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Autocrine Regulation of Prolactin Secretion by Endothelins

A Permissive Role for Estradiol

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We have previously found that lactotrophs express and secrete endothelin-like peptides that influence prolactin (PRL) secretion in an autocrine fashion. We have also observed that the incidence of endothelin-immunoreactive lactotrophs is markedly affected by ovarian steroids. In this study, we examined how the ovarian steroid background determines the efficiency of the endothelin-mediated autocrine feedback regulation of PRL secretion. Ovariectomized adult female rats were used throughout these studies. Steroid replacements were made by sc implantation of Silastic capsules immediately following ovariectomy. Eight to 10 wk later, three animals from each treatment group (no steroid control, estradiol, progesterone, estradiol plus progesterone) were sacrificed by decapitation, and the anterior pituitary cells were enzymatically dispersed using collagenase and hyaluronidase. A PRL-specific reverse hemolytic plaque assay was used to measure PRL secretion at the single-cell level. BQ123, a synthetic cyclic pentapeptide with distinctive endothelin-A receptor antagonist quality, caused only a modest elevation of PRL secretion in the control group. Endothelin antagonism did not affect PRL secretion in cells obtained from progesterone-implanted animals. Endothelin antagonism did, however, increase overall PRL secretion in the estradiol and estradiol plus progesterone groups by five- and threefold, respectively. Frequency distribution of PRL plaques in these same two BQ123-treated groups revealed two subpopulations, indicating that lactotrophs differ in their response to endogenous endothelin feedback and that this difference is steroid dependent. These observations clearly suggest that the ovarian steroid milieu (estrogens in particular) can have a profound influence on the self-regulatory mechanisms of lactotrophs. Our results also emphasize that endogenous endothelins may play an important role in the negative feedback regulation of PRL secretion in female rats.

Key Words: Prolactin; endothelin; estradiol; progesterone; pituitary; autocrine.

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Introduction

The presence of endothelin-like immunoreactivities in the neuroendocrine magnocellular neurons of the paraventricular and supraoptic nuclei suggested that endothelins may be involved in the regulation of pituitary function *(1–3)*. Endothelins are also intrinsic to the anterior lobe of the pituitary gland *(4–6)*, indicating that endothelins subserve a role as paracrine and/or autocrine regulators of pituitary hormone secretion *(7–9)*. However, the physiologic (or pathologic) context of these endothelin-mediated auto/paracrine feedback mechanisms remained elusive.

The influence of endothelins on prolactin (PRL) secretion is the most extensively studied among all pituitary hormones *(10–14)*. Lactotrophs express endothelin ET_A receptors *(5,15)* and are capable of synthesizing and releasing endothelin-like peptides *(16)*. Since the half-maximal concentration of endothelin-1 on PRL secretion is in the picomolar range *(15)*, even a modest amount of endothelin can profoundly influence the secretory activity of lactotrophs. Indeed, we have earlier reported that endothelins, presumably endothelin-1 *(17)*, released by lactotrophs, is sufficient to affect PRL secretion in an autocrine fashion *(16)*. Moreover, we have observed that the presence of ET-1-like immunoreactivity in lactotrophs is markedly influenced by the ovarian steroid background *(17)*. All these observations indicate strongly that endogenous endothelins are important local regulators of PRL secretion.

In the present study, we sought to determine the influence of ovarian steroids on the endothelin-mediated autocrine regulation of PRL secretion.

Results

Secretory activity of individual lactotrophs was determined by the size of hemolytic plaques, which is directly proportional to the amount of PRL secreted over time *(18–21)*. In our hands, regardless of treatment, the overall distribution of plaque sizes did not pass a normal distribution test. Therefore, all statistical analysis and comparisons were made using logarithmically transformed data that conformed normality criteria.

The endothelin-mediated autocrine feedback on PRL secretion was assessed through the changes caused by suspending the action of endogenous endothelins with a specific endothelin ET_A receptor antagonist, BQ-123 *(22)*. In

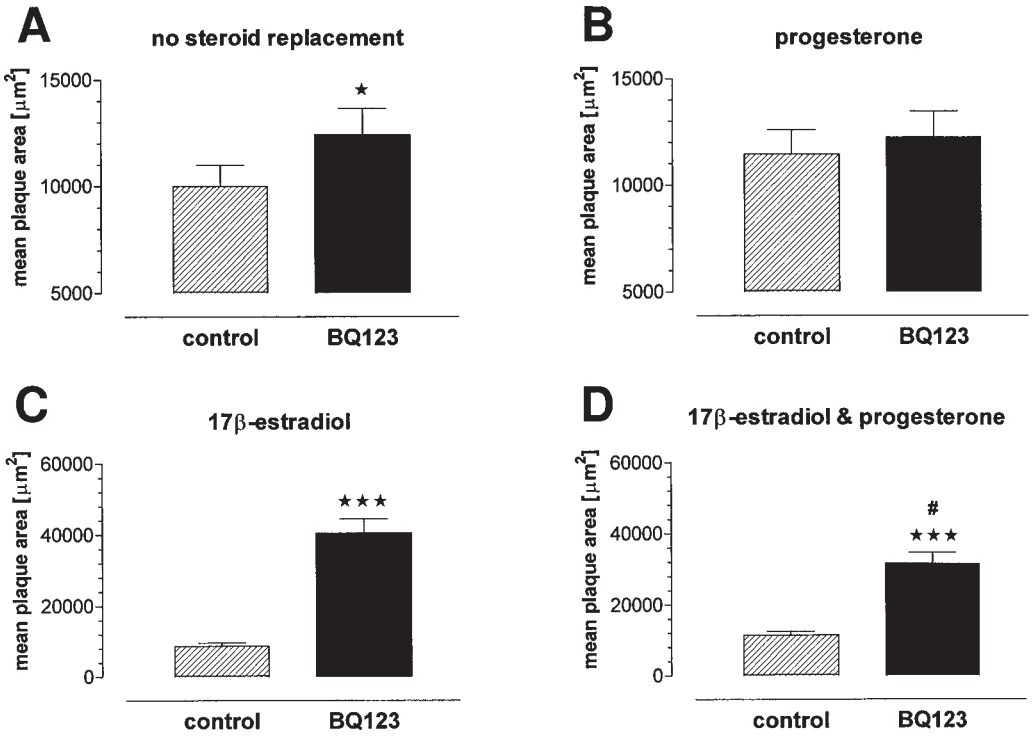

Fig. 1. Effects of 17β-estradiol and progesterone implants on endothelin receptor antagonist-induced PRL secretion. Lactotrophs were obtained from (**A**) control, (**B**) progesterone, (**C**) 17β-estradiol-, and (**D**) 17β-estradiol and progesterone–treated animals. PRL secretion of individual lactotrophs was measured by reverse hemolytic plaque assay as described in Materials and Methods. Cells were challenged for 3 h in the absence (▨) or presence (■) of BQ-123 at 10^{-7} *M*. Bars represents mean plaque area (±SEM). Averages were calculated on a large number of individual plaques (at least 300 plaques in each group). *$p < 0.05$; ***$p < 0.001$; #significant difference ($p < 0.05$) from estradiol alone (two-way analysis of variance [ANOVA] followed by Dunnet multiple comparisons).

our attempt to reveal the influence of in vivo ovarian steroid treatments on the endogenous endothelin-mediated regulation, freshly dispersed cells were used to minimize the influence of in vitro culture conditions. When cells were obtained from long-term ovariectomized (OVX) animals without steroid replacement, 10^{-7} *M* BQ-123 induced only a modest elevation of PRL secretion, which was reflected by a 24% increase in average plaque size (Fig. 1A). BQ-123 did not affect PRL secretion of cells obtained from progesterone-implanted animals (Fig. 1B). In the estradiol and estradiol plus progesterone groups, however, endothelin antagonism by BQ-123 did increase overall PRL secretion by 4.6- and 2.8-fold, respectively (Fig. 1C,D). The BQ-123-induced increase in PRL secretion was significantly less when estradiol replacement was accompanied by progesterone (Fig. 1D).

The PRL-secreting cell populations were further analyzed using logarithmically binned data (Fig. 2, Table 1). BQ-123 caused a small shift from the lowest secretory cells toward the larger plaque-forming population (Fig. 2A). When animals were pretreated with estradiol (Fig. 2C) or with estradiol and progesterone (Fig. 2D), ET_A receptor antagonism robustly increased the proportion of large plaque-forming cells at the expense of the smaller plaque-forming population. In the absence of endothelin antagonist, frequency distributions of PRL plaque sizes were homogeneous in all four groups, since they can be best fitted with a first-order Gaussian function (Fig. 2A–D, Table 1). BQ-123 treatment did not affect the frequency distribution of PRL plaque sizes when only progesterone implants were present (Fig. 2B). However, in the case of OVX animals without steroid replacement (Fig. 2A) and the estradiol replacement groups (Fig. 2C), frequency distribution of PRL plaques can be fitted best with a second-order Gaussian function (Table 1), indicating that BQ-123 treatment unmasked two distinct populations of lactotrophs that differ in terms of their response to endogenous endothelins. Interestingly, progesterone given in combination with estradiol prevented BQ-123 from unmasking the heterogeneity of the lactotroph population (Fig. 2D, Table 1). Conversely, progesterone did not prevent an overall BQ-123-induced shift of the proportion of lactotrophs from the small secretors to the large secretors (Fig. 2D).

Discussion

By using the ovariectomy/steroid replacement paradigm, we provide evidence that the autocrine regulation of PRL secretion by endothelins can be affected profoundly by the ovarian steroid background. Further interpretation of our data is somewhat hampered by the fact that lactotrophs can perceive the effect of endogenous endothelins as either inhib-

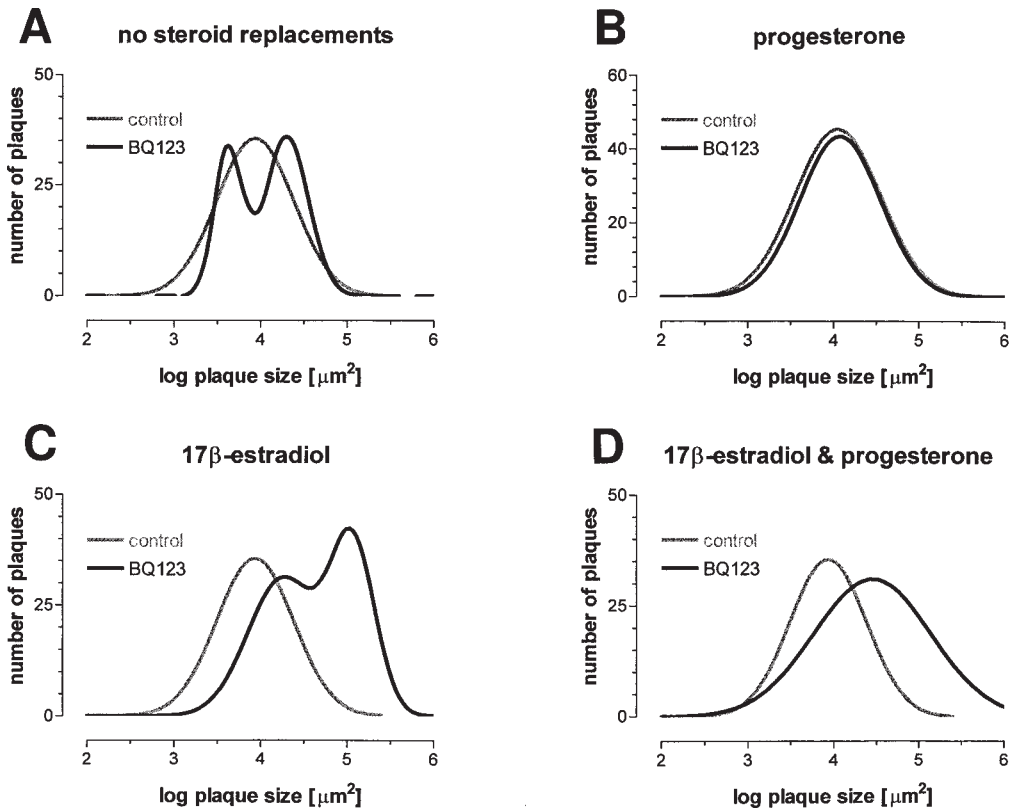

Fig. 2. Effects of long-term exposure to ovarian steroids on distribution of PRL plaque sizes. Lactotrophs were obtained from OVX rats, (**A**) bearing empty, (**B**) progesterone, (**C**) 17β-estradiol-, and (**D**) 17β-estradiol and progesterone–containing implants. Logarithmically binned data (0.2 bin width) were fitted with one- or two-order Gaussian functions. The two-order Gaussian function was accepted only when it resulted in a significantly better ($F > 5.0$) fit (see Table 1). When lactotrophs from control (A) or 17β-estradiol-treated (C) groups were treated with endothelin antagonist BQ-123, the distribution of plaque sizes could be fitted best with a second-order Gaussian function, indicating two distinct populations of lactotrophs.

Table 1
Summary of Analysis
of Frequency Distributions of PRL Plaque Sizes[a]

In vivo steroid replacement	In vitro treatment	*F* value	Model accepted
None	Control	0.01	1
	BQ-123	26.35	2
Progesterone	Control	1.13	1
	BQ-123	0.92	1
Estradiol	Control	0.93	1
	BQ-123	15.10	2
Estradiol + progesterone	Control	1.13	1
	BQ-123	0.82	1

[a] We applied two models to describe the distribution of lactotrophs in terms of their PRL secretion. Model 1 assumes a homogeneous population of lactotrophs in which the distribution of plaque sizes can be fit best with a first-order Gaussian function, whereas model 2 assumes heterogeneity of lactotrophs with at least two distinct populations. Model 2 was only accepted when a second-order Gaussian function resulted in a significantly better fit (pSTAT; $F > 5.0$).

itory or stimulatory *(16,23,24)*. However, the fact that endothelin antagonism markedly increased PRL secretion in the estradiol and estradiol plus progesterone groups indicates clearly that long-term exposure to 17β-estradiol enhances the inhibitory nature of the autocrine regulation of PRL secretion by endothelins.

Frequency distribution of the PRL plaques in BQ-123-treated OVX and estradiol replacement groups revealed two subpopulations, indicating that lactotrophs differ in their response to endogenous endothelins. A similar functional heterogeneity of lactotrophs in their responsiveness to dopamine and various PRL secretagogues has already been reported *(25–30)*. However, the physiologic implication of the detected diversity among lactotrophs is uncertain. Taken together, our data suggest that the efficiency of the endogenous endothelin-mediated autofeedback is steroid dependent. It appears that estradiol enhances the lactotrophs' response to endogenous endothelins, while progesterone diminishes it. Interestingly, progesterone seems to potentiate endothelin-induced luteinizing hormone secretion of estradiol-treated gonadotrophs *(31)*, indicating a phenotype-

specific action of ovarian steroids on the endothelin-mediated regulation of pituitary hormone secretion.

We have previously found that only a combined treatment with estradiol and progesterone increases the incidence of endothelin-immunoreactive lactotrophs *(17)*. Our immunocytochemical observation *(17)* seems consistent with our present pharmacologic data, assuming, that in lactotrophs, long-term exposure to estradiol elevates both the release and synthesis of endogenous endothelins, while progesterone reduces the release without affecting the synthesis of endothelins. The mechanism by which estradiol and progesterone modulates the action of endogenous endothelins is not yet known. It is equally conceivable that estradiol enhances endothelin ET_A receptor expression of the lactotrophs, or increases the lactotrophs' responsiveness to their own endothelins by promoting a more efficient coupling of the inhibitory signaling complex to the endothelin ET_A receptor.

Signal transduction aside, the most intriguing question concerns the physiologic context of the ovarian steroid–modulated autocrine feedback regulation of PRL secretion. Our present data, taken together with our previous observations *(16,17)*, clearly predict an important role for endogenous endothelins in regulating PRL secretion during the estrous cycle, pregnancy, or lactation.

Several clinical observations indicate that ovarian steroids can modify the level of endothelin gene expression in the vascular endothelium *(32–39)*. Little is known, however, how ovarian steroid replacement therapy might affect endothelin-mediated auto/paracrine regulations outside the cardiovascular system. The experimental paradigm applied in the present study offers an animal model to investigate the possible consequences of long-term estradiol and/or progesterone treatments on the local regulatory mechanisms of the pituitary gland.

Materials and Methods

Animals

Female Sprague-Dawley rats from Charles River (Raleigh, NC) were used throughout the 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 OVX and implanted subcutaneously with Silastic capsules (Dow Corning, Midland, MI) filled with either crystalline 17β-estradiol or progesterone as described previously *(40,41)*. One group of rats received both progesterone and 17β-estradiol implants, and a group of OVX rats bearing empty implants served as the control. These implants deliver steroid hormones at a steady rate for several weeks, resulting in 50–60 pg/mL of 17β-estradiol and 10–20 ng/mL of progesterone in the serum *(40)*. The animals were sacrificed 8–10 wk after surgery.

Preparation of Pituitary Cells

Pituitary glands were collected from each treatment group after rapid decapitation. The anterior pituitary cells were enzymatically dispersed using collagenase (700 U/mL) (Type I; Worthington, Freehold, NJ) and hyaluronidase (520 U/mL) (Type III; Sigma, St. Louis, MO) according to the procedure described in detail elsewhere *(42,43)*.

Hemolytic Plaque Assay

A PRL-specific reverse hemolytic plaque assay *(19)* was used to measure PRL secretion at the single-cell level as described previously *(16)*. In essence, protein A–coupled ovine red blood cells were mixed with enzymatically dispersed pituitary cells (approx 50,000 pituitary cells/mL of 2% ovine red blood cells) and loaded into Cunningham chambers *(18)*. After the unattached cells were rinsed out, the cells were incubated in the presence of polyclonal anti–rat PRL serum (rabbit anti–rat PRL IC-5 from the National Institute of Diabetes and Digestive and Kidney Diseases, at a 1:80 final dilution). To assess the autocrine effects of endogenous endothelins, we applied a specific and reversible ET_A receptor antagonist, BQ-123 (Peptide International, Louisville, KY), at a concentration of 10^{-7} *M*. Following 3 h of incubation with or without the ET_A receptor antagonist, hemolytic plaques around PRL-secreting cells were developed with guinea pig complement (1:30) (Life Technologies, Gaithersburg, MD). It is assumed that the size of the hemolytic plaque is directly proportional to the hormone secreted by the cell in the center *(18)*. Cells were fixed with 4% paraformaldehyde containing 2% glutaraldehyde for 30 min at room temperature. The slides were immersed in 0.1 *M* phosphate-buffered saline (pH 7.4) and stored at 4ºC until quantitation.

Data Collection and Analysis

Digital images of plaques were collected using a Leica DMLB light microscope (Leica, Wetzlar, Germany) equipped with a 5X phase-contrast objective and a Spot camera (Diagnostic Instruments, Sterling Heights, MI). Numerical values for each plaque area (μm^2) were obtained individually by a Metamorph Image Analysis System (Universal Imaging, Downingtown, PA) and transported to a Microsoft Excel spreadsheet. For statistical analysis, two-way ANOVA followed by Dunnett multiple comparisons test was applied using the Prism 3.0 program from Graphpad (San Diego, CA); $p < 0.05$ was considered as the threshold of significance.

For further analysis of cell populations, logarithmically bined data were analyzed by the pSTAT program (pCLAMP6 software package, version 6.03; Axon, Foster City, CA). Each data set, representing different treatment groups, was fitted with Gaussian curves using the Levenberg-Marquart least-squares method. The first, second, and third order of fitted Gaussian curves within each group were compared

using pSTAT, and the higher order was accepted only when it resulted in a significantly improved fit (signified by *F* values >5.0).

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