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Endothelin-like Immunoreactivity in Lactotrophs, Gonadotrophs, and Somatotrophs of Rat Anterior Pituitary Gland Are Affected Differentially by Ovarian Steroid Hormones

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It has been previously found that all hormone-producing phenotypes of the anterior lobe of the pituitary gland are capable of producing endothelin (ET)-like substances. The aim of this study was to determine whether the expression of ET-1-like peptides in lactotrophs, gonadotrophs, and somatotrophs is influenced by different in vivo ovarian hormonal conditions. Anterior lobes of the pituitary gland were harvested from ovariectomized and ovarian steroid–replaced adult female rats 10–12 d after surgery. Quantitative immunocytochemistry was performed on enzymatically dispersed pituitary cells. The presence of ET-1-like immunoreactivity in prolactin-, luteinizing hormone–, or growth hormone–producing cells was demonstrated by double-label immunocytochemistry. The incidence of ET-1 immunopositive pituitary cells was unaffected by progesterone treatment alone. Estradiol replacement caused a modest decrease in the number of lactotrophs and somatotrophs expressing ET-1 but increased the incidence of ET-1 immunopositive cells among gonadotrophs. Combined treatment with estradiol and progesterone robustly increased the incidence of ET-1 immunopositive lactotrophs and gonadotrophs but had no effect on somatotrophs. These data reveal that the synthesis of ET-1-like peptides in lactotrophs and gonadotrophs (and, to a lesser extent, in somatotrophs) is sensitive to ovarian steroids. Furthermore, these findings predict that ovarian steroids modulate ET-1 biosynthesis during the estrous cycle, suggesting a possible mechanism by which the ovarian steroid milieu may regulate the responsiveness of lactotrophs and gonadotrophs to their hypothalamic secretagogues.

Key Words: Endothelin; pituitary; luteinizing hormone; growth hormone; estradiol; progesterone.

Introduction

Endothelins (ETs) have an important role in the autocrine and paracrine regulation of different cellular functions such as secretion, cell cycle, and differentiation *(1)*. It is generally assumed that these peptides play a similar role in the pituitary gland as well *(2–4)*. Indeed, ET-1 and ET-3 *(5)* as well as ET_A receptors are expressed in the anterior lobe of the pituitary gland *(6,7)*, and ET-like peptides are potent modulators of pituitary hormone secretion in vitro *(8–11)*. Furthermore, lactotrophs synthesize and secrete ETs (ET-1 in particular), and endogenous ETs regulate prolactin (PRL) secretion in an autocrine fashion *(12)*. Since the rest of the hormone-producing cells of the anterior lobe of the pituitary gland are also capable of producing ET-like peptides *(13)*, it seems likely that ETs play an important local (autocrine or paracrine) regulatory function for all major anterior lobe hormones.

Ovarian steroids modulate ET-induced luteinizing hormone (LH) secretion from cultured rat pituitary cells *(14)*, indicating that the regulatory functions of ET-like peptides in the pituitary gland may be integrated into the overall regulatory mechanisms by which peripheral hormones influence pituitary hormone secretion. However, the regulation of the biosynthesis and secretion of ET-like peptides in the anterior lobe of the pituitary gland is poorly understood. The central aim of the present study was to determine whether the expression of ET-like peptides is influenced by different ovarian steroid backgrounds in vivo. Using the ovariectomy-steroid replacement paradigm and double-label immunocytochemical techniques, we provide evidence that 17β-estradiol and progesterone modulate ET gene expression in the anterior lobe of the pituitary gland in a cell-type-specific manner.

Results

ET-like immunoreactivity could be readily detected in various hormone-producing phenotypes of the pituitary gland (Fig. 1). Although we have not chemically characterized the ET-like immunoreactivity detected herein, based on our previous observations using a wide array of different ET antisera *(12,13),* we infer that the majority of the

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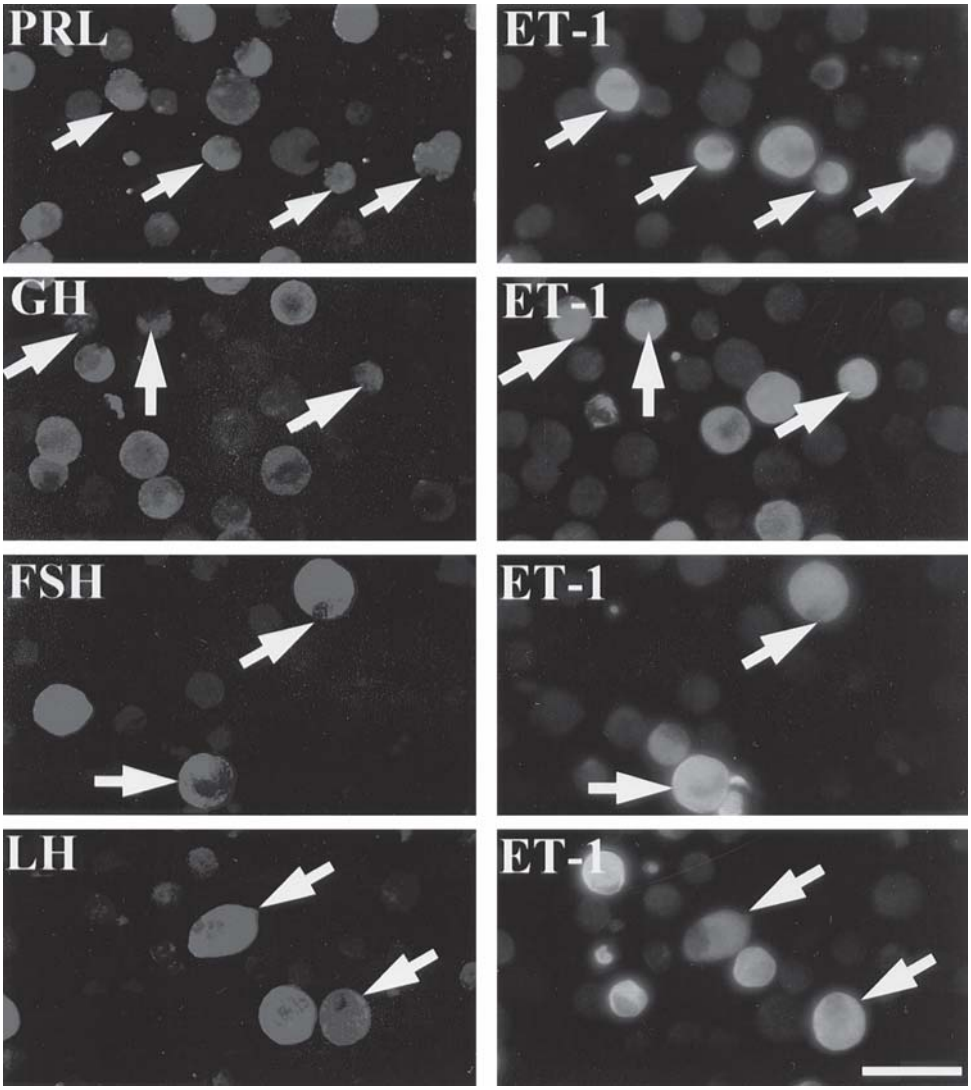

Fig. 1. Representative double-labeled immunofluorescence images for cellular colocalization of PRL, GH, FSH, and LH with ET-1 immunoreactivities. Pituitary hormones were visualized with secondary antibodies conjugated to CY3 (PRL, FSH, and LH, appears in red) or fluorescein isothiocyanate (GH, appears in green). Immunoreactive ET-1 was detected with biotinylated anti-mouse IgG and AMCA-streptavidin conjugate (appears in light blue). Arrows indicate the phenotypically identified pituitary cells, which also express ET-1 immunoreactivity. (Bar = 20 μm).

ET-like immunoreactivity detected in these pituitary cells represents ET-1 (Fig. 1).

Following ovariectomy, lactotrophs, gonadotrophs, and somatotrophs (identified by their respective PRL, LH/follicle-stimulating hormone [FSH], or growth hormone [GH] immunoreactivity) constituted 44.3 ± 8.1, 3.3 ± 0.3, and 39.5 ± 0.7% of the total cell population of the anterior lobe, respectively (Fig. 2A,C,E). In the presence of a 17β-estradiol implant (alone or in combination with progesterone implants), the relative abundance of lactotrophs increased significantly (Fig. 2A), apparently at the expense of somatotrophs (Fig. 2E).

The overall incidence of colocalization of ET-1 with PRL, LH/FSH, or GH indicated differential sensitivity toward ovarian steroid treatment. In ovariectomized (OVX) animals, expression of ET-1 was low and constituted only 4.6 ± 1.4, 8.7 ± 1.2, and 0.9 ± 0.2% of the lactotrophs, gonadotrophs, and somatotrophs, respectively (Fig. 2B,D,F). The relative incidence of ET-1 immunopositive cells was unaffected by progesterone treatment alone (Fig. 2B,D,F). 17β-Estradiol treatment decreased the incidence of ET-1 immunopositive cells among lactotrophs and somatotrophs (Fig. 2B,F). On the other hand, the incidence of ET-1 immunopositive cells was increased among gonadotrophs by 17β-estradiol treatment (Fig. 2D). Simultaneous treatment with 17β-estradiol and progesterone dramatically increased the percentage of ET-1-expressing cells among lactotrophs and gonadotrophs (Fig. 2B,D). In somatotrophs, although pro-

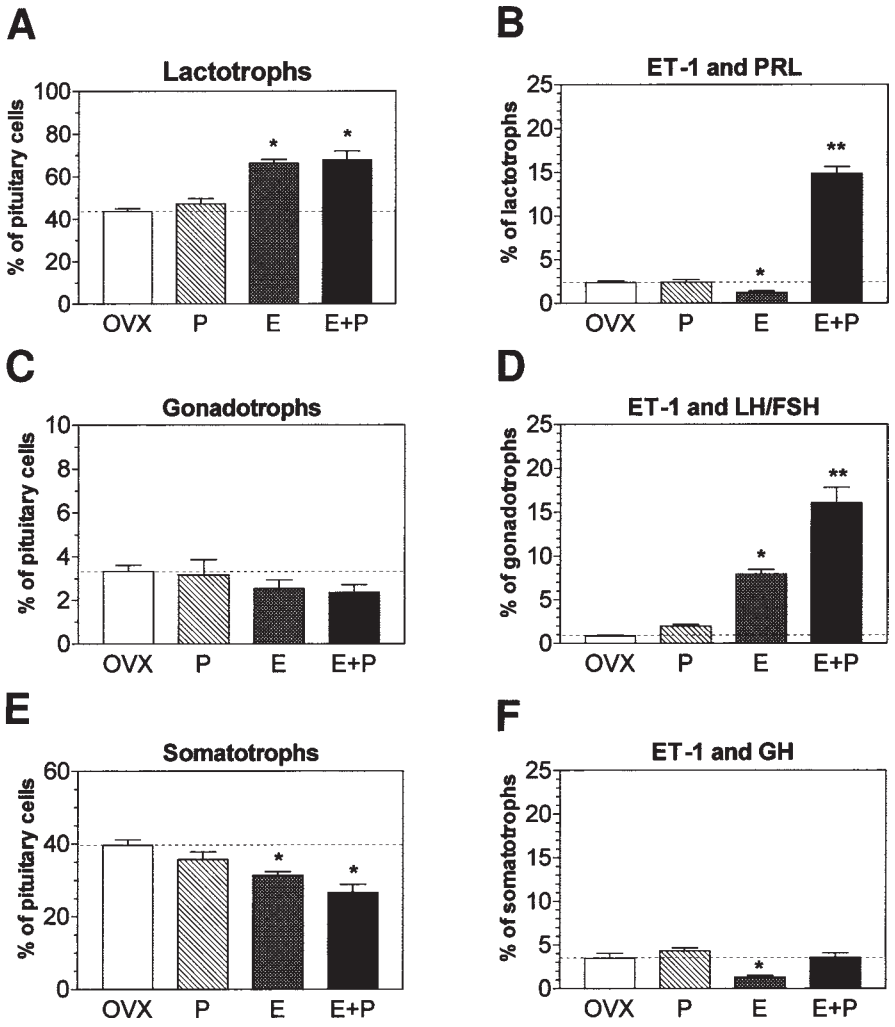

Fig. 2. Effects of ovarian steroid replacements on the prevalence of lactotrophs, gonadotrophs, and somatotrophs among all hormone-producing cells of the anterior lobe of the pituitary gland (**A, C, E**), and on the relative incidence of ET-1 immunopositive cells among lactotrophs, somatotrophs, and gonadotrophs (**B, D, F**). Animals were ovariectomized and treated with placebo (OVX), progesterone (P), estradiol (E), or a combination of estradiol and progesterone (E+P), as described in Materials and Methods. For each hormonal phenotype, 300 cells were selected at random, and the incidence of ET-1 immunopositive cells is expressed as a percentage of PRL (B), GH (D), and LH (F) immunoreactive cells, respectively. Asterisks indicate significant differences from the control OVX group (*$p < 0.05$, **$p < 0.01$).

gesterone prevented the 17β-estradiol-induced decrease in ET-1 expression, the combined steroid treatment did not elevate the number of ET-1 immunopositive cells when compared to the OVX group (Fig. 2F).

Discussion

The quantitative double-label immunofluorescence technique applied enabled us to evaluate the effect of ovarian steroids on ET-1 expression in phenotypically identified cell populations of the anterior lobe of the pituitary gland. We have found that lactotrophs, gonadotrophs, and somatotrophs have the capacity to express ET-1-like immunoreactivity and that the expression of ET-1 in the pituitary is sensitive to ovarian steroids. The three hormonal phenotypes showed characteristically different responses to estradiol and progesterone treatment. In general, it appears that the ovarian steroid sensitivity is more robust in gonadotrophs and lactotrophs than in somatotrophs. Whereas 17β-estradiol alone increased the expression of ET-1-like immunoreactivity among gonadotrophs, it had the opposite effect on lactotrophs and, to a lesser degree, on somatotrophs. Progesterone treatment alone did not significantly change the incidence of ET-1-expressing cells among lactotrophs, gonadotrophs, and somatotrophs. Interestingly, simultaneous treatment with the two steroids caused a robust increase in the population of ET-1-expressing lactotrophs and gonadotrophs. The latter effect was largely

absent among somatotrophs, in which the addition of progesterone only reversed the inhibitory effect of estradiol.

Synthesis of ET-1 in lactotrophs and somatotrophs appears to be under negative control by 17β-estradiol, whereas in gonadotrophs, estradiol has the opposite effect. An inhibitory effect of estrogens on ET-1 gene expression has previously been observed in nonpituitary tissues. For instance, pregnant women have lower plasma ET-1 concentrations than nonpregnant women, while postmenopausal estrogen replacement therapy decreases the plasma level of ET-1 *(15–18)*. Consistent with these clinical observations, ovariectomized animals have higher levels of plasma ET-1 than cycling female rats, and the administration of estradiol reduces plasma ET-1 levels of these animals *(19)*. Since 17β-estradiol attenuates ET-1 secretion as well as ET-1 mRNA production of cultured bovine endothelial cells in vitro *(20)*, it seems likely that endogenous estrogen modulates ET-1 concentration in the blood by affecting the vascular endothelium directly.

Estrogens are powerful regulators of gene expression in the periphery as well as in the central nervous system *(21)*. In the pituitary gland, 17β-estradiol regulates the expression of PRL and LH/FSH genes through well-established nuclear signaling pathways *(22–24)*. The mechanism by which 17β-estradiol regulates ET-1 expression is less straightforward, because no estrogen-responsive element in the 5' upstream region of the ET-1 gene has yet been found *(25,26)*. Although it is not altogether impossible that the ET-1 promoter contains a heretofore unidentified estrogen response element *(26)*, it seems more likely that the regulation of ET-1 gene expression by estrogens is indirect *(20)*. Clearly, further experiments using in vitro systems are required to elucidate the mechanisms by which 17β-estradiol and progesterone modulate ET gene expression in the pituitary.

Even less clear is the mechanism by which progesterone interacts with 17β-estradiol, especially in lactotrophs, because progesterone receptors have not been detected in lactotrophs *(27–29)*. One possibility is that progesterone acts centrally *(29–31)*, perhaps by altering the output of neuroendocrine dopaminergic neurons in the hypothalamus *(32,33)*. Alternatively, since gonadotrophs express progesterone receptors *(27–30,34)*, it is also conceivable that a paracrine interaction between gonadotrophs and lactotrophs *(35)* is required for the marked reversal of estradiol's action on lactotrophs by progesterone.

Although double-label immunocytochemistry can readily detect ET-like material in defined cell populations with a high degree of specificity, it gives no indication of the rate of ET secretion. From a functional point of view, it would be of interest to establish whether the increased expression of ET-1 by the combined treatment of estradiol and progesterone is accompanied by an increase or decrease in ET-1 secretion. Experiments applying reverse hemolytic plaque assay to assess ET-1 secretion *(12)* from single immunocytochemically identified lactotrophs and gonadotrophs are currently under way to address this.

Ontogenetically, lactotrophs originate from somatotrophs through an intermediate cell type often referred to as mammosomatotrophs *(36–38)*, and these hormonal phenotypes remain functionally interconvertible in mature animals *(37–42)*. It has been well recognized that 17β-estradiol is a major factor responsible for the recruitment of lactotrophs in the pituitary gland, because the presence of a high estradiol concentration during differentiation of somatomammotrophs favors their commitment toward lactotrophs *(41, 43)*. It seems unlikely, however, that ETs *per se* play a decisive role in estradiol-induced lactotroph recruitment, since 17β-estradiol and 17β-estradiol plus progesterone both increase the number of lactotrophs, whereas the effects of these treatments on ET biosynthesis in lactotrophs were opposite in nature.

Taken together, these data reveal that the synthesis of ET-1-like immunoreactivity in lactotrophs, gonadotrophs, and, to a lesser extent, somatotrophs is sensitive to ovarian steroids. This indicates that under physiologic conditions, 17β-estradiol and progesterone may act in concert to regulate ET-1 expression in the anterior lobe of the pituitary gland in a cell-specific manner. The characteristic changes in ET-1 expression brought about by different hormonal treatment suggest a role for ET-1 in the autocrine or paracrine regulation of cellular functions within the pituitary gland. The remarkable effect of the combined treatment with 17β-estradiol and progesterone on lactotrophs and gonadotrophs predicts that ovarian steroids modulate ET-1 biosynthesis during the estrous cycle. Because ET-like peptides are established modulators of PRL and LH secretion *(2–4)*, these findings suggest a possible mechanism by which the ovarian steroid milieu affects the responsiveness of lactotrophs and gonadotrophs.

Materials and Methods

Animals

Female Sprague-Dawley rats from Charles River (Raleigh, NC) were used throughout these experiments. Animals were adapted to a daily 12-h light cycle (6:00 AM to 6:00 PM) and maintained under controlled temperature and humidity. Rat chow and water were provided *ad libitum*. Rats were ovariectomized and immediately implanted subcutaneously with Silastic capsules (Dow Corning, Midland, MI) filled with either crystalline 17β-estradiol or progesterone as described previously *(44,45)*. One group of rats received both progesterone and 17β-estradiol implants, and a group of OVX rats bearing empty implants served as control. The animals were sacrificed 10–12 d after surgery. These implants deliver steroid hormones at a steady rate for several weeks, resulting in 70–100 pg/mL of 17β-estradiol and 30–40 ng/mL of progesterone in the serum *(44)*.

Pituitary Cell Preparation

Four to six pituitary glands were collected from each treatment group after rapid decapitation. The anterior pituitary cells were enzymatically dispersed according to the procedure described in detail elsewhere *(46,47)*. In brief, the intermediate and posterior lobes were removed, the anterior lobes were rinsed and diced into approx 1-mm^3 cubes and the cells were dissociated using collagenase (700 U/mL, Type I; Worthington, Freehold, NJ) and hyaluronidase (520 U/mL, Type III; Sigma, St. Louis, MO). The monodispersed cells were resuspended in HEPES-buffered saline (pH 7.35) at a concentration of approx 10^6 cells/mL and plated on glass cover slips coated with poly-L-lysine (0.1 mg/mL in distilled water for 10 min) (Sigma). Twenty minutes later the attached cells were fixed with 4% paraformaldehyde in isotonic sodium phosphate-buffered saline (PBS) (pH 7.34) at room temperature for 10 min followed by repeated rinses with PBS. The cover slips were stored at 4ºC immersed in PBS until immunocytochemistry.

Immunocytochemistry

Quantitative immunocytochemical analysis was performed as described previously *(12)*. To detect ET-like immunoreactivities, monoclonal anti-ET-1 antibody (ME-6901-01; Peninsula, Belmont, CA) was used throughout these studies. Following a 30-min incubation with 10% normal horse serum in PBS containing 0.3% Triton X-100 (TPBS) at room temperature, the cells were incubated with the anti-ET-1 antibody (1:5000 in TPBS) for 48 h at 4ºC. The ET-1 immunoreactivity was visualized by using biotinylated antimouse IgG and AMCA-streptavidin D conjugate (both from Vector).

To identify the specific hormonal phenotype, pituitary cells were stained for PRL, LH, FSH, and GH, using antirat PRL (αrPRL-IC5), antirat LH (αrLH-S11), antirat FSH (αrFSH-IC1), and antirat GH (αrGH-IC1) pituitary hormone antisera. The PRL, FSH, and LH-like immunoreactivities were visualized by antirabbit IgG-CY3 conjugate (Chemicon), and the GH immunoreactivity was detected by antimonkey IgG-FITC conjugate (Sigma ImmunoChemicals). To detect all gonadotrophs for the quantitative immunocytochemical analysis, a simultaneous staining with both LH and FSH antisera was employed.

Statistical Analyses

Approximately 1200 pituitary cells were counted to determine the percentage of PRL, LH/FSH, and GH immunopositive cells in each experimental group. Subsequently, a minimum of 300 PRL, LH/FSH, and GH immunopositive cells were examined, in randomly chosen fields, to determine the percentage of double-labeled (hormone vs ET-1) cells. Data obtained from at least three separate enzymatic dispersions for each treatment group were combined and analyzed by one-way analysis of variance followed by Fisher's test for multiple comparisons (GraphPad Prism 3.0 software). Differences were considered significant at $p < 0.05$.

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