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—Full Paper—

Estrogen Regulates the Serum Level of Phosphorylated Prolactin in Mice

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Abstract. Phosphorylated prolactin (PPRL) is considered to be the most quantitatively important post-translationally modified form of prolactin (PRL) in rodents. We recently detected two different types of PPRL in the mouse pituitary gland; one was phosphorylated at serine and the other was phosphorylated at serine/threonine. Furthermore, we showed that there are obvious differences in the ratios between PPRLs and non-phosphorylated PRL in the pituitary gland based on age and sex and that estrogen influences PRL phosphorylation at serine in female mice. In the present study, we examined whether estradiol (E2) increases serine PPRL in the male pituitary gland in the same manner as in the female pituitary gland and examined whether PPRL is released into serum. We first determined the relative amounts of intrapituitary PPRLs in male mice under different pharmacological conditions that increased PRL secretion. The results indicated that treatment with E2 increases serine PPRL. We then performed two-dimensional electrophoresis and immunoblotting analysis after immunoprecipitation with anti-mouse PRL antibody using male and female sera under different pharmacological conditions that increased PRL secretion. The results of this experiment indicated that there were PRLs phosphorylated at serine and serine/threonine in the female serum but not in the male serum. The levels of PPRLs in sera were greatly increased with the E2 treatment for both male and female sera. Furthermore, we examined the effect of E2 on PPRL synthesis in cultured male pituitary glands. In this experiment, we observed increased serine PPRL synthesis and stronger immunohistochemical staining of PRL cells with E2 treatment. These findings suggested that serine PPRL synthesis and secretion were influenced by estrogen.

Key words: Immunoprecipitation, Mouse, Phosphorylated prolactin, Serum

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Phosphorylation of PRL in the anterior pituitary gland has been demonstrated in the human, rat [1], chicken, turkey [2] and bovine [3]. An analysis of phosphorylation sites demonstrated that the serine residue at amino acid position 179 in human PRL is a site of phosphorylation [4]. Previous studies have shown that purified rat PRL is phosphorylated *in vitro* by γ-PAK [5]. This phosphorylated PRL (PPRL) antagonizes the growth effect of unmodified PRL in the Nb2 T lymphoma assay [4, 6]. In a recent study, we identified three different charge types of PRL in mice [7]. Isoform 1 is non-phosphorylated PRL, while isoforms 2 and 3 are phosphorylated at serine or serine and threonine, respectively. The ratio between serine PPRL and non-phosphorylated PRL in adult female mice is higher than that of adult

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male mice; however, the ratio between serine/threonine PPRL and non-phosphorylated PRL is higher in adult male mice than in adult female mice. Furthermore, we suggested that estrogen might influence serine phosphorylation of PRL [7]. However, it is unclear whether PPRLs were secreted into the blood circulation and which factors, besides estrogen, promote phosphorylation of PRL.

The present study demonstrated that PPRL could be detected by two-dimensional electrophoresis and immunoblotting analysis with anti-PRL antibody after immunoprecipitation of mouse serum. We also noted that serum PPRL was altered under different physiological conditions. Furthermore, treatment with E2 increased PPRL synthesis in the *in vitro* cultured pituitary glands.

Materials and Methods

Animals

The experimental design of this study was in compliance with the guidelines for animal experiments of this institution. ICR mice were housed under a controlled temperature (22 ± 2 C) in an artificially illuminated room (12-h light/12-h dark). Food and tap water were available *ad libitum*. At 70–90 days of age, females were mated with males, and the day a vaginal plug was identified was designated as day 0 of pregnancy. Parturition regularly occurred on day 19 of pregnancy. The day of birth was designated as day 0 of infancy. Blood was collected from male mice at 60 days old and from cycling female mice during each stage of the estrous cycle. Some of the 60-day-old female mice were ovariectomized under ether anesthesia and 7 euthanized days later. Sham operations were performed as a control. Two days after OVX, silastic medical grade tubes (Kaneka, Osaka, Japan) filled with 10 mg of 17β-estradiol (E2; Sigma-Aldrich, St. Louis, MO, USA) were implanted into some of the OVX and male mice for 7 days. Silastic tubes without E2 were implanted into the control mice. Some of the 60-day-old male mice were given tap water containing metoclopramide (20 mg/l; Wako Pure Chemicals, Osaka, Japan) for 4 weeks. Water intake was monitored throughout the experiment, and the calculated dose of metoclopramide was approximately 0.2 mg per 100 g body weight per day. The estrous cycles of female mice were determined by examination of vaginal smears obtained between 0900 and 1000 h each day. Blood samples were collected between 1600 h and 1800 h under ether anesthesia, stored at room temperature for 30 min and centrifuged at 15,000 *g* for 5 min. The supernatants were collected as serum samples and stored at –20 C until further analysis. Each pituitary was ultrasonically disrupted in sample buffer containing 9.8 M Urea, 4% (w/v) Triton X-100 and 40 mM Tris base and centrifuged at 15,000 *g* for 15 min. The supernatant was stored at –80 C.

Immunoprecipitation

We performed immunoprecipitation to evaluate the level of PPRL in mouse serum using an Immunoprecipitation Kit (Roche Diagnotics, Mannheim, Germany) in accordance with the manufacturer's protocol. Serum samples from three mice were pooled together to form samples of 600 μl for each group. The samples were mixed with 400 μl buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40 and 0.5% sodium deoxycholate) to obtain 1 ml of sample solution, and this was then incubated with 0.5 μl of primary rabbit antiserum raised against recombinant PRL [8] at 4 C for 1 h with continuous shaking. Protein A beads were then incubated with the samples at 4 C overnight with continuous shaking. After incubation, the beads were collected by centrifugation at 12,000 *g* and washed with wash buffer and beads were added to 70 μl sample buffer containing 9.8 M urea, 4% (w/v) Triton X-100 and 40 mM Tris base and denatured by heating to 100 C for 3 min. They were then removed by centrifugation at 12,000 *g* at 20 C, and the supernatants were used for two-dimensional electrophoresis.

Two-dimensional electrophoresis (2-DE)

Two-DE was performed in a horizontal 2-DE setup (IPG Multiphore; GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA), as described in detail previously [9] and essentially according to the procedure of Gorg [10]. The pituitary (containing 125 μg protein) and immunoprecipitation samples were applied by in-gel rehydration (IPG pH 3–10) for 12 h according to the manufacturer's instruction. The proteins were then focused for up to 91,000 Vh at a maximum voltage of 3,500 V.

IPGs were either used immediately for a second

electrophoretic analysis or stored at –80 C until further analysis. The second dimension of electrophoresis was resolved on 12.5% SDS-polyacrylamide gels. After electrophoresis, proteins were fixed in 50% methanol/7% acetic acid for 1 h and then stained in 0.1% coomassie brilliant blue (CBB) in 50% methanol/1% acetic acid for 1 h. Gels were destained in 30% methanol/1% acetic acid overnight and then placed in deionized water for 30 min. The staining and washing steps were performed with continuous gentle agitation. The visualized 2-DE protein patterns were analyzed using ImageMaster 2D Platinum ver. 5.0 (GE Healthcare Bio-Sciences).

Immunoblotting

After 2-DE, separated proteins were electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA) and immersed in primary rabbit antiserum raised against recombinant PRL. Antigen-antibody complexes were detected using a biotinylated secondary antibody and streptavidin-biotin-horseradish peroxidase complex using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). The specific proteins detected by each antibody were stained with 3,3′-diaminobenzidine as a chromogen.

Organ culture

The anterior pituitary glands of 60-day-old male mice were cut at the isthmus into two equivalent halves. These glands were preincubated in synthetic medium 199 for 1 h at 37 C in an atmosphere of 95% O_2/5% CO_2. The experimental group was incubated in medium 199 supplemented with 10^{-6} M 17β-estradiol (E2). The control group was incubated in medium 199 without E2. These pituitary glands were used for 2-DE and Immunohistochemical analyses after 2 or 6 h of incubation. The pituitary glands were dissolved in sample buffer and resolved by 2-DE. The media were added to four volumes of acetone, and the mixtures were kept at –80 C for 6 hours. The protein precipitate was separated and dissolved in sample buffer by 2-DE. IPG gels of pituitary gland from E2-treated and control mice were resolved simultaneously by 2-DE on 12.5% SDS-polyacrylamide gels and stained with CBB. The CBB-stained gels were used to compare the PRL density ratio of the control group to that of the E2-treated group. IPG gels of media were resolved simultaneously by 2-DE on 12.5% SDS-polyacrylamide gels and used for immunoblotting with rabbit antiserum raised against recombinant PRL.

Statistics

All experiments were performed on at least three animals. The data is presented as the mean ± SEM where applicable. The statistical significance of differences was examined using one-way analysis of variance.

Results

Densitometric analysis of the ratio of PPRL to isoform 1 in the pituitary gland

The ratio of isoform 2 to isoform 1 was 0.4 and that of isoform 3 to isoform 1 was 0.6 in the 60-day-old male mice (Fig. 1A and D). Implantation of silastic tubes containing E2 into 60-day-old male mice resulted in a large increase in the ratio of isoform 2 to isoform 1 (increased to 0.8); however, the ratio of isoform 3 to isoform 1 decreased from 0.6 to 0.2 (Fig. 1B and D). There were no differences between the sham-operated and 60-day-old mice (data not shown). Administration of the dopamine antagonist metoclopramide, which is known to increase prolactin secretion [11] in male mice, did not increase serine or serine/threonine PPRL in the pituitary gland (Fig. 1C and D).

PPRL in mouse serum

We examined whether mouse PRL exists in serum as a phosphorylated form under different physiological conditions. In the case of the male mouse, PPRLs were found in the pituitary gland (Fig. 1A), but only non-phosphorylated PRL was found in the serum (Fig. 2A). However, we identified the appearance of PPRL in the pituitary gland and serum of male mice treated with E2 (Fig. 1A and 2B). There was an increase in PPRL at serine and serine/threonine in the 60-day-old mice after administration of metoclopramide for 4 weeks (Fig. 2C). Serine/threonine PPRL was absent from the serum of the 60-day-old female mice during proestrus (Fig. 2D); however, serine and serine/threonine PPRL were present in their serum during the other stages of the estrous cycle (Fig. 2E). In the case of the OVX mice, PPRLs were greatly reduced,

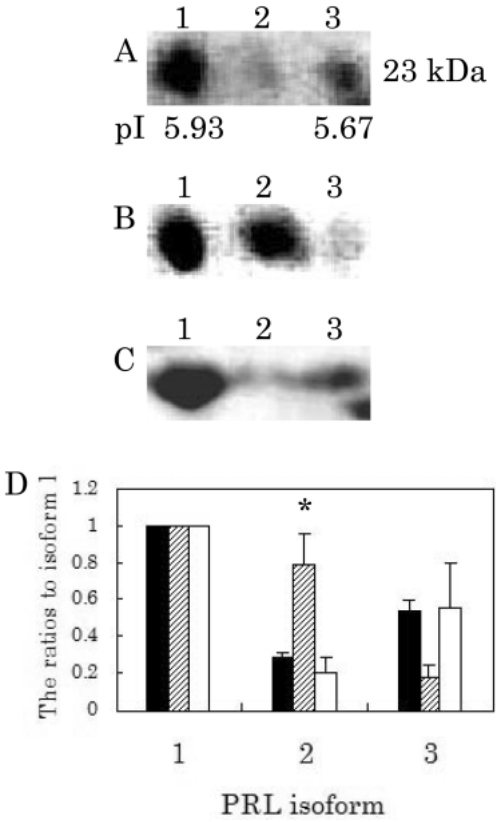

Fig. 1. The effect of E2 and metoclopramide on the ratio of PPRL to PRL in the male pituitary gland. A: 60-day-old male mice. B: 7 days after E2 implantation in 60-day-old male mice. C: 60-day-old male mice after drinking tap water containing metoclopramide for 4 weeks. D: Densitometric analysis of CBB staining. These graphs indicate relative values for isoforms 2 and 3 to isoform 1, the value of which was designated as 1. Closed bars: 60-day-old male. Hatched bar: 7 days after E2 implantation in 60-day-old male mice. Open bars: 60-day-old male drinking tap water containing metoclopramide for 4 weeks (n=4). 1, 2 and 3: isoforms 1, 2 and 3, respectively. An asterisk indicates a significant difference from 60-day-old male mice ($P<0.05$).

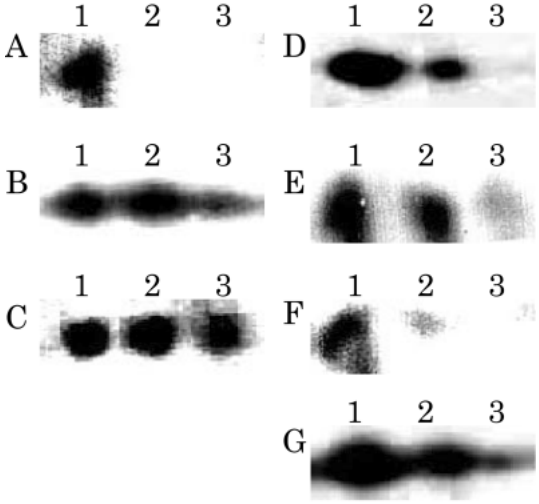

Fig. 2. Two-dimensional electrophoresis and immunoblotting after immunoprecipitation for the PRL isoforms in serum. A: 60-day-old male. B: 60-day-old male with E2 implantation for 7 days. C: 60-day-old male after drinking tap water containing metoclopramide for 4 weeks. D: 60-day-old female at proestrus. E: 60-day-old female at estrus. F: 60-day-old female 7 days after OVX. G: 60-day-old female 2 days after OVX and 7 days after E2 implantation. 1, 2 and 3: isoforms 1, 2 and 3, respectively.

and no serine/threonine PPRL was identified in their serum (Fig. 2F). Furthermore, implantation of silastic tubes containing E2 for 7 days 2 days after OVX increased the serum levels of serine PPRL in the OVX mice (Fig. 2G).

Organ culture of male mouse pituitary glands

There was no difference between the ratios of isoform 2 to isoform 1 and isoform 3 to isoform 1 for the control pituitaries after 2 h of incubation; both were 0.2 (Fig. 3A and B). However, the ratio of isoform 2 to isoform 1 in E2-treated pituitaries after incubation for 2 h increased to 0.3, and the ratio of isoform 3 to isoform 1 was similar to that of the control (Fig. 3A′ and B). Similarly, the ratio of isoform 2 to 1 in the E2-treated pituitaries increased to 0.4 after incubation for 6 h, whereas the ratio of isoform 3 to isoform 1 did not change (Fig. 3C, C′ and D).

Implantation E2 greatly induced an increase in both non-phosphorylated PRL and PPRL content in the pituitary gland. After treatment with E2 for 2 h, isoforms 1 and 3 increased to about 1.2 times higher than the levels of the control, respectively. However, isoform 2 increased to about 1.7 times the level of the control (Fig. 3E). Similarly, isoform 2 increased by more than isoform 1 or 3 after incubation for 6 h. In regard to the three isoforms in the pituitary glands i*n vitro*, treatment with E2 only influenced the increase in isoform 2.

No PPRLs were found in the media after incubation for 2 h (data not shown), but serine PPRL was found in the media treated with E2 after incubation for 6 h. However, only non-phosphorylated PRL was present in the control

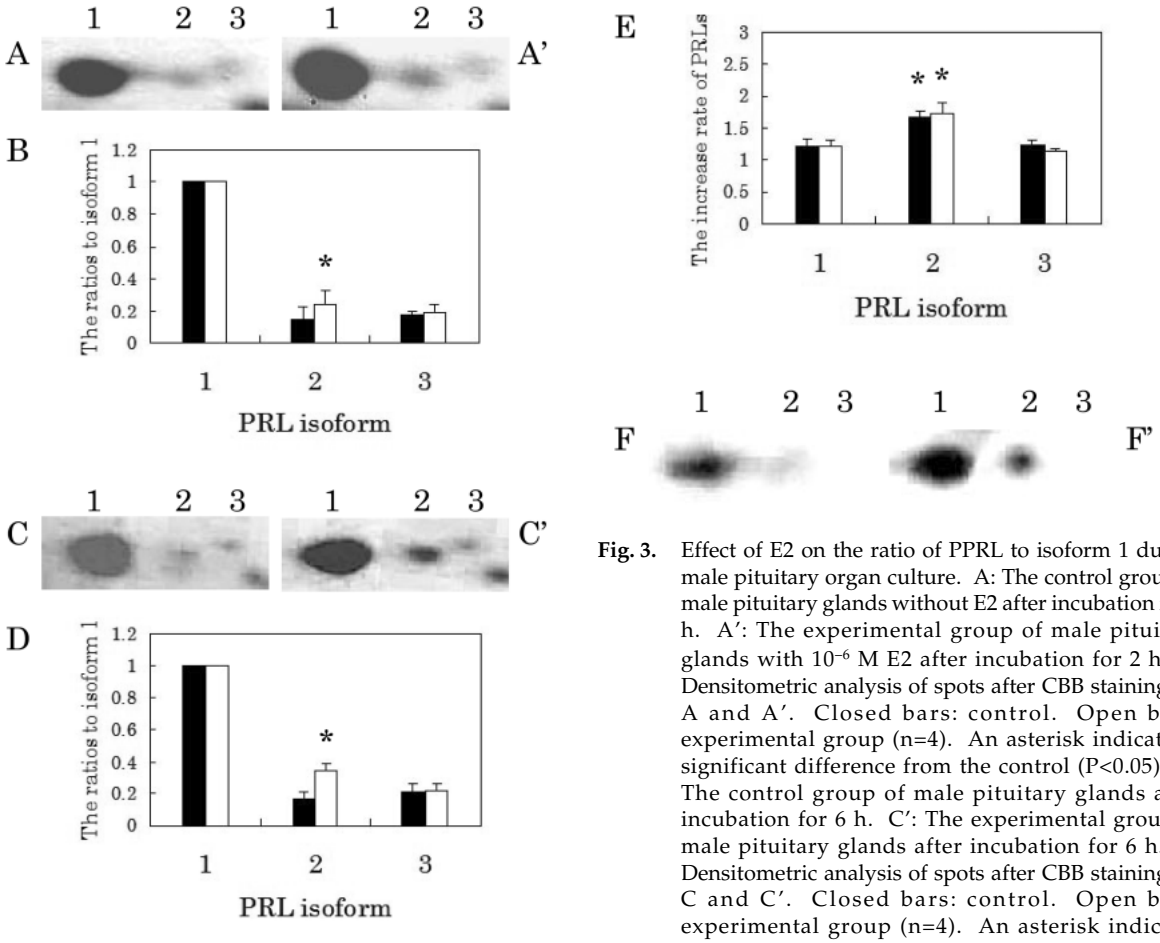

Fig. 3. Effect of E2 on the ratio of PPRL to isoform 1 during male pituitary organ culture. A: The control group of male pituitary glands without E2 after incubation for 2 h. A′: The experimental group of male pituitary glands with 10^{-6} M E2 after incubation for 2 h. B: Densitometric analysis of spots after CBB staining for A and A′. Closed bars: control. Open bars: experimental group (n=4). An asterisk indicates a significant difference from the control ($P<0.05$). C: The control group of male pituitary glands after incubation for 6 h. C′: The experimental group of male pituitary glands after incubation for 6 h. D: Densitometric analysis of spots after CBB staining for C and C′. Closed bars: control. Open bars: experimental group (n=4). An asterisk indicates significant difference from the control ($P<0.05$). E: Increase in the rates of isoform 1, 2 and 3 with E2 treatment compared with each isoform of the control. Closed bars: 2 h incubation. Open bars: 6 h incubation (n=4). Asterisks indicate significant differences in the increase rates with E2 treatment compared with that of the control ($P<0.05$). F: The control group of media after incubation for 6 h. F′: The experimental group of media after incubation for 6 h.

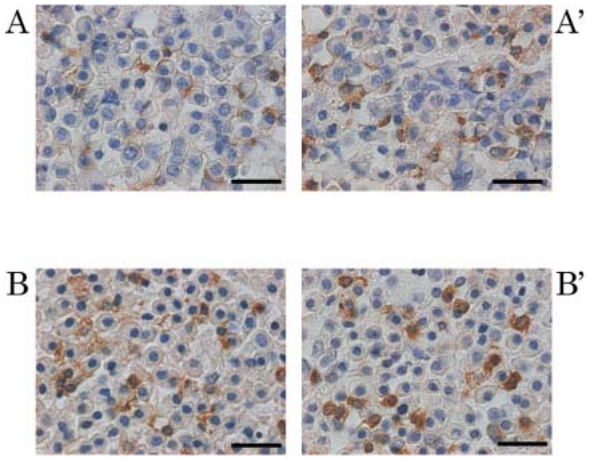

Fig. 4. Effect of E2 on the morphological change in PRL cell type during male pituitary organ culture. A: Immunohistochemistry with anti-PRL antibody for the control group after incubation for 2 h. A′: Immunohistochemistry with anti-PRL antibody for the experimental group after incubation for 2 h. B: Immunohistochemistry with anti-PRL antibody for the control group after incubation for 6 h. B′: Immunohistochemistry with anti-PRL antibody for the experimental group after incubation for 6 h. Scale bar=20 μm.

media (Fig. 3F and 3F′).

PRL-immunopositive cells were unchanged after treatment with E2 for 2 h (Fig. 4A and A′). However, the cytoplasm of the PRL-immunopositive cells was densely stained and thick compared with that of control cells after incubation for 6 h (Fig. 4B and B′).

Discussion

Sex differences have been observed in regard to the ratio of PPRL to PRL in the mouse pituitary gland [7]. However, the ratio of PPRL in serum has not been determined. In this study, we identified PPRL in the serum of female mice; however, PPRL was not present in male serum. The ratio of isoform 2 to isoform 1 in the adult female mouse pituitary gland was higher than that in the adult male, but the opposite was true for the ratio of isoform 3 to isoform 1. There might not have been any serine PPRL in the serum because its production was low in the male pituitary gland. Several studies have demonstrated the physiological action of serine PPRL; that is, it is an effective antagonist for unmodified PRL-induced proliferation of Nb2 cells [4, 12]. Walker *et al*. also found that serine PPRL increases transcripts for β-casein compared with non-phosphorylated PRL in a normal murine mammary cell line. They demonstrated serine PPRL is a PRL receptor antagonist and that it antagonizes PRL-induced proliferation in many tissues [13, 14]. However, no one has been able to show the function of serine/threonine PPRL. We previously identified that serine/threonine PPRL is relatively abundant in the pituitary gland of the male mouse [7]; however, it was not present in male serum in the present study. Thus, serine/threonine PPRL might have a role in storage in secretory granules or an affect on neighboring PRL cells through an autocrine and/or paracrine pathway.

Furthermore, we showed that administration of E2 to male and OVX female mice raised the levels of non-phosphorylated PRL and serine PPRL in their sera. These results were also consistent with our previous report showing that the level of serine PPRL in the pituitary was increased by E2 [7]. On the other hand, E2 did not increase the level of serine/threonine PPRL in the male pituitary gland. The absence of serine/threonine PPRL in pituitary glands of proestrus 60-day-old female mice [7] seems to suggest that E2 induces dephosphorylation at threonine of serine/threonine PPRL in pituitary gland. In the present study, E2 increased the levels of both serine and serine/threonine PPRL in the serum of 60-day-old male mice. Organ culture of male pituitary gland showed that the levels of non-phosphorylated PRL and serine/threonine PPRL increased to about 1.2 times those of the control after E2 treatment. Furthermore, E2 treatment for 7 days increased the levels of non-phosphorylated PRL and serine/threonine PPRL to about three times those of the OVX mice; serine PPRL was further increased to about six times the level of the OVX mice *in vivo* [7]. Although serine/threonine PPRL was not found in the media treated with E2 after incubation for 6 h during organ culture, we did identify the appearance of both serine and serine/threonine PPRL in the serum of mice treated with E2 *in vivo*. Excess and long-term E2 treatment may increase the levels of both non-phosphorylated PRL and PPRLs in the pituitary gland and may induce secretion into serum.

Conversely, dopamine is the major PRL inhibiting factor. Metoclopramide is a potent dopamine antagonist that enters the central nervous system. It acts on the dopamine 2 receptor and induces an increase of PRL levels in serum [15, 16]. We examined whether the levels of PPRL in the pituitary gland and serum were changed by inhibiting dopamine action using its antagonist. In this regard, we determined that the levels of PPRLs did not increase in the pituitary glands of the male mice. However, PPRLs were present in male serum. Secretion of PPRLs from the pituitary gland into serum may have been induced by the dopaminergic antagonist metoclopramide, and they may not have been promoted to phosphorylate PRL because the level of serine PPRL was not increased in the pituitary gland. We concluded that estrogen not only induced an increase in PRL synthesis, but it also induced phosphorylation of PRL at serine.

Our hypothesis, based upon our previous findings [7], is that phosphorylation of PRL seems to be related not only to PRL cell type of but also to the shape of the secretory granules, since the granules are frequently polygonal in certain PRL-producing cells. Prolactin-producing cells concentrate secretory products within the dense

matrix of membrane-bound secretory granules, which are stored and aggregated in the cytoplasm, and respond to specific secretagogues by releasing prolactin via exocytosis [17]. It has been suggested that PRL has the capacity to self-aggregate in the trans-golgi network without aggregation factors, like granins [17]. Phosphorylation of PRL occurs in pituitary secretory granules as a result of γ-PAK [5]. PRL cells are classified into types I, II and III based upon the size and shape of secretory granules as follows: 100 nm in diameter is type I, 150–200 nm is type II and 300 nm is type III, respectively. In both male and female mice, E2 implants also increase the proportion of type III PRL cells and decrease the proportion of type I and II PRL cells [18, 19]. Furthermore, OVX mice have a decreased proportion of type III PRL cells [20]. In this regard, we observed that the level of PPRL was increased in E2-treated cultured pituitary glands, and immunohistochemistry showed that the PRL cells were densely stained. However, little is known about the direct morphological effects of dopamine on the secretory granules of PRL-producing cells. The variation in the levels of serine PPRL in the pituitary glands and sera seemed consistent with that of type III cells in pituitary glands in our previous report [7]. These results suggest that PPRL is strongly related to accumulation of secretory granules, which induces the polymorphic change in granules.

Acknowledgments

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