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Effects of Estrogen and Dopamine Agonists on the Expression of Argyrophilic Nucleolar Organizer Regions in Prolactin Cells of Rats

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ABSTRACT—The number of nucleolar organizer regions reflects nuclear and cellular activity, such as the proliferation and differentiation of cells. Prolonged administration of estrogen (E_2) induces the hyperplasia of prolactin (PRL) cells and the development of PRL-secreting pituitary tumors in rats. Dopamine agonists are known to reduce the effects of E_2 treatment. The purpose of this study was to investigate whether the changes of argyrophilic nucleolar organizer regions (AgNORs) in PRL cells are related to circulating levels of PRL or to the proliferative activity of PRL cells during the administration of stimulatory (E_2) or inhibitory (dopamine agonist) treatment in rats. E_2 increased the size and number of AgNORs per nuclear profile in PRL cells in rats. Bromocriptine and cabergoline , which are dopamine agonists, each reduced the number and size of AgNORs in PRL cells treated with E_2 for 10 weeks. In rats treated with E_2 alone or dopamine agonists followed by E_2, the number and size of AgNORs were correlated more closely with serum levels of PRL than with the proliferative activity of PRL cells. However, neither the number nor size of AgNORs in PRL cells was related with these parameters in different ages of the control. The number and size of AgNORs may be useful in evaluating the secretory activity of pituitary cells during the administration of stimulatory or inhibitory agents.

INTRODUCTION

Nucleolar organizer regions, which are loops of DNA that encode for ribosomal RNA, are associated with argyrophilic nonhistone proteins (Rüschoff *et al.*, 1989). The ribosomal RNA genes that are transcribed by polymerase I, are involved in the synthesis of ribosomes and proteins. The number of nucleolar organizer regions is thought to reflect nuclear and cellular activity (Busch *et al.*, 1979; Ochs and Busch, 1984). Ploton *et al.* (1986) reported a simple silver-staining method which improves the visualization of the nucleolar organizer regions in paraffin sections. The size and number of argyrophilic nucleolar organizer regions (AgNORs) have been shown to be related to the proliferation or differentiation of cells (Smetana and Likovsky, 1984; Ploton *et al.*, 1986; Hall *et al.*, 1988; Rüschoff *et al.*, 1989), although the exact significance of changes in the expression of AgNORs is not known. Several investigators have studied AgNORs in normal and tumorous pituitary cells of humans (Stefaneanu *et al.*, 1989; Bayindir *et al.*, 1992; Shibuya *et al.*, 1992). Nevertheless, it has not been established whether the changes in AgNORs are related to cellular proliferative activity or hormone production due to the lack of a well-characterized subset of cells in which to analyze such changes. Moreover, most of these studies have been restricted to human pathological tissues.

Long-term treatment of rats with high doses of estrogen (E_2) induces hyperplasia and adenomatous transformation of prolactin (PRL) cells accompanied by an increase in the proliferative activity of such cells and in the serum concentration of PRL (Meyer and Clifton, 1956; Lloyd, 1983; Phelps and Hymer, 1983).

We conducted the present study to evaluate changes in the size and number of AgNORs in PRL cells during the stimulation by E_2, and following the administration of a dopamine agonist, bromocriptine (BC) or cabergoline (CG), in rats with chronically E_2-stimulated pituitary glands. An additional goal was to investigate the relationships between the changes of AgNORs in PRL cells and the proliferative activity of PRL cells or the serum PRL level in rats.

MATERIALS AND METHODS

Animals

Four-week-old female Fischer 344 rats (Charles River Japan Co. Ltd.,Yokohama, Japan) were used. They were housed in a light- and temperature-controlled room, and were fed laboratory chow (Oriental Yeast Co., Tokyo, Japan), with tap water given *ad libitum*.

Estrogen treatment (Experiment I)

The objective of this first experiment was to examine the effects of E_2 on the expression of AgNORs in PRL cells of rats. Intact rats were given a subcutaneous injection of estradiol-17β (E_2) (Sigma, St.

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Louis, MO, USA), 2 mg, dissolved in 0.2 ml sesame oil, each week for a period of 3 or 10 weeks. Two groups of age-matched control rats received an injection of sesame oil according to the same schedule. Control rats were sacrificed by decapitation on the day of diestrous phase. Three hours before sacrifice, all rats received an intraperitoneal injection of 5-bromo-2'deoxyuridine (BrdU) (Sigma), 50 mg/kg body weight (BW), in saline.

Treatment of dopamine agonist followed by E_2 (Experiment II)

The objective of this second experiment was to examine the effect of dopamine agonists on the expression of AgNORs in PRL cells. Rats were given a subcutaneous injection of 2 mg E_2 in 0.2 ml oil weekly for 10 weeks. No additional E_2 treatment was given thereafter. One week later, rats were administered either bromocriptine (BC) orally at a dose of 0.6 mg/kg BW in 0.2 ml sesame oil, the same dose of cabergoline (CG), or the vehicle alone, every third day. Animals were killed by decapitation after 30 days. BC was kindly supplied by Sandoz Ltd. (Basel, Switzerland), while CG was kindly supplied by Farmitalia Carlo Erba (Milan, Italy). CG, a recently developed compound derived from ergot, possesses long-lasting dopaminergic effects (Ferrari *et al.*, 1989; Cooper, 1990) and several advantages in terms of PRL-lowring effect and antitumor effect on E_2 -induced rat pituitary tumor cells (Eguchi *et al.*, 1995a, b).

Collection of blood and preparation of tissue

Rats were anesthetized with ether and decapitated. Blood was sampled from the trunk. The sera were collected and frozen at -20°C for assay of PRL. Pituitary glands were removed, weighed and fixed in Bouin's fluid. Horizontal paraffin sections were cut 5 μm thick.

Measurement of serum PRL

Serum PRL levels were determined by radioimmunoassay using the double antibody method. The PRL standard (rPRL RP-3) was supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Bethesda, MD, USA). Anti-rat PRL serum (HAC-RT26-01RBP85) was a gift from Dr. Wakabayashi (Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Japan). Radioiodinated rat PRL was purchased from Du Pont / NEN Research Products (Boston, MA, USA) and anti-rabbit IgG from Bio Makor (Rehovot, Israel).

Proliferative activity of PRL cells

Immunostaining for BrdU or proliferating cell nuclear antigen (PCNA) was done to evaluate the proliferation of cells in different sections of the same pituitary gland (Carbajo-Pérez and Watanabe, 1990; Oishi *et al.*, 1993). BrdU incorporated into cellular DNA was detected with a cell proliferation kit using a monoclonal anti-BrdU (Amersham, Arlington, Heights, IL, USA). Mouse monoclonal anti-PCNA (Novocastra, Newcastle, UK) was used at a final dilution of 1: 100. The avidin-biotin peroxidase complex (ABC) kit (Vector Labs., Burlingame, CA, USA) was used for immunohistochemical study. After final incubation for immunostaining of BrdU or PCNA , the reaction products were visualized with 0.01 % 3,3'-diaminobenzidine (DAB) (Sigma) in phosphate buffered saline (PBS) containing 0.005 % H_2O_2.

After washing in PBS, sections were further reacted with anti-rat PRL serum (1: 3,000 dilution). Immunostaining was performed with a streptavidin-biotin-alkaline phosphatase kit (Nichirei Co. Ltd. Tokyo, Japan). Fast red was used as the color substrate. Positive immunostaining for PRL was visualized as a red color, which was easily distinguished from the nucleus stained brown by BrdU or PCNA antiserum. About 3,000 pituitary cells were counted per anterior lobe and the percentage of these that were PRL-immunoreactive was calculated. The proliferative activity of PRL cells was expressed as the percentage of these PRL-immunoreactive cells that were doubly immunostained with BrdU or PCNA antiserum.

The specificities of immunostaining for BrdU and PCNA were evaluated by replacing the primary antisera with normal mouse serum. The specificity of immunostaining for PRL was examined by preabsorption of the antiserum (1:3,000 dilution, 1 ml) with 10 μg of PRL (NIDDK rat PRL I-6) at 4°C for 24 hr.

Combined AgNOR staining and PRL immunostaining

Staining procedures were performed according to the methods described (McMeekin *et al.*,1989). Tissue sections were stained with a freshly prepared solution that consisted of 1 volume of 1% gelatin dissolved in 1% aqueous formic acid and 2 volumes of 50% aqueous silver nitrate solution . Sections were incubated in the dark at room temperature for 30 min. Slides were rinsed three times in distilled water and dipped in PBS prior to immunostaining. Immunostaining for PRL cells was performed with the ABC kit and visualized with DAB. AgNORs appeared as black dots within the nucleus, a color that was easily distingushed from the cytoplasm stained brown with anti-PRL.

The number of AgNORs per nuclear profile in 200 PRL-immunopositive cells per anterior lobe was counted in each experimental group. Counting was performed under oil immersion at a magnification of × 1,000. The microscope was carefully focused to count all AgNORs within the nuclear profile. The diameters of AgNORs were measured on photographic prints using a digimatic caliper (Mitsutoyo, Tokyo, Japan). The sizes of AgNORs were calculated according to the formula for an ellipsoid $S = ab\ \pi/4$, where a is the large axis , and b is the small axis. The size of the nuclear profiles of PRL cells was measured with an ocular micrometer and calculated according to the formula for an ellipsoid.

Statistical analysis

Analysis of differences between groups was performed by Kruskal-Wallis test followed by the Mann-Whitney U test. The correlation between two parameters was examined by linear regression analysis.

RESULTS

Experiment I

E_2 for 3 or 10 weeks significantly increased the pituitary weight, serum levels of PRL, and the percentage of PRL cells as compared with age-matched control groups (Table 1). The pituitary gland of each animal showed an essentially uniform distribution of BrdU- and PCNA-stained cells throughout the pars distalis. Double immunostaining with anti-PRL and anti-BrdU or anti-PCNA allowed the clear identification of PRL cells in the proliferative stage. There was a significant correlation between the percentages of BrdU-immunoreactive PRL cells and those of PCNA-immunoreactive PRL cells in all rats ($r = 0.8$, $p<0.001$).

The proliferative activity of PRL cells and serum PRL levels did not differ significantly between the two control groups in the diestrous phase (Table 1). After E_2 treatment, the proliferative activity of PRL cells and serum PRL levels increased , and significant differences were detected between E_2-treated groups and age-matched control groups. The duration of E_2 treatment (3 or 10 weeks) did not significantly alter the proliferative activity of PRL cells but did alter serum PRL levels (Table 1).

The PRL cells generally contained one or more rounded AgNORs within the nuclear profile (Fig. 1). There was no difference in size of AgNORs between the two control groups. In contrast, the mean size of the AgNORs increased

Table 1. Effects of chronic E_2 on pituitary PRL cells in female rats

Weeks of E_2 treatment	3 weeks		10 weeks	
	Control (n=4)	E_2 (n=5)	Control (n=5)	E_2 (n=5)
Pituitary weight (mg)	10.85 ± 0.73	21.24 ± 0.90**	13.60 ± 0.52#	209.4 ± 21.7**+
Serum PRL levels (ng/ml)	11.03 ± 3.87	243.4 ± 42.3**	11.87 ± 1.02	3135 ± 473**+
% of PRL cells	34.18 ± 1.93	44.40 ± 2.25**	35.36 ± 2.61	65.37 ± 1.63**+
Proliferation of PRL cells				
BrdU (%)	0.22 ± 0.14	1.34 ± 0.23**	0.36 ± 0.14	1.91 ± 0.14**
PCNA (%)	1.67 ± 0.19	11.02 ± 1.56**	1.59 ± 0.45	12.36 ± 1.34**
Size of nuclear profile (μm^2)	28.76 ± 1.14	32.70 ± 0.61*	27.41 ± 1.52	31.35 ± 1.07*
Size of AgNORs (μm^2)	1.03 ± 0.05	2.02 ± 0.07**	1.25 ± 0.13	2.57 ± 0.15*+
No. of AgNORs / nuclear profile	1.41 ± 0.03	1.65 ± 0.04**	1.73 ± 0.06#	2.07 ± 0.06**+

Data are expressed as the mean ± standard error of the mean.
Differences to matched control: *p<0.05, **p<0.01.
Differences between control groups: #p<0.05.
Differences to group treated with E_2 for 3 weeks: +p<0.005.

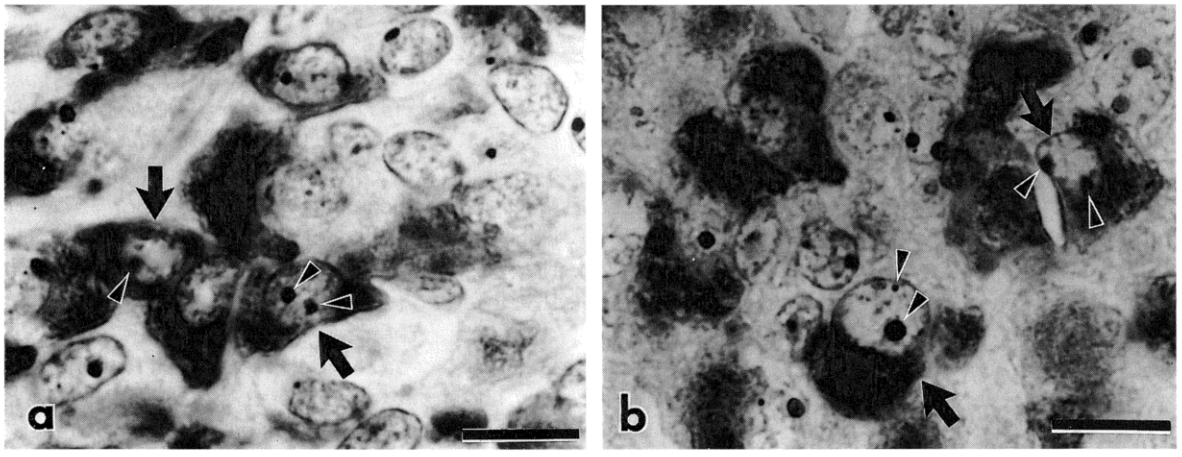

Fig. 1. Argyrophilic nucleolar organizer regions (AgNORs) in pituitary cells of the rat. Combined AgNOR staining and immunohistochemistry for PRL. (**a**) Control (14 weeks old). (**b**) E_2 treatment for 10 weeks (14 weeks old). Note the enlargement of AgNORs in pituitary PRL cells treated with E_2. Arrows and arrowheads indicate PRL-immunoreactive cells and AgNORs, respectively. Bar: 10 μm.

significantly after E_2 treatment (Table 1). The size was significantly greater in the rats treated with E_2 for 10 weeks than for 3 weeks. Thus, the size of AgNORs was dependent on the duration of E_2 treatment. The size of nuclear profile in PRL cells was also increased following 3 and 10 weeks of E_2 treatment relative to that in each matched control group (Fig. 1, Table 1). The size, however, was almost constant between two E_2-treated groups. Although the number of AgNORs in the controls increased significantly with age between 7 and 14 weeks, the treatment with E_2 further increased the mean number of AgNORs per nuclear profile. This increase was also dependent on the duration of E_2 treatment (Table 1).

On the other hand, although the size of AgNORs in PRL-immunonegative pituitary cells significantly increased in E_2-treated rats, changes in the number of AgNORs were not observed.

Experiment II

After treatment with BC or CG for 30 days, serum levels of PRL significantly were reduced compared with the vehicle-treated (control) group but did not reduce the percentage of PRL-immunoreactive cells. CG treatment also significantly

Table 2. Combined effects of E_2 and dopamine agonists on pituitary PRL cells in female rats

Ttreatment[a)]	Control (n=4)	Dopamine agonists	
		Bromocriptine (n=5)	Cabergoline (n=5)
Pituitary weight (mg)	360.9 ± 23.2	312.4 ± 38.5	136.4 ± 19.5**+
Serum PRL levels (ng/ml)	5269 ± 278	4184 ± 283*	153.6 ± 38.3**++
% of PRL cells	56.03 ± 1.38	55.95 ± 1.27	58.10 ± 1.03
Proliferation of PRL cells PCNA (%)	5.24 ± 1.40	4.42 ± 1.27	1.05 ± 0.42*+
Size of nuclear profile (μm^2)	29.13 ± 0.93	29.36 ± 0.50	25.44 ± 0.84*+
Size of AgNORs (μm^2)	1.97 ± 0.04	1.68 ± 0.07*	1.24 ± 0.06**+
No. of AgNORs / nuclear profile	1.82 ± 0.09	1.45 ± 0.04**	1.27 ± 0.02**+

[a)] After 10 weeks treatment with E_2, animals received a dopamine agonist or vehicle (control) every third day for 30 days.
Differences to control: *p<0.05, **p<0.01.
Differences to group treated with bromocriptine: +p<0.05, ++p<0.01.

reduced the weight of pituitary glands and the proliferative activity of PRL cells. In contrast, BC treatment did not alter these parameters compared with controls (Table 2).

Each dopamine agonist significantly reduced the mean size of AgNORs and the degree was more marked in the CG- than in the BC-treated group (Table 2). This situation was similar for the number of AgNORs per nuclear profile in PRL cells. The decrease in size and number of AgNORs induced by each dopamine agonist was more pronounced than that in the size of the nuclear profile. In contrast, there were no differences in the number and size of AgNORs between the control and dopamine agonist-treated groups in PRL-immunonegative cells.

Regression analysis in experiments I and II

In control groups of the experiment I, no correlation was observed between the number of AgNORs per nuclear profile in PRL cells and the serum PRL levels or the proliferative activity of PRL cells. There was also no such correlation for the size of AgNORs. In E_2-treated groups of the experiment I and in the experiment II, the number and the size of AgNORs were significantly correlated with serum levels of PRL ($p<0.05$). However, these changes of AgNORs were not related to the proliferative activity (both BrdU and PCNA) of PRL cells, except for a significant correlation observed between the size of AgNORs and the proliferative activity of PRL cells in the experiment II ($p<0.05$).

DISCUSSION

An increase in the size and number of nucleoli implies the amplified synthesis of ribosomal RNA in response to the demand for more cellular proteins (Boldy *et al.*, 1989). Marked changes in the size and number of AgNORs in PRL cells were induced under conditions increasing or decreasing cellular function. These changes were specific for PRL cells and more closely related with serum PRL levels than the proliferation of PRL cells. However, there was no correlation between serum PRL levels and the number or size of AgNORs in PRL cells in control rats. The close relationship between PRL secretion and E_2 has been well documented. Stefaneanu *et al.* (1989) have shown that the number of AgNORs in PRL cells increased with the advance of pregnancy in human pituitary glands, suggesting that changes of AgNORs may related with the progressive rise in circulating E_2. Therefore, the temporal rise in circulating E_2 levels may fail to induce the changes of AgNORs in PRL cells under a physiological condition (during the estrous cycle) in female rats.

E_2 stimulates PRL secretion and the proliferation of PRL cells (Lloyd *et al.*, 1975). The marked elevation in serum levels of PRL induced by prolonged treatment was due in part to a direct action of E_2 on the pituitary, which is in agreement with the increase in PRL gene transcription (Seo *et al.*, 1979) and synthesis and release of PRL (Phelps and Hymer, 1983; Maurer, 1982). Furthermore, E_2 increased the number of ribosomes and rough endoplasmic reticula in PRL cells (Aumüller *et al.*, 1978), suggesting elevated ribosomal gene transcription.

On the other hand, a dopamine agonist, BC, suppressed

the mitosis of PRL cells as well as PRL gene transcription and secretion of PRL in the anterior pituitary gland (Lloyd *et al.*, 1975; Maurer, 1980). BC decreased the incidence of ribosomes, rough endoplasmic reticulum, and Golgi complexes in PRL cells (Rengachary *et al.*, 1982; Tindall *et al.*, 1982). Therefore, protein synthesis involves the changes in the size and number of AgNORs in PRL cells. However, why the changes of AgNORs in PRL cells were more closely related to hormonal activity than to the proliferative activity of PRL cells, of which are prerequisite for protein synthesis, remains unclear. Similar findings have been reported by previous investigators (Peebles and McNicol, 1989; Shibuya *et al.*, 1992). Peebles and McNicol (1989) reported that adrenalectomy, which stimulates the secretion of adrenocorticotropic hormone (ACTH), increased the number of AgNORs in ACTH-producing cells in rat pituitaries.

In contrast, several investigators reported that the number of AgNORs depended on the proliferation of tumor cells but not on the hormonal activity of pituitary nontumorous or adenoma cells (Bayindir *et al.*, 1992; Stefaneanu *et al.*, 1989). These inconsistent results may reflect technical problems, for they did not assess AgNORs in a specific type of pituitary cells followed by immunocytochemical evaluation.

Previous investigators using the reverse hemolytic plaque assay and the cell immunoblot assay have found that basal PRL secretion is higher at the single cell level in chronically E_2-treated rats than in control rats (Lloyd *et al.*,1987; Kendall and Hymer, 1987). Using a cell immunoblot assay, we found that basal PRL secretion from single cells is lower in dopamine agonist-treated rats than in control rats (unpublished data). These findings indicate that the circulating PRL level is influenced by the rate of hormone secretion from PRL cells. The changes in the number and size of AgNORs, therefore may reflect the the secretory activity of pituitary cells.

The number of AgNORs per nuclear profile in PRL cells was significantly greater in vehicle-treated control rats at 14 weeks old than at 7 weeks old, although the serum level of PRL and the proliferation of PRL cells did not differ significantly between the two control groups in diestrous phase. Therefore, the changes of AgNORs in PRL cells under the normal growth condition may be related to cellular differentiation or maturation (Smetana and Likovsky, 1984) rather than the hormonal or proliferative activity. Carbajo *et al.* (1993) also concluded that the expression of AgNORs in pituitary cells was related more to cellular maturation than to proliferative activity during postnatal development of rats. The majority of PRL cells in the pituitary of 7-week-old rats (control rats comparable to E_2 treatment for 3 weeks) used in this study, therefore may not have fully matured from the immature or intermediate types (Takahashi and Miyatake, 1991).

The present study has shown that the size of AgNORs changes more markedly than the nuclear profile under conditions increasing or decreasing cellular function. However, the increase in size of AgNORs cannot be dismissed as a result of enlargement of nuclear structures by E_2, for PRL-immunonegative cells also showed such changes. Alternatively, we did not directly demonstrate a relationship between the number and size of AgNORs and the secretory activity of PRL cells. We therefore could not exclude other possible influences, such as cellular transformation (Crocker,1990). Thus, the argyrophilic method for demonstrating the proteins associated with the nucleolar organizer region permits an additional approach to study of pituitary cells, although the precise biochemical nature of AgNORs is unclear.

In conclusion, the present findings suggest that the number and size of AgNORs in PRL cells were associated with serum levels of PRL, probably secretory activity rather than with the proliferatiion of PRL cells under conditions stimulating and inhibiting cellular function, but the cause of an increase in the number of AgNORs under normal growth conditions remains unknown.

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