuclear accumulation of ERs and the appearance of PRs reaching a peak at 12 h, then declined to a plateau near the control level. If P was administered to the animals at the peak of PRs, subsequent nuclear accumulation of ERs caused by estradiol (E2) injection was suppressed. This was observed only in the anterior pituitary, not in the hypothalamus. They concluded that P affected the response to E2 in the pituitary gland by a well defined temporal pattern and using a same protocol it had no effect in the hypothalamus.

It seems that there is a cross-talk between ERs and PRs. In our protocol, we ensured a consistently higher blood level of both E and P than in controls by the implanted silastic capsules for 5 months. It was found in our unpublished experiments that in the anterior pituitary E extremely enhanced the density of ER studied by in situ hybridization and P prevented the enhancement of its level. In the hypothalamus there was no significant difference between the control and steroid treated rats.

We suggested that the protective role of P on the effect of E causing undesired changes would be mediated through the cross-talk between the ERs and PRs at the pituitary level.

Materials and methods

Animals

Sprague-Dawley male rats were used for our experiments. They were kept in a light (light on at 5 h and off at 19 h) and temperature (22 ± 2°C) controlled vivarium and fed with standard lab chow and water ad libitum. All together 150 animals were used. The treatment of the animals was in accordance with the rules of the "European convention for the protection of vertebrate animals used for experimental and other scientific purposes", Strasbourg, 1986. Our protocol was approved by the Department of Animal Health Care, Budapest. Permission number: 37/1999.

When the animals were 25 day-old they received DES, DES + P, just P containing or empty (ECAP) silastic capsule implanted under the skin of the neck in the subcutaneous tissue under ether anesthesia (Reanal, Budapest, Hungary).

Preparation of the hormone containing capsules

Silastic capsules (id 1.55 mm, od 3.13 mm, length 10 mm) (Dow Corning Corporation, Midland, MI) were filled with DES (Sigma, St. Louis, Mo) or P (Sigma) and the ends of the capsules were sealed by Szilorfix (Finomvegyszer Szövetkezet, Budapest, Hungary), a silicon based glue. A day after drying, the capsules were used for implantation. The steroids can pass through the wall of the capsule and can exert its effect through the general circulation [36]. At the end of the experimental period the animals were sacrificed in the morning.

Experimental protocol

Five groups of animals were used in the experiments.

1. Intact rats.
2. Sham operated rats (they received empty capsule sealed with glue, ECAP).
3. DES implanted rats.
4. DES + P implanted rats.
5. P implanted rats

Radioimmunoassay

The animals used for RIA study were decapitated, the trunk blood was collected. The coagulation was prevented by EDTA (ethylene diamine tetraacetic acid sodium salt, Serva, Heidelberg, Germany) solution. The pituitaries were removed and weighted, then immersed in Bouin solution (75% saturated picric acid, 25% formaldehyde, and 5% acetic acid). The acids were purchased from Reanal, Budapest Hungary and the formaline from Merck, Darmstadt, Germany.

The trunk blood was centrifuged at 4°C. Plasma was stored at −20°C until determination of pituitary hormones. Iodination was carried out by Chloramin T (Mallinckrodt Baker, Inc., Phillipsburg, N.J.) method. Separation of the free and bound antigens was made by double antibody method. Hormone Kit was obtained from the National Hormone and Peptide Program (NHPP), NIDDK, and Dr. Parlow. Hormone levels were expressed in ng/ml plasma.

Statistical analysis

The mean of two parallel determinations for each animal was subjected to one-way analysis of variance (ANOVA). Tukey's test was used as post test.

Immunohistochemistry

At the end of the experiments three animals of each group were anesthetized by ketamine-hydrochloride (75 mg/100 g bw) (Sigma, St. Louis, MO) and perfused with 4% paraformaldehyde (Merck, Darmstadt, Germany) in potassium phosphate buffer (KPB) (pH 7.4, 0.1 M). The components were purchased from Sigma (St. Louis, MO). Pituitaries were removed and postfixed overnight. After washing in KPB, the pituitaries were immersed in ascending sucrose solution (10–20–30%), then frozen on dry ice. Two parallel series of 20 μm thick sections were cut on cryostate (Shandon, Pittsburg, PA). The pituitary sections were rinsed in KPB. PRL was visualized by indirect fluorescence technique. VIP was stained using ABC Kit. The final complex was visualized by nickel-DAB (diaminobenzidine tetrahydrochloride) reaction. The following primary antisera were used in our experiment: PRL antiserum (dilution 1:500) was raised in rabbit by Nagy and characterized by DeMaria et al. [37]. VIP (dilution 1:10,000) was raised in rabbit by Görcs and characterized by Gulyás et al. [38]. For fluorescence labeling the secondary antibody was raised in goat conjugated to Cy3 (DAKO A/S, Glostrup, Denmark). Vectastain ABC Elite Kit was purchased from Vector Laboratories (Burlingame, CA). DAB was purchased from Sigma (St. Louis, MO). Nickel-ammonium sulfate was purchased from Reanal (Budapest, Hungary).

Quantitative analysis of the cell number

All VIP ir cells were counted in three mid-level horizontal sections of the pituitary glands of three animals from each experimental group. The size of horizontal sections was very different because the weight of pituitaries was also very different. The results were subjected to ANOVA. Tukey's test was used as post test.

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