The Effect of Estrogen on Phosphorylation of Prolactin in the Mouse Pituitary Gland

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Abstract. Several studies have indicated that prolactin (PRL) assumes oligomeric, proteolytically cleaved, phosphorylated and glycosylated forms. Phosphorylated PRL (PPRL) is considered to be the most important posttranslationally modified form in the rat. In the present study, we examined whether or not PRL is present in the mouse pituitary gland in the phosphorylated form. Mouse pituitary PRL was digested with acid phosphatase, resolved by two-dimensional gel electrophoresis, stained with Coomassie brilliant blue, and then immunoblotted against the anti-PRL, anti-phosphoserine and anti-phosphothreonine antibodies. We also examined whether PRL is phosphorylated by protein kinases and semi-quantified the ratios of PPRL to PRL in the pituitary gland. The results indicated that three types of PRL are present in the pituitary glands of both male and female mice. One was non-phosphorylated (isoform 1), and the other two were immunoreactive to anti-phosphoserine (isoform 2) and/or anti-phosphothreonine (isoform 3) antibodies. The ratio between isoforms 2 and 1 of the 30-day-old female mice was higher than that of the 20-day-old female mice. However, the ratios among the three isoforms in the male pituitary glands did not differ with age. The ratio of PPRL to isoform 1 was obviously reduced after ovariectomy (OVX), and it recovered with estrogen replacement. These results suggest that estrogen influences PRL phosphorylation in female mice.

Key words: Mouse, Ovariectomy (OVX), Phosphorylated prolactin, Pituitary, Two-dimensional electrophoresis (2-DE)

(J. Reprod. Dev. 53: 515–523, 2007)

Prolactin (PRL) is a polypeptide hormone that is synthesized by and secreted from PRL cells in the anterior pituitary gland. The important feature of PRL is that it assumes several forms in addition to the major 23 kDa form in the pituitary glands of many species. Proteolytic cleavage and other posttranslational modifications of the amino acid chain result in these various forms [1]. The 16 kDa N-terminal proteolytic cleavage product of PRL (16K-PRL) potently inhibits angiogenesis [2, 3]. Anterior pituitary PRL undergoes phosphorylation in rats, humans [4], chickens, turkeys [5] and cattle [6]. Phosphate analysis of phosphorylated PRL (PPRL) has shown that the ratio of non-phosphorylated PRL to PPRL is species-specific [5, 6]. Analysis of phosphorylation sites has demonstrated one at serine 179th (177th in rat) in human PRL [7]. A minor phosphorylation site has also been identified at threonine 58rd or 63rd in rat PRL [7]. Bovine PRL is primarily phosphorylated at serine 90th with minor phosphorylation sites at serine residues 26th and 34th [8]. PPRL in the rat antagonizes the growth effect of
unmodified PRL in the Nb2 T lymphoma assay [9, 10]. PPRL might play a unique role as an autocrine regulator of PRL secretion since it suppresses the release of non-phosphorylated PRL from GH3 cells [11].

In the present study, electrophoretic mobility on two-dimensional electrophoresis and immunoblotting with anti-PRL, anti-phosphoserine, or anti-phosphothreonine antibodies showed that mouse PRL is phosphorylated on either a serine or threonine residue in the pituitary gland and that mouse PRL is phosphorylated by some protein kinases. We also determined the relative amounts of intrapituitary PRL isoforms under various physiological conditions.

Materials and Methods

Samples

The experimental protocol proceeded according to our institutional guidelines for animal experiments. We housed ICR mice at 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 that a vaginal plug appeared was designated as day 0 of pregnancy. Parturition regularly occurred on day 19 of pregnancy. The day of birth was designated as newborn day 0. Pituitary glands were collected from 20-, 30- and 60-day-old male and female mice and at each estrus stage for 60-day-old cycling female mice. Some of the 60-day-old female mice were ovariectomized (OVX) under ether anesthesia and euthanized 2 or 7 days later. Control mice underwent a sham operation. Silastic® medical grade tube (Kaneka, Osaka, Japan) containing 10 mg of 17β-estradiol (E2: Sigma-Aldrich, St. Louis, MO, USA) were implanted into some of the mice 2 days after OVX and were left in place for 7 days. Silastic tube without E2 were implanted into the control mice. Blood samples were collected from O VX- and E2-treated mice under ether anesthesia, stored at room temperature for 30 min, and centrifuged at 15,000 g for 5 min. The serum E2 concentration was measured by RIA. The estrous cycles of female mice were determined from vaginal smears examined daily between 0900 h and 1000 h. All mice were euthanized between 1600 h and 1800 h under ether anesthesia. The pituitary gland was removed and used immediately or stored at −80 C. Each pituitary was ultrasonically disrupted in sample buffer [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.

Two-dimensional electrophoresis (2-DE)

Samples were resolved by horizontal 2-DE (IPG Multiphore; GE Healthcare Bio-Sciences, Piscataway, NJ, USA) as described previously [12] and essentially according to the procedure of Gorg [13]. The samples (containing 125 µg protein) were applied by in-gel (IPG, pH 3–10) rehydration for 12 h according to the manufacturer’s instructions. The proteins were then focused for up to 91,000 Vh at a maximum voltage of 3,500 V.

The IPGs were used immediately for a second electrophoretic analysis or stored at −80 C. Proteins were resolved in the second dimension on 12.5% sodium dodecyl sulfate SDS-polyacrylamide gels, fixed in 50% methanol/7% acetic acid for 1 h, and then stained with 0.1% Coomassie brilliant blue (CBB) in 50% methanol/1% acetic acid for 1 h. The gels were destained in 30% methanol/1% acetic acid overnight and soaked in deionized water for 30 min. Staining and washing proceeded under continuous gentle agitation. The visualized 2-DE protein profiles were analyzed using ImageMaster 2D Platinum ver. 5.0 (GE Healthcare Bio-Sciences).

Immunoblotting

After 2-DE, the separated proteins were electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). Non-specific binding was blocked with 0.01% normal goat serum in Tris buffer for 30 min at room temperature. The membranes were subsequently immersed in primary rabbit antiserum against recombinant PRL [14], phosphoserine (Chemicon International, Temecula, CA, USA), or phosphothreonine (Stressgen Biotechnologies, San Diego, CA, USA). Antigen-antibody complexes were detected using the biotinylated secondary antibody and streptavidin-biotin-horseradish peroxidase complex in a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA). The specific proteins detected by each antibody were visualized using 3,3′-diaminobenzidine (DAB) as the chromogen.
Dephosphorylation

After 2-DE and CBB staining, PRL spots were extracted with Tris buffer [20 mM Tris-HCl, 1% (w/v) SDS] and precipitated with acetone. The precipitates (1 µg) were incubated with or without (as a control) acid phosphatase from human semen (Sigma-Aldrich) at a ratio of 1 µg to 1 U of enzyme in 0.1 M sodium citrate buffer, pH 4.8, at 37°C for 4 h. Thereafter, the samples were precipitated with acetone and resolved once again by 2-DE.

Phosphorylation of non-phosphorylated PRL

Non-phosphorylated PRL (1 µg) was treated with the following protein kinases: casein kinase I (100 units; Promega, Madison, WI, USA) in 25 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, and 0.1 mM ATP; casein kinase II (100 units; Promega) in 25 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 200 mM NaCl, and 0.1 mM ATP; the catalytic subunit of cAMP-dependent protein kinase (100 units; Promega) in 40 mM Tris-HCl (pH 7.4), 20 mM magnesium acetate, 0.2 mM ATP, and 3 µM cAMP; and p21-activated protein kinase 2 (PAK2; GloboZymes, Carlsbad, CA, USA) in 20 mM Tris-HCl (pH 7.5), 8 mM MgCl₂, 3 mM EDTA, 30 mM 2-mercaptoethanol, 0.008% Triton X-100 and 0.2 mM ATP. Thereafter, all samples were precipitated with acetone and resolved by 2-DE.

Densitometric analysis of the ratio between PPRL and PRL

The density of each PPRL with CBB staining was determined after 2-DE by a densitometry (ImageMaster 2D Platinum ver. 5.0), and the ratio to the density of non-phosphorylated PRL in the pituitary gland of mice was calculated under various physiological conditions.

Semi-quantitative analysis

We semi-quantified the relative amounts of PPRLs in pituitary glands of the mice by comparing the CBB staining intensities of the PPRLs and non-phosphorylated PRL based to 1 µg bovine serum albumin (BSA; Protein standard solution, Fermentas, Burlington, Ontario, Canada) spots after 2-DE using ImageMaster 2D Platinum ver. 5.0.

BSA (1 µg) was dissolved in sample buffer and resolved by 2-DE to be used as standard protein. The IPG gels for the pituitaries of the 7-day OVX or E2-treated mice 2 days after OVX were resolved together with BSA gels at 2-DE on 12.5% SDS-polyacrylamide gel and stained with CBB. Three spots were detected for the BSA sample, and the most alkaline spot was used as the standard for semi-quantitative analysis.

Statistics

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

Results

Two-DE and immunoblotting for PRL

The results of 2-DE and immunoblotting using blots of pituitary gland samples on membranes with the antibody against mouse PRL confirmed
that there are three isoforms of about 23 kDa (1, 2 and 3, with isoform 3 being the most acidic; Fig. 1 A and B) in the pituitary of 60-day-old female mice at estrus. Isoforms 2 and 3 were immunoreactive against anti-phosphoserine (Fig. 1C), and isoform 3 was immunoreactive against both the anti-phosphoserine and anti-phosphothreonine antibodies (Fig. 1D). These findings indicate that PRL is primarily phosphorylated on serine or threonine and that it assumes three forms in the mouse pituitary gland.

**Dephosphorylation of PRL**

Three spots (isoforms 1, 2 and 3) extracted with Tris-buffer (pH 8.0) and precipitated with acetone were incubated with or without acid phosphatase for 4 h and resolved by 2-DE. Although the control showed all isoforms (Fig. 2A), acid phosphatase resulted in the absence of isoforms 2 and 3 (Fig. 2B). This indicated that isoform 1 was non-phosphorylated, while isoforms 2 and 3 were dephosphorylated and modified with phosphate.

**Phosphorylation of isoform 1**

Isoform 1 was extracted after 2-DE and CBB staining, and migration of its phosphorylated derivatives after kinase treatment was monitored using 2-DE. Incubation with PAK2 resulted in phosphate incorporation into two isoforms that had the same net charge as isoforms 2 and 3 (Fig. 2D). Incubation with casein kinase I and cAMP-dependent protein kinase resulted in phosphate incorporation into isoform 1 and the same net charge as isoform 2 (data not shown). However, casein kinase II did not phosphorylate isoform 1 (data not shown).
The ratio of isoform 2 to isoform 1 was 0.4 and that of isoform 3 to isoform 1 was 0.6 in the 20- and 30-day-old (Fig. 3A, C, D and F) and 60-day-old male mice (data not shown), indicating that age did not affect the distribution of PRL isoforms in these mice. The ratios in the 20-day-old female mice were similar to those of the male mice (Fig. 3B and C). However, the ratio of isoform 2 to isoform 1 in the 30-day-old female mice increased to 0.6 and that of isoform 3 to isoform 1 decreased to 0.4 (Fig. 3E and F). Furthermore, isoform 3 was absent during the proestrus stage in the 60-day-old female mice, but there were no differences in the ratios of isoforms 2 and 3 to isoform 1 for any other stage of the estrus cycle between the 60- and 30-day-old female mice (data not shown). Because the ratios of isoforms 2 and 3 to isoform 1 differed with gender, we further determined these ratios in OVX mice before and after estrogen replacement. In this experiment, the serum E2 concentrations were 7.1 ± 1.7 pg/ml (n=5) 7 days after OVX and 425.9 ± 248.5 pg/ml (n=5) after estrogen replacement in the OVX mice. These ratios were remarkably lower 2 and 7 days after OVX compared with the diestrus stage in the 60-day-old female mice (Fig. 4B, C and E).

Sham-operated mice were no different from the 60-day-old female mice during the diestrus stage (Fig. 4A and E). Furthermore, estradiol implantation for 7 days starting 2 days after OVX indicated that the ratio of isoform 2 to isoform 1 increased to a level equivalent to that of the sham operation mice. The ratios of isoform 2 and 3 did not increase in the control mice without E2 (data not shown). However, estradiol replacement had no effect on the ratio of isoform 3 to isoform 1, which remained identical to that of the OVX mice (Fig. 4D and E).

Semi-quantitative analysis of the pituitary contents of PRL

E2 implantation greatly induced increases in both the non-phosphorylated PRL and PPRL contents of the pituitary gland. The contents of isoform 1, 2 and 3 against the BSA standard were 55.4 ± 11.8, 8.8 ± 3.3 and 7.3 ± 2.6 of 7 days after OVX, respectively. On the other hand, the contents in the E2 treated mice were 148.0 ± 19.1, 52.7 ± 10.9 and 25.1 ± 0.5, respectively (Fig. 5A). Isoform 1 and 3 after E2 treatment increased to about three times that of the OVX mice, and isoform 2 was further increased to about six times that of the OVX mice (Fig. 5B). E2 implantation influenced the ratio of...
isoform 2 to isoform 1 content among the three isoforms in the pituitary gland.

Discussion

Greenan et al. [15] have shown that rat PRL is phosphorylated and that there are at least two acidic PPRL isoforms. Ho et al. [16] showed that four isoforms (isoforms 1, 2, 3, and 3'; 3' has the most acidity) exist in the rat pituitary. Isoform 1 and 2 are non-phosphorylated PRLs and 3 and 3' are phosphorylated PRL. In the present study, we discovered three isoforms in the mouse pituitary gland. Isoform 2 and 3 were two of the types discovered; isoform 2 is phosphorylated at serine and isoform 3 is phosphorylated at both serine and threonine. Serine residue is the primary site of phosphorylation in PRL. The phosphorylation site of PRL include serine 177th in the rat, serine 179th and 180th in the bovine, and serine 179th in the human [7]. On the other hand, threonine is a minor phosphorylation site of rat PRL. Phosphorylation has been identified in rat PPRL at both serine and threonine. Although we were able to show that PRL isoform 3 from the mouse pituitary was phosphorylated at threonine, we could not identify the precise phosphorylation site in this study.

We also showed that PRL is phosphorylated in vitro by protein kinases. Other studies [4, 17] have shown that rat PRL is phosphorylated by casein kinase I, calcium/phospholipid-dependent kinase, cAMP-dependent protein kinase, and PAK2, all of which result in phosphate incorporation by isoform 1 to become isoforms 2 and 3. We showed here that phosphorylation in vitro changes the electrophoretic mobility of PRL isoform 1 to those of isoforms 2 or 3 when it is separated by 2-DE on the basis of net charge. Isoform 1 was phosphorylated by casein kinase I and cAMP-dependent protein kinase to isoform 2, but not isoform 3. Only PAK2 phosphorylated isoform 1 to isoforms 2 and 3. We were unable to identify a protein kinase that phosphorylated only isoform 1 to isoform 3. PAK2 is a mammalian homologue of yeast Ste20-like protein kinase and is part of the PAK-I subfamily. Signaling via PAK2 is involved in regulation of apoptosis and malignant transformation [18–20]. However, the mechanism by which PAK2 enters secretory granules to cause PRL phosphorylation and by which it distinguishes whether to phosphorylate isoform 1 to isoform 2 or 3 remains unknown.

Other researchers have shown that the ratios of PPRL to PRL are constant within various species, such as 0.7:1.0 in turkeys and 0.2:1.0 in sheep and rats [5]. We compared the staining intensity of PPRL and non-phosphorylated PRL spots. The ratios of isoform 2 to isoform 1 and of isoform 3 to isoform 1 did not differ in the pituitaries of the male mice at any age or in those of the 20-day-old female mice. However, the ratio of isoform 2 to isoform 1 (0.6:1.0) increased and that of isoform 3 to isoform 1 (0.4:1.0) decreased at estrus in the 60-day-old female mice. Of all the stages of the estrous cycle, isoform 3 was only absent from mice during proestrus, and the ratios of isoforms 2 and 3 to isoform 1 did not differ during any other stage of estrous. Ho et al. [16] previously reported that the rat at proestrus is deficient in one PPRL isoform. Thus, the differences in the ratios of PPRL to non-phosphorylated PRL between male and female
mice have become interesting subjects of study. However, our results suggest that isoform 2 is dominant in female mice and that isoform 3 is dominant in male mice.

Estrogen is essential for regulation of PRL release, which it stimulates by inhibiting the activity of the tuberoinfundibular dopaminergic system (TIDA). Furthermore, estrogen directly stimulates PRL gene expression because of an estrogen-responsive element localized in the distal promoter region of the PRL gene [21]. We examined whether estrogen regulates PRL phosphorylation and release. We found that the ratios of isoforms 2 and 3 to isoform 1 in OVX mice were remarkably decreased compared with those in normal female mice and that administering estradiol replacement to OVX mice recovered only PPRL isoform 2. These results indicated that estrogen stimulates serine phosphorylation of PRL to increase the level of isoform 2. Although an elevated estrogen concentration stimulated PRL biosynthesis, phosphorylation at threonine in PRL was not promoted. This notion is supported by the findings that isoform 3 was absent in the afternoon of proestrus in 60-day-old mice. The level of serum PRL increases in patients with hyperprolactinemia, and serum PPRL is phosphorylated at serine but not threonine in patients with macroprolactinemia [22]. Histologically, PRL cells are classified into three types based mainly upon the size of the immunoreactive prolactin granules. All three types undergo sexual dimorphic changes during postnatal development in the mouse pituitary gland [28]. Type I PRL cells are usually oval or polygonal and scarce in the pituitary gland, and they usually contain small spherical granules of PRL that are about 100 nm diameter. Type II PRL cells are usually triangular or cup-shaped, and they usually contain medium, spherical PRL granules ranging from 150 to 200 nm in diameter. Type III cells are polygonal, oval, or irregularly-shaped and have a well-developed Golgi apparatus and endoplasmic reticulum. They contain polymorphic PRL granules with a maximal diameter of 300 nm.

Type II PRL cells are predominant in the pituitaries of both male and female mice, and the ratios between all types of PRL cells with any of the others do not differ with gender until 4 weeks of age. However, after 5 weeks of age, the number of type III PRL cells appreciably increases in the pituitary glands of female mice [28, 29]. The increase in type III PRL cells at this age corresponded to that of isoform 2 in the current study, which arose due to phosphorylation of isoform 1. Furthermore, estrogen slightly increased the total numbers of PRL cells in the pituitary glands of OVX female mice compared with those of OVX control mice. The ratio of type III to total PRL cells increased, while the ratios of type I and II PRL cells to total PRL cells decreased [30]. The present study showed that the relative amount of isoform 2 was remarkably reduced after OVX and estradiol replacement. These results seem to suggest that PRL is phosphorylated at serine or threonine mainly in type II or type III PRL cells. Phosphorylation at threonine might be predominant in the type II cells of both male and female mice until 4 weeks of age. Thereafter, phosphorylation at serine might be predominant in type III cells, which increase in number with puberty. Phosphorylation of PRL seems to be related to not only PRL cell type, but also to the shape of the secretory granules, since the granules...
are frequently polygonal in type III cells. In conclusion, we identified three charged types of PRL. Isoform 1 is non-phosphorylated PRL, isoform 2 is phosphorylated at serine, and isoform 3 is phosphorylated at both serine and threonine. The ratios between isoforms 2 and 1 were higher in the 30-day-old female mice than in the male mice; the opposite was the case for the ratio between isoforms 3 and 1. Furthermore, our results suggest that estrogen is associated with the serine phosphorylation of PRL. Thus, PPRL in the various types of PRL cells may play different roles that are related to the ratios of its 2 PRL isoforms, which differ with age and gender.

Acknowledgment

This research was supported by a Collaboration with Bio-Venture Companies’ Projects for Private Universities: matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology of Japan (2001–2005).

References


