Changes in mRNA levels of a pituitary-specific trans-acting factor, Pit-1, and prolactin during the rat estrous cycle

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The present study examined the changes in mRNA levels of a pituitary-specific trans-acting factor, Pit-1, and prolactin during the rat estrous cycle. Total cytoplasmic RNA was analyzed by Northern blot and slot-blot hybridization to examine the prolactin mRNA level. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to examine the Pit-1 mRNA level. Proestrous and estrous prolactin mRNA levels were significantly higher than the metestrous and diestrous levels, whereas Pit-1 mRNA levels of the estrous and metestrous stages were about two- to threefold higher than those of the proestrous and diestrous stages. Proestrous Pit-1 mRNA levels increased gradually from 10.00 h to 20.00 h, while prolactin mRNA levels slightly decreased until 14.00 h but increased later until 20.00 h. During the rat estrous cycle, especially in the afternoon of the proestrous day, changes of prolactin mRNA levels seem to follow a prior increase of Pit-1 mRNA. Therefore, Pit-1 may be partly involved in the regulation of prolactin gene expression according to the rat estrous cycle. Estradiol administration to ovariectomized rats significantly increased both the mRNA levels of prolactin and Pit-1, which suggests that the gene expression of Pit-1 is regulated by estrogen through indirect extracellular pathways.

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Prolactin (PRL) is secreted by lactotrophs of the anterior pituitary and regulates the normal development and differentiation of female breast and milk production. Blood levels of PRL fluctuate as a function of the estrous cycle and a major surge of PRL level occurs in the late afternoon of the proestrous day (1, 2).

Gene expression of PRL is regulated by a number of extracellular factors, such as dopamine (DA), thyrotropin-releasing hormone (TRH), estrogen and vasoactive intestinal peptide. The PRL mRNA levels change during the rat estrous cycle (3, 4). In the lactotroph, the pituitary-specific transcription factor, Pit-1, is the pivotal regulator for PRL gene expression (5–7). Regulation of PRL gene expression by TRH and DA has been well known to be mediated by Pit-1 (7–9). However, so far, the gene expression of PRL has not been correlated with changes in Pit-1 mRNA during the estrous cycle. Therefore, the aims of the present investigation were to assess the changes of Pit-1 and PRL mRNA levels during the rat estrous cycle and to study the effect of estrogen on the gene expression of Pit-1 and PRL.

Materials and methods

Animals

Adult female rats of Sprague-Dawley strain (weighing 200–250 g) were used throughout the experiments. Animals were housed under a controlled temperature and light schedule (14-h light, 10-h darkness; lights on at 07:00 h). Standard food and water were freely available. Vaginal smears were checked daily and rats showing at least three consecutive 4-day regular estrous cycles were sacrificed at 10.00 h and used. In order to study the effect of estrogen on the gene expression of Pit-1 and PRL, animals were ovariectomized (OVX) under a light ether anesthesia. After 2 weeks, OVX rats were implanted with Silastic capsules (10 mm in length, inner diameter 1.575 mm, outer diameter 3.575 mm; Dow Corning, Silastic Medical Grade Tubing) containing 17β-estradiol (0.86 mmol/l in oil, Sigma) or vehicle. At 10.00 h after 2 days, animals were sacrificed and anterior pituitaries were dissected rapidly and assigned to experiments as described below.

Ribonucleic acid extraction and Northern blot hybridization

Total cytoplasmic RNA was extracted using the guanidinium thiocyanate–acid phenol–chloroform method (10). The RNA content was quantified at A260 absorbance. The optical density ratio of A260 to A280 ranged from 1.8 to 2.0. To determine mRNA levels of
PRL. Northern blot and slot-blot hybridization were employed. For Northern blot analysis, RNA (10 μg) from the same number of anterior pituitaries (two or three per experimental group) was electrophoresed on an agarose gel. The RNA was transferred to a Nytran membrane (0.45-μm pore size; Schleicher and Schuell) using the capillary transfer method. The RNA blotted membrane was dried and baked at 75°C for 2 h. The membrane was then prehybridized and hybridized with a 32P-labeled PRL cDNA probe (specific activity >1.0 x 10⁹ cpm/ml) at 42°C overnight. Following hybridization, the membrane was washed twice, dried and exposed to X-ray film (β-max, Amersham) at −70°C for 3–4 days.

Reverse transcription-polymerase chain reaction (RT-PCR)
The Pit-1 mRNA levels were measured by RT-PCR. For RT-PCR analysis a set of two Pit-1 specific primers—an upstream primer (5’-CACCTCGGTGA-TACCTTT-3’) at exon 1 and a downstream primer (5’-GTITGTGCCACTTTITC-3’) at exon 5—was synthesized based on the rat Pit-1 cDNA and mouse Pit-1 genomic DNA structure (6, 11). The amplified fragment is predicted to be 616 bp in size. Ribonucleic acid (1 μg) was reverse transcribed with Moloney Murine leukemia virus (MMLV) RNaseH reverse transcriptase kit (Promega). The PCR was carried out with an automated thermocycler (Ericomp). Five microliters of cDNA products were amplified with AmpliTaq DNA polymerase (Perkin-Elmer Cetus) using the buffer and reaction conditions recommended by the manufacturer. Thirty-five cycles of PCR amplification were run with thermofile of denaturation at 94°C for 1 min and annealing and extension at 57°C for 1 min, respectively. Resulting products were electrophoresed on an agarose gel with TRIS acetate–EDTA buffer at 100 V for 1.5 h. To confirm that the PCR bands were Pit-1 mRNA-specific, PCR products were transferred to a Nytran membrane and hybridized with Pit-1 cDNA probe labeled with an enhanced chemiluminescence (ECL) random priming kit (Amersham). Hybridization signals were amplified by an ECL detection system (Amersham) and autoradiographed onto X-ray film (Hyperfilm-ECL, Amersham). Autoradiographed bands of Southern blot signals were scanned with a laser densitometer (Biomed Instruments).

Validation of RT-PCR technique
To validate the measurement of Pit-1 mRNA with the RT-PCR technique, serially diluted total cytoplasmic RNAs were reverse transcribed and amplified for 35 cycles. A linear relationship with a correlation coefficient of 0.99 was observed as a function of increasing amounts of cytoplasmic total RNA (Fig. 1A). Effects of increasing cycles on the amplification products are shown in Fig. 1B. The possibility that RT-PCR products might originate from genomic DNA contamination was excluded by reverse transcription using oligo d(T) primer. Southern blot hybridization of PCR products with the rat Pit-1 cDNA

Fig. 1. Validation of reverse transcription-polymerase chain reaction (RT-PCR) amplification of cytoplasmic total RNA. Pit-1 PCR products were size fractionated on an agarose gel and visualized under an ultraviolet illuminator after ethidium bromide staining. Bands of PCR products were exactly the same size as expected at 616 bp. Southern blot hybridization signals of PCR products with Pit-1 cDNA probe labeled using enhanced chemiluminescence random priming system confirmed the positive signals of PCR products. (A) Reverse transcription-PCR amplification from serially diluted RNAs generated increasing amounts of PCR products, and their Southern hybridization signals showed a linear relationship with a correlation coefficient of 0.99 (r). (B) Increasing thermal cycles produced increasing densities of PCR and Southern blot bands. As DNA marker, Hae III-digested φX 174 DNA was used.
probe confirmed the positive signals of Pit-1 mRNA. Our RT-PCR conditions did not amplify any other Pit-1 variant forms described previously (12–14).

Statistics
Statistical comparison between groups was analyzed by either an unpaired Student's t-test for two groups or a one-way analysis for variance for more than two groups. Fisher's least significant difference test was used for the post hoc comparison. The level of statistical significance was set at \( p < 0.05 \).

Results

Alterations of Pit-1 and PRL mRNA during the rat estrous cycle

Figure 2 shows the Northern blot analysis of PRL mRNA (Fig. 2A) and RT-PCR (Fig. 2B) followed by Southern blot analysis (Fig. 2C) of Pit-1 mRNA during the rat estrous cycle.

Changes in PRL and Pit-1 mRNA levels during the rat estrous cycle are described in Fig. 3. Levels of PRL mRNA of the proestrous and estrous stages were significantly higher than those of the metestrous and diestrous stages. Changes of Pit-1 mRNA levels during the same estrous cycle were much greater than those of PRL mRNA levels. In particular, Pit-1 mRNA levels of the estrous and metestrous stage were about twofold and threefold higher than those of the proestrous and diestrous stages, respectively.

Changes of Pit-1 and PRL mRNA during the proestrous day

Changes of Pit-1 and PRL mRNA levels during the proestrous day, detected by RT-PCR and slot-blot analysis, are summarized in Fig. 4. Over the course of time, PRL mRNA levels were slightly decreased until 14.00 h and then increased in accordance with time. The overall profile of PRL mRNA levels during proestrus appears to be similar to that of previous reports (3, 4). In the same time period, Pit-1 mRNA levels were increased substantially with time. More than fourfold increases of Pit-1 mRNA levels were observed at 20.00 h compared with those at 10.00 h.

Effect of in vivo administration of estrogen on the Pit-1 and PRL mRNA levels

In order to determine the changes in PRL and Pit-1 mRNA levels in response to estrogen administration, PRL and Pit-1 mRNA levels were determined by Northern blot analysis and RT-PCR followed by Southern blot hybridization. Estrogen treatment led to significant increases of PRL and Pit-1 mRNA levels (Fig. 5).
Discussion

The present study examined the changes of Pit-1 and PRL mRNA levels during the rat estrous cycle and with the administration of estrogen. Changes of PRL and Pit-1 mRNA levels were not parallel during the rat estrous cycle. For instance, PRL mRNA levels of the proestrous and estrous stages were higher than those of the metestrus and diestrous stages, whereas Pit-1 mRNA levels were much higher in metestrus than other stages of the estrous cycle. In contrast to PRL mRNA, the lowest Pit-1 mRNA levels were found in proestrus. This discrepancy is not yet fully understood. In part, it may be explained by the suggestion that Pit-1 has a relatively long half-life (15). Therefore, the increased amount of Pit-1 in metestrus might be maintained in the proestrous stage and activate PRL gene expression in combination with an increased input of estrogen. Another possibility may be the differential response to the regulatory input between Pit-1 and PRL. During the rat estrous cycle, high levels of estrogen and progesterone occur at different moments (16). The blood estrogen concentration peaks in proestrus, while the progesterone level is high in metestrus and forenoon of diestrous. Recently, Cho et al. (17) reported that progesterone inhibits the estrogen-induced gene expression of PRL. The effect of progesterone on Pit-1 is still unclear, but it is possible that progesterone may regulate Pit-1 differently than PRL.

Although minor changes in PRL mRNA levels were observed between the proestrous and estrous day, a more detailed examination of the proestrous stage with 2-h intervals showed that PRL mRNA levels fluctuated greatly as a function of time. After 14.00 h, the increase of PRL mRNA levels paralleled the increase of Pit-1 mRNA levels. Whereas before 14.00 h, the decrease of PRL mRNA contrasted with increase of Pit-1 mRNA. The reason for this discrepancy is unclear. It is possible that Pit-1 is required for the activation of PRL gene expression by estrogen (18). The forenoon rise of estrogen may induce the increase of intracellular Pit-1 level through other extracellular inputs or intracellular regulating molecules, and Pit-1 itself may activate its own gene expression (5, 9). Sufficient intracellular Pit-1 may cooperate with estrogen and activate PRL gene expression after 14.00 h.

In the present study, estrogen administration increased the mRNA levels of both PRL and Pit-1. Previous studies reported that estrogen regulates the expression of PRL gene through binding to the estrogen response element in the 5' flanking region of the PRL gene (19–22). Day et al. (18) suggested that both Pit-1 and the estrogen receptor are required for the effect of

Fig. 4. Changes of prolactin and Pit-1 mRNA levels with time on the proestrous day. Prolactin mRNA levels were detected by slot-blot hybridization (A) and Pit-1 mRNA levels were detected by reverse transcription-polymerase chain reaction (RT-PCR) and Southern blot hybridization (B). Each point represents the mean (±sem) of Pit-1 mRNA (closed circle) and prolactin mRNA (open circle) levels from three repeated experiments (C). Each value is expressed as relative units over the 10.00-h value of 1.0; *p < 0.05 and **p < 0.01 vs the 10.00-h value.

Fig. 5. Effect of in vivo administration of estrogen on the mRNA levels of Pit-1 and prolactin. Representative results of Northern blot analysis of prolactin mRNA (A) and Southern blot hybridization of Pit-1 reverse transcription-polymerase chain reaction (RT-PCR) products (B). Photography for 18S ribosomal RNA (18S) was used to certify equal loading of RNA onto a Northern blot gel. In vivo administration of 0.86 mmol of estradiol (E) to the ovariectomized rats markedly augmented the mRNA levels of both prolactin and Pit-1. Each bar represents the mean (±sem) of prolactin (open bar) and Pit-1 mRNA levels (closed bar) from four repeated experiments (C). Values of estrogen-treated groups are described as relative units over the value of the vehicle (V) group (1.0): *p < 0.05 and **p < 0.01 vs the vehicle group value.
estrogen on PRL gene expression. The present observation that estrogen increased Pit-1 mRNA as well as PRL mRNA coincides with this previous suggestion. However, Zhang et al. (23) reported that estrogen had no effect on the Pit-1 mRNA levels in the GH3 cell culture system. The discrepancy between the two results might be due to different experimental conditions. Indeed, our in vivo approach with estrogen can act via the hypothalamus and then factors such as TRH and dopamine might influence pituitary Pit-1 mRNA levels (7–9).

The presence of the estrogen response element in the 5′ flanking region of Pit-1 gene has not been reported yet. Therefore, one may speculate that regulation of Pit-1 gene expression by estrogen is mediated by other extracellular inputs. The direct action of steroids at the level of the anterior pituitary has been well described: estrogen can directly stimulate PRL release (24) as well as PRL mRNA accumulation in the anterior pituitary cultured in vitro (25, 26). Estrogen also blocks the inhibitory action of dopamine on PRL release (27, 28). In addition, indirect pathways through hypothalamic mediation are likely to occur. Indeed, there is an inverse relationship between dopamine concentration in hypophyseal portal blood and the circulating estrogen levels at the proestrous stage of the adult rats (29, 30). In the tuberoinfundibular dopamine system, estrogen can bind to 10–15% of tyrosine hydroxylase-containing nerve terminals (31). Estrogen decreases tyrosine hydroxylase activity (32, 33) and dopamine release (30, 34). Taken together, these data suggest that estrogen may inhibit tuberoinfundibular dopamine release; the subsequent low dopamine inputs to the pituitary lactotrophs may lead to increased Pit-1 gene expression.

In summary, the present study suggests that the gene expression of Pit-1 is regulated by estrogen through indirect extracellular pathways. During the rat estrous cycle, especially in the afternoon of the proestrous day, changes of PRL mRNA levels seem to follow a prior increase of Pit-1 mRNA. Therefore, Pit-1 may be partly involved in the regulation of PRL gene expression according to the rat estrous cycle. More detailed mechanisms of Pit-1 gene regulation during the estrous cycle remains to be clarified.

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