The importance of dopamine in the pathogenesis of experimental prolactinomas

MUSTAPHA EL-Azouzi, M.D., DORA W. HSU, M.Sc., PETER McL. BLACK, M.D., PH.D., FERENC JOLESZ, M.D., E. TESSA HEDLEY-WHYTE, M.D., ANNE KLIDANSKI, M.D., and NICHOLAS T. ZERVAS, M.D.

Neurosurgical Services, Massachusetts General Hospital and Brigham and Women’s and Children’s Hospitals; Magnetic Resonance Imaging Center, Brigham and Women’s Hospital; Divisions of Neuropathology and Endocrinology, Massachusetts General Hospital; and Departments of Surgery, Pathology, Radiology, and Medicine, Harvard Medical School, Boston, Massachusetts

The factors responsible for the production of prolactin-secreting tumors are obscure. One hypothesis, that chronic loss of dopamine control from the hypothalamus may be associated with prolactinoma formation, was tested. Female adult Fischer 344 rats were subjected to ovariectomy and were then given subcutaneous implants of diethylstilbestrol (DES). Sequential studies assessed the neuronal activity of the tuberoinfundibular dopaminergic neurons of the arcuate nucleus of the hypothalamus (A12) during and after this estrogen-induced pituitary growth. Immunocytochemical staining for tyrosine hydroxylase was used as a marker for dopamine synthesis, plasma radioimmunoassay provided plasma prolactin levels, and magnetic resonance imaging and histological studies were performed to examine the structural changes occurring in the pituitary gland. Animals were sacrificed from 3 to 67 days after DES implantation. To determine the reversibility of the estrogen-induced changes, rats were also sacrificed at different time intervals after the removal of 30-, 40-, or 60-day DES implants.

After 30 days of DES treatment, plasma prolactin levels increased 40-fold and pituitary weight increased more than threefold. Tyrosine hydroxylase immunoreactivity diminished gradually and was almost completely depleted at 30 days. Pituitary histology revealed marked prolactin cell hyperplasia. These changes were completely reversible; removal of the capsule after 30 days resulted in eventual normalization of plasma prolactin levels and pituitary size and in restoration of tyrosine hydroxylase immunoreactivity in the A12 region.

Sixty days of DES treatment produced large hemorrhagic tumors with sustained high plasma prolactin levels and an irreversibly distorted A12 area. These observations suggest that in these animals loss of dopamine regulation secondary to estrogen stimulation initially produces prolactin hyperplasia but that prolonged loss leads to adenoma formation.

KEY WORDS • diethylstilbestrol • dopamine • immunocytochemistry • adenoma • prolactin • pituitary tumor • tyrosine hydroxylase • rat

Tuberoinfundibular dopaminergic (TIDA) neurons located in the arcuate nucleus of the hypothalamus are known to play a major regulatory role in tonic prolactin secretion from the anterior pituitary gland.13,20,21,25 Through their terminals in the median eminence, these neurons secrete dopamine into the hypophyseal portal vasculature, activate dopamine receptors on lactotrophs, and inhibit prolactin secretion.20

Chronic estrogen administration in rats has been shown to induce hyperprolactinemia and prolactin cell hyperplasia.19,25 Estrogen treatment for 3 to 5 days causes a significant increase in plasma prolactin levels and turnover of dopamine in the median eminence and arcuate nucleus.15,34 The increased prolactin secretion from the anterior pituitary gland has been thought to be the result of estrogen-induced reduction in the capacity of prolactin cells to internalize dopamine and incorporate it into prolactin secretory granules.15 Long-term administration of estrogen results in impaired TIDA neuronal activity14 and pituitary tumor formation.8,11,25,32 The biochemical mechanism by which long-term estrogen treatment causes these alterations is still unclear: whether the effect of estrogen on TIDA is...
permanent or reversible also remains a controversial subject.36,7,11,13,26,28,32,34

The present study uses tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthetic pathway of dopamine, as a marker to examine the neuronal activity of the TIDA system in estrogen-implanted rats. Magnetic resonance (MR) imaging and histological studies provide methods to follow the anatomical and structural changes occurring in the pituitary gland and TIDA neurons during the development of tumors induced by estrogen.

Materials and Methods

Animal Preparation

Young adult female Fischer 344 rats weighing 125 to 150 gm each* were housed in a room equipped with a regulated 12:12-hour photoperiod and a temperature maintained at 23±2°C. The rats were routinely housed four to five per cage and allowed water and Purina Rat Chow ad libitum. Under ether anesthesia, the animals were subjected to ovariectomy. Silastic capsules containing 20 mg diethylstilbestrol (DES) and enclosed by silicone type A medical adhesive were then implanted subcutaneously. This capsule preparation has previously been shown to produce very large prolactin-secreting pituitary tumors in this strain of rats.37,38

The animals were randomly divided into two groups. The first group consisted of pairs of rats sacrificed 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 40, 60, or 67 days after DES implantation; this group allowed an evaluation of estrogen-induced changes with time. In the second group, pairs of rats were treated with DES for a given period and were then sacrificed at different time intervals following the removal of the DES capsule as follows: rats with 30 days of DES implantation were sacrificed 5, 10, 20, 30, 60, or 150 days after removal of the capsule; those with 40 days of DES implantation were sacrificed 30 days after removal; and those with 60 days of DES implantation were sacrificed at 7, 30, or 45 days after removal. These animal preparations allowed an analysis of reversibility. Four age-matched rats with ovariectomy but no implantation of DES capsules were used as a control group. Tissue collection was performed between 11:00 a.m. and noon. The animals were decapitated under ether anesthesia and the brains and pituitaries were taken, weighed, and fixed in 10% phosphate-buffered formalin.

Magnetic Resonance Imaging

Magnetic resonance imaging was performed with a 30-cm bore superconductive magnet† operating at 1.9 tesla, with an 81.05-MHz proton Larmor frequency. In the coronal plane, T1-weighted images (TE 10 msec, TR 0.46 sec, four acquisitions) and T2-weighted images (TE 60 msec, TR 1.92 sec, two acquisitions) were obtained with an 80-mm field of view (pixel size 314 × 314 μm). Eight contiguous slices, 2 mm thick, were made. The pituitary size was measured in the coronal plane on the 30th and the 60th days of DES treatment. The tumor was outlined electronically at the level of the sella. The cross-sectional area of the tumor was determined by the number of pixels within the region of interest.

Hormone Radioimmunoassay

Blood samples were obtained either by an external jugular tap or immediately after the animal was sacrificed. After centrifugation, plasma was collected and stored at -70°C. The concentration of rat prolactin in the plasma was determined by radioimmunoassay by previously described methods,15 using reagents obtained from the National Institute of Arthritis, Diabetes and Kidney Disease.

Statistical Analysis

Student’s t-test was used to compare the pituitary weight and plasma prolactin levels of the normal rats versus the groups with 30-, 40-, and 60-day DES treatment. Differences with a p value < 0.05 (one-tailed t-test) were considered significant. All mean values are expressed ± standard error of the mean.

Histological and Immunocytochemical Studies

Fixed rat brains and pituitaries were processed, embedded in paraffin, serially sectioned 6 μm thick, and placed on gel-coated slides. At each 100-μm interval, a section was stained with hematoxylin and eosin for histological review. Immunostaining was performed on unstained sections using the avidin-biotin-peroxidase complex technique.4,16 Antiserum to TH was used at 1:8000 to 1:10,000.‡ The characteristics of this antiserum have been described by Joh, et al.17 For cellular differentiation in the pituitary gland, antisera to rat prolactin, human growth hormone (GH), and adrenocorticotropic hormone (ACTH) were used at 1:1500, 1:1000, and 1:200 dilutions, respectively.§ Sections were incubated at room temperature with anti-TH overnight and with antibodies to the pituitary hormones for 45 minutes. Antigen-antibody binding was visualized with 3,3’-diaminobenzidine/H2O2 incubation. Omission of the primary antibodies produced no staining. The GH and ACTH immunoreactivity were used to gauge the extent of replacement of the anterior pituitary by prolactin cells.

*M. El-Azouzi, et al.

‡ Antiserum obtained from Eugene Tech International, Allendale, New Jersey.
§ Antiserum to rat prolactin, GH, and ACTH obtained from Dr. S. Raitt of the National Hormone and Pituitary Program, Baltimore, Maryland.

Fischer rats obtained from Charles River Laboratories, Wilmington, Massachusetts.
† Superconductive magnet manufactured by Oxford Instruments North America, Inc., Bedford, Massachusetts.
Dopamine in the pathogenesis of experimental prolactinomas

Results

Pituitary Weight

Mean weights of pituitary glands increased from 13.0 ± 1.0 mg in the control group to 43.6 ± 2.5 mg after 30 days of DES implant (p < 0.005) and to 131.9 ± 6.2 mg after 60 days (p < 0.001). Thus, the mean weights in the estrogen-treated groups were approximately three times greater than those of control rats after 30 days of estrogen implant and 10 times greater after 60 days (Table 1). The pituitary glands of the rats treated with estrogen for 60 days were markedly enlarged and dark red in color with intratumoral hemorrhage visible grossly.

Plasma Prolactin Levels

Pretreatment levels of plasma prolactin were less than 50 ng/ml. Treatment with DES elevated circulating prolactin to 1941.5 ± 442.7 ng/ml after 30 days (p < 0.001) and to 4460.0 ± 714.3 ng/ml after 60 days (p < 0.001) (Table 1). In rats whose DES capsules were removed following 30 days of implantation, plasma prolactin levels remained elevated for 10 days, then progressively decreased and returned to a normal basal level at 5 months (Fig. 1 and Table 2). Conversely, the plasma prolactin levels in those rats with DES capsules in place for 60 days before removal remained greatly elevated as compared to those observed in any of the other experimental groups (Fig. 1 and Table 2).

Magnetic Resonance Studies

Magnetic resonance imaging demonstrated an increase in size of the pituitary during implantation. The reversibility of the DES effects on pituitary expansion was further demonstrated with the reduction of pituitary size to the normal range following removal of the DES capsule after 30 days of implantation (Fig. 2). Magnetic resonance imaging also demonstrated the

<table>
<thead>
<tr>
<th>Days of Treatment</th>
<th>Pituitary Weight (mg)†</th>
<th>Plasma Prolactin Level (ng/ml)</th>
<th>No. of Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.0 ± 1.0</td>
<td>&lt; 50.0</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>15.4 ± 0.2</td>
<td>107.0 ± 6.0</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>16.1 ± 0.8</td>
<td>107.5 ± 10.5</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>17.8 ± 1.7</td>
<td>260.0 ± 24.0</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>26.4 ± 0.4</td>
<td>441.6 ± 11.8</td>
<td>3</td>
</tr>
<tr>
<td>15</td>
<td>27.8 ± 0.2</td>
<td>343.7 ± 58.0</td>
<td>2</td>
</tr>
<tr>
<td>18</td>
<td>27.5 ± 0.4</td>
<td>809.0</td>
<td>1</td>
</tr>
<tr>
<td>21</td>
<td>34.5 ± 6.1</td>
<td>1377.0 ± 328.0</td>
<td>2</td>
</tr>
<tr>
<td>24</td>
<td>38.7 ± 1.7</td>
<td>894.5 ± 5.5</td>
<td>2</td>
</tr>
<tr>
<td>27</td>
<td>30.2 ± 5.5</td>
<td>1498.0 ± 492.0</td>
<td>2</td>
</tr>
<tr>
<td>30</td>
<td>43.6 ± 2.5</td>
<td>1941.5 ± 442.7</td>
<td>4</td>
</tr>
<tr>
<td>40</td>
<td>—</td>
<td>4557.0 ± 504.4</td>
<td>3</td>
</tr>
<tr>
<td>60</td>
<td>131.9 ± 6.2</td>
<td>2988.0</td>
<td>1</td>
</tr>
<tr>
<td>67</td>
<td>—</td>
<td>4460.0 ± 714.3</td>
<td>4</td>
</tr>
</tbody>
</table>

* All means are expressed ± standard error of the means.
† Values represent the mean of two animals per group.

Fig. 1. The effect of estrogen on plasma prolactin (PRL) levels in rats after implantation of diethylstilbestrol (DES) capsules. Open circles indicate continuous DES treatment (upper graph). Closed circles represent rats sacrificed at different intervals following 30 days of DES administration (center graph). Closed squares indicate data from rats with DES capsules removed after 60 days of administration (lower graph). Animals indicated by open circles are the same ones in all graphs. Each point represents one pair of rats.
FIG. 2. Magnetic resonance images demonstrating changes in size and contour of pituitary and brain in rats after treatment with diethylstilbestrol (DES). A: A control rat. Arrow points to the pituitary. B: Rat after a 30-day exposure to DES giving rise to an enlarged pituitary (arrow). C: The same animal as in A, 150 days after the removal of a 30-day implant. Both the brain and pituitary have returned to normal size. The third ventricle and hypothalamic region are indicated by an arrow. D: Rat after a 60-day exposure of DES resulting in an enormous pituitary tumor (arrow) and severely compressed brain. E: The same animal as in D, 45 days following the removal of a 60-day DES capsule. The tumor has enlarged, with hemorrhage (arrow) on the top and a necrotic area in the center (arrowhead).

<table>
<thead>
<tr>
<th>Duration of Treatment</th>
<th>Days After Capsule Removal</th>
<th>Plasma Prolactin Level (ng/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2437.0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3394.0</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1153.0</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>266.0</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>61.0</td>
<td></td>
</tr>
<tr>
<td>60 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>3587.2</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>1844.5</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>3039.0</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers represent the mean of two animals per group.

TABLE 2

Plasma prolactin measurements after the removal of estrogen implants

continued growth of the pituitary tumor with hemorrhage, compression, and distortion of the brain (Fig. 2). Rats treated with DES for 60 days did not survive longer than 45 days following removal of the estrogen stimulus.

In one representative animal, the enlarged pituitary was measured at the level of the sella in the coronal plane on the 30th and 60th days of DES implantation. The suprasellar tumor was outlined electronically. Within the defined region of interest, only tumor tissue was seen. The cross-sectional area of the tumor on the 30th day of DES treatment was 873 pixels. On the 60th day of DES treatment, the tumor was significantly larger; the cross-sectional area was 2060 pixels, representing a 236% increase in measured areas.
Dopamine in the pathogenesis of experimental prolactinomas

**Histological and Immunocytochemical Findings**

For the first 6 days of DES treatment, the slightly enlarged rat pituitaries retained normal gland architectural characteristics, as demonstrated by hematoxylin and eosin and reticulin staining. Prolactin immunoreactive cells were abundant, with numerous GH- and ACTH-immunoreactive cells present throughout the glands. In the pituitaries treated with DES for 12 to 30 days, there was an increase in vascularization with dilation of the vessels and a decreased number of GH- and ACTH-positive cells. Prolactin immunoreactive cells appeared hyperplastic with large vacuolated cytoplasm (Fig. 3).

In the pituitaries treated with DES for 60 days, there was marked derangement of the reticulin pattern together with cellular and nuclear pleomorphism, suggesting neoplastic changes. Cells immunoreactive to GH and ACTH were sparsely present with focal areas containing only prolactin-positive cells (Fig. 3).
In the rats with DES capsules removed following 30 days of treatment, the pituitary remained hyperplastic until 20 days after removal. By 30 days after removal, there was an apparent reduction in the size of the pituitary mass and some restoration of the normal gland architecture. In a rat treated with DES for 30 days and sacrificed 60 days after capsule removal, the pituitary was reduced to the size of the untreated control pituitaries and normal gland histology was completely restored. There was normal distribution of GH-, ACTH-, and luteinizing hormone-positive cells among the prolactin cells.

**Tyrosine Hydroxylase Immunoreactivity**

Specific TH-like immunoreactivity (TH-LI) was found in all dopaminergic neurons in the brain. These included the cell groups located in the substantia nigra and their projections to the striatum, the cells in the zona incerta and their projections to the dorsal hypothalamus, and (most important for the present studies) the cells located in the periventricular and arcuate nuclei of the hypothalamus (A12), which belong to the TIDA system and their axon terminals in the external layer of the median eminence.

In the untreated rats, strong TH-LI was observed in the neuronal cell bodies in A12 and in their projections and terminals on the capillary loops in the external layer of the median eminence (Fig. 4). The intensity of TH-LI in these areas decreased over the duration of DES treatment, became very weak by 22 days, and was almost absent in the brains treated with DES for 30 days except for a few weakly positive neuronal perikarya (Fig. 4). No neuronal degeneration was observed in the A12 area in any of the DES-treated brains.

In the rats with DES capsules removed after 30 days of implantation, TH-LI in the A12 area reappeared as early as 5 days following capsule removal and was restored to the same intensity as in the untreated rats after 60 days (Fig. 4). This indicated a reestablishment of the TH-dependent dopamine synthetic activity. In contrast, TH-LI was not restored in the brains of rats treated for 60 days even as long as 45 days after removal of the DES capsule.

**Discussion**

Estrogen-induced hyperprolactinemia, pituitary hyperplasia, and alterations in TIDA neuronal activity in rats are reversible processes following 30 days of estrogen treatment but not after 60 days of treatment. This has been demonstrated by plasma prolactin measurements, MR imaging, and pituitary histological and TH immunoreactivity studies. Furthermore, the occurrence of frank tumor is associated with complete loss of TH staining in the TIDA neurons.

There is some evidence that estrogen exposure may lead to prolactinomas in humans. Prolactinomas occur 2.8 times more often in women than in men, and exposure of women to higher levels of estrogen may account for this prevalence. A recent report on the development of a prolactinoma in a man receiving massive doses of estrogen suggests that high doses of exogenous estrogen may represent a risk factor for developing prolactinomas in humans. There is a genetic predisposition for tumor development in rats, with Fischer 344 rats shown to be the most susceptible strain in the formation of pituitary tumors as a result of chronic estrogen stimulus.

Deficient hypothalamic dopaminergic regulation of prolactin release during long-term estrogen treatment has already been reported, and progressive changes in TIDA neurons have been reported in chronic hyperprolactinemia. This is in contrast with short-term (3-day) estrogen implantation, which increases dopamine release from the median eminence and arcuate nucleus. Numerous studies have shown that prolactin and prolactin-releasing drugs stimulate the turnover of TIDA neurons and the release of dopamine into the hypophysial portal blood.

Tyrosine hydroxylase, the rate-limiting enzyme in dopamine and noradrenaline synthesis, can be used as a specific marker for catecholaminergic neurons and their projections. The distribution of TH-immunoreactive neurons and their fibers in the present study is comparable to the distribution of the dopaminergic system observed by others. Sar has shown the lack of immunoreactivity to dopamine β-hydroxylase in the A12 region, verifying that these cells are dopaminergic rather than noradrenergic. With combined autoradiographic and immunohistochemical techniques, he has also demonstrated that estradiol is localized in the TH-immunoreactive neurons in the TIDA region, suggesting a direct effect of estradiol on these dopaminergic neurons.

In the present study, the complete involution of the pituitary mass and the normalization of plasma prolactin levels following the removal of DES capsules were closely related to the reestablishment of TH-immunoreactivity in the arcuate and median eminence region in the short-term treated animals. Contrary to the reports made by others, we did not observe neurotoxic effects of hyperprolactinemia in the TIDA areas of these brains. The total restoration of TH-immunoreactivity suggests that the synthetic activity of these dopamine neurons was merely suppressed in the presence of estrogen. The rapid reappearance of TH-LI upon the removal of this stimulus and prior to the decline of plasma prolactin levels is an indication that the suppression of neuronal TH was directly related to estrogen rather than to the hyperprolactinemic state, since the latter persisted for at least 10 days after capsule removal.

In contrast, the persistent high prolactin levels and the massive hemorrhagic pituitary tumors produced by long-term DES treatment could result from a permanently disrupted hypothalamic dopamine supply to the pituitary, possibly due to distortion, compression, and mechanical damage to the median eminence and the pituitary stalk by the large tumor mass.
Dopamine in the pathogenesis of experimental prolactinomas

Fig. 4. Tyrosine hydroxylase-like immunoreactivity (TH-LI) in the arcuate nucleus (ARC) of the hypothalamus and median eminence (ME). Immunoperoxidase, × 92. A–D: The TH-LI diminishes over the duration of diethylstilbestrol (DES) treatment and is nearly absent at 30 days. A normal specimen is shown in A. Specimens after 12, 22, and 30 days of DES treatment are shown in B, C, and D, respectively. E–H: Removal of the DES capsules resulted in restoration of TH-LI, which eventually reached the normal level of intensity. Specimens obtained at 10, 20, 30, and 60 days after DES removal are shown in E, F, G, and H, respectively. Note the expansion of the third ventricle due to hydrocephalus caused by tumor compression in B to F. The nuclei of the arcuate neurons are visible in each panel while the cytoplasmic immunoreactivity of these neurons has disappeared in C and D and reappeared in E to H.
Histological preparation of the pituitaries in our study revealed proliferation of prolactin cells with a relative decrease in number of GH and ACTH cells, probably due to compression by the hyperplastic lactotrophs. The increase of pituitary vascularization was revealed by histological study, and frequent hemorrhages were detected by MR imaging — especially after 60 days of implantation. These observations may support the proposal made by Weiner, et al., that these tumors could have escaped dopamine regulation by changes in the pituitary vascular supply to a direct arterial vascularization of estrogen-induced prolactin-secreting tumors in rats. This would effectively dilute the dopamine concentration delivered to the pituitary.

Long-term (60-day) DES implantation caused disarray of the normal pituitary architecture and focal adenoma formation, with pronounced intratumoral hemorrhage. Few GH and ACTH cells were present, consistent with observations of others. The seemingly neoplastic change observed in these pituitaries with 60 days of DES treatment could also account for the irreversibility following long-term estrogen stimulation.

Adding to the complexity of the mechanisms by which estrogen induces transformation in the rat pituitary is the possible involvement of neuropeptides such as vasoactive intestinal peptide (VIP) and galanin, a newly-identified 29-amino acid peptide. It has been shown that estrogen administration stimulates pituitary lactotrophs by increasing the pituitary concentration of VIP and induces a high level of expression of galanin messenger ribonucleic acid in these rat pituitaries. Both estrogen and VIP have been shown to activate cyclic adenosine monophosphate, a possible factor in cell proliferation. In addition, estrogen receptors on lactotrophs have been demonstrated in rats, in cell lines in culture, and in human pituitary tumors.

Whether the depletion of dopamine in TIDA neurons observed in our long-term estrogen-treated animals was the immediate cause for tumorigenesis, however, cannot be answered by the data obtained in the present study. Further studies are needed to answer this question.

Acknowledgments

The authors thank Dr. Salvatore Raiti of the National Hormone and Pituitary Program for supplying antisera against the pituitary hormones, Ms. Kathleen Dashner and Ms. Kathleen Riley for their assistance in animal surgery, Mr. Vincent Colucci for his help in the MR study, Ms. Helen Bikkal for performing the radioimmunoassay, and Ms. Rose Hand for typing the manuscript.

References

Dopamine in the pathogenesis of experimental prolactinomas


Manuscript received November 7, 1988. Accepted in final form July 17, 1989. Dr. Jolesz is the recipient of Grant 5R04-NS01083-03 from the National Institutes of Health. Dr. El-Azouzi is supported by a fellowship from H. M. Hassan II, King of Morocco. 

Address reprint requests to: Peter McL. Black, M.D., Ph.D., Neurosurgical Service, Brigham and Women's Hospital and Children's Hospital, 300 Longwood Avenue, Boston, Massachusetts 02115.