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Estrous Cycle Dependent Regulation of Peptidylarginine Deiminase Transcripts in Female Rat Pituitary*

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SUMMARY - Northern blot hybridization demonstrated 4.5-5.0 kilobase peptidylarginine deiminase mRNA in poly (A)+ RNA-enriched fractions of both male and female pituitaries. Dot blot analysis of total RNA fractions showed more than 50-fold sex difference in the mRNA content. The female pituitary mRNA content showed at least a 50-fold variation during the estrous cycle characterized by elevation at diestrus and proestrus followed by a rapid decline at estrus. The data were discussed in comparison with the sex difference and estrous cycle dependence of the pituitary enzyme content reported previously.

Peptidylarginine deiminases (protein-L-arginine iminohydrolase, EC 3.5.3.15) are a group of enzymes which convert arginine residues in proteins to citrulline residues(1-6). There are at least three types of peptidylarginine deiminases in mammalian tissues, i.e. a skeletal muscle type, an epidermal type, and a hair follicle type (6). The skeletal muscle type enzyme is present in the widest range of tissues among them. The enzyme proteins from bovine brain (4), rabbit (5), rat (6), and mouse (7) muscles have been purified to apparent homogeneity. The complete primary structure of the rat muscle enzyme has been deduced from its cloned cDNA sequence in our laboratory (8). It is localized in some prolactin producing cells (9), and shows female-dominant sex difference and estrous cycle dependence(10). Here we show a clear sex difference in the peptidylarginine

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deiminase content in rat pituitaries. We also show a striking contrast between the variation patterns of the mRNA and enzyme protein contents during the estrous cycle

EXPERIMENTAL PROCEDURES

Animals- About 4-month-old male and female Wistar rats were kept in an environmentally controlled room (22 ± 1°C, lights on 8:00-20:00) with free access to food and water. The estrous cycle positions of female rats were estimated by the vaginal smear test unless stated otherwise. All the animals were killed by cervical dislocation in the early afternoon (13:00-15:00).
Isolation of RNA - Extraction of total RNA was performed by the method of Chirgwin et al.(11). Enrichment of poly (A)$^+$ RNA from total pituitary RNA was performed by a single passage through an oligo (dT)-cellulose column (Pharmacia Type 7).
Northern and Dot Blot Hybridization - Poly (A)$^+$ RNA-enriched samples were electrophoresed on 1 % agarose containing formaldehyde (12) for Northern blot analysis. The gel was soaked successively in 50 mM NaOH, 10 mM NaCl for 30 min, 100 mM Tris-HCl, pH 7.5, for 30 min, and 1 M ammonium acetate for 1 h. The RNAs were transferred to nitrocellulose filter (Schleicher & Schuell, BAS-85) in 1 M ammonium acetate overnight.

Dot blotting was performed with a Bio-Dot™ Apparatus (Bio-Rad). Total RNA samples were incubated in 0.2 M morpholinopropanesulfonic acid, pH 7.0, 50 mM sodium acetate, 5 mM EDTA, 2.2 M formaldehyde, 50 % formamide at 65°C for 15 minutes. They were then mixed with 3 volumes of 20 X SSC (1X SSC: 150 mM NaCl, 15 mM trisodium citrate), and the mixtures were serially diluted with 20 X SSC. The resulting solutions were applied to the wells of the filter-mounted apparatus, and allowed to pass slowly by gentle suction.

The RNA-mounted filters were baked at 80°C for 2 h in vacuum to immobilize RNAs. They were next incubated at 42°C for 3 h in 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 10 mM EDTA, 0.2 % SDS, 5 X Denhaldt's solution, 50 % formamide, containing denatured salmon sperm DNA (100 μg/ml) for prehybridization. Hybridization was performed with ^{32}P-labeled DNA probes prepared by the random priming method (13) from a 1 kb fragment excised from λPAD1 by EcoRI digestion (8), or from chicken β-actin cDNA (Oncor, Inc.) at 42°C overnight in the prehybridization buffer. The former probe covers a C-terminal third of the sequence encoding peptidylarginine deiminase. The filters were first washed with 2 X SSC, 0.1 % SDS for 10 min three times at room temperature, and then with 0.1 X SSC, 0.1 % SDS at 55°C for 20 min twice (for filters incubated with peptidylarginine deiminase cDNA), or with 0.2 X SSC, 0.1 % SDS at 52°C for 20 min twice (for filters incubated with chicken β-actin DNA), and subjected to autoradiography without drying. Autoradiography was performed at -70°C using Kodak XAR films with an intensifying screen. In some cases, the filters incubated with peptidylarginine deiminase cDNA were washed with 10 mM Tris-HCl, pH 7.6, 1 mM EDTA at 65°C to remove bulk of the labeled probe, and allowed to stand for additional several weeks. The filters became virtually free of radioactivity by then, and could be used for hybridization with chicken β-actin cDNA.

RESULTS

Sex Difference in Pituitary Peptidylarginine Deiminase mRNA Contents - First we performed Northern blot analysis of poly(A)$^+$

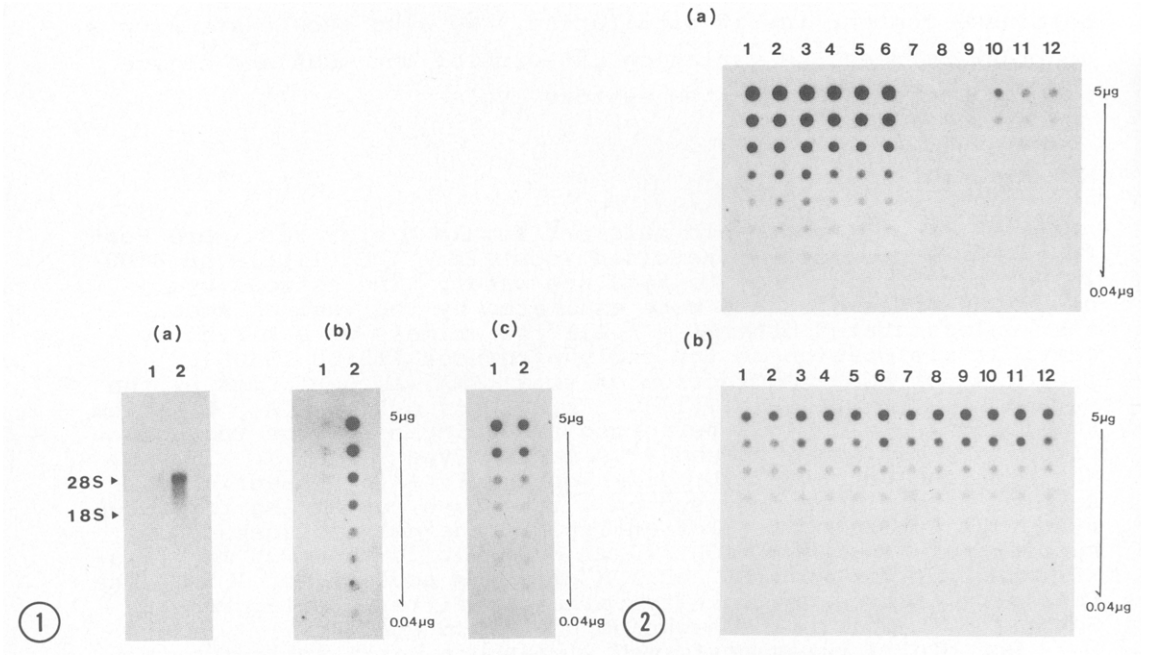

Fig. 1. Northern blot and Dot blot analyses of male and female rat pituitary RNAs. Pituitaries from six animals were pooled. Those from female rats were used regardless of their estrous cycle positions. (a) Northern hybridization with peptidyl-arginine deiminase cDNA using poly (A)$^+$RNA (2 μg). (b) Dot hybridization with peptidylarginine deiminase cDNA. Decreasing amounts of total RNAs ranging from 5 to 0.04 μg were applied by serial twofold dilution of the samples. (c) Dot hybridization with chicken β-actin cDNA. The filter used in (b) was deprobed as described in the text for the hybridization with β-actin cDNA. Lane 1, male RNA; lane 2, female RNA.

Fig. 2. Estrous cycle dependent variation of peptidylarginine deiminase contents in female pituitaries. Decreasing amounts of total RNA samples (5 to 0.04 μg) extracted from individual pituitaries were applied by serial twofold dilution. (a) Hybridization with peptidylarginine deiminase cDNA. (b) Hybridization with chicken β-actin DNA to the deprobed filter used in (a) as described in the text. Lanes 1-3, diestrus; lanes 4-6, proestrus; lanes 7-9, estrus; lanes 10-12, metestrus.

RNA-enriched fractions extracted from male and female pituitaries. The extraction was performed from six pooled pituitaries, and those of female rats were used regardless of their estrous cycle positions. The male RNA sample yielded a faint band upon hybridization with peptidylarginine deiminase cDNA (Fig. 1a). This band was detected in the female RNA sample at much higher intensity. Its size was estimated to be 4.5-5.0 kilobases from its relative mobility to that of 28 S rRNA. 4.5-5.0 Kilobase mRNAs were also detected in rat skeletal muscle, spinal cord, cerebellum, cerebrum, and submaxillary gland (8).

Next we made a semiquantitative comparison of the peptidylarginine deiminase mRNA contents between male and female

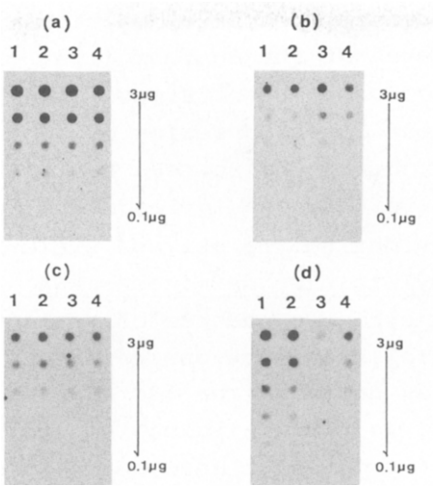

Fig. 3. Examination of peptidylarginine deiminase mRNA contents of spinal cord (a), submaxillary gland (b), cerebellum (c), and uterus (d) during the estrous cycle. Pooled total RNA samples derived from three animals staged at the corresponding estrous cycle phases were applied in decreasing orders ranging from 3 to 0.1 μg by serial twofold dilution. Hybridization was conducted with peptidylarginine deiminase mRNA. Lane 1, diestrus; lane 2, proestrus; lane 3, estrus; lane 4, diestrus.

pituitaries by dot blot analysis using total RNAs extracted from pooled pituitaries (Fig. 1b). Serially decreasing amounts of RNA samples ranging from 5 to 0.04 μg were applied to a nitrocellulose sheet. The male RNA sample gave only a faint signal even at the maximum input. By contrast, the female RNA showed a readily detectable spot at the minimum input. This means more than 50-fold difference in the peptidylarginine deiminase mRNA content between male and female pituitaries. Such marked difference was not noted when the deprobed series of spots were hybridized with chicken β-actin cDNA as a control (Fig. 1c).

Estrous Cycle Dependence of Pituitary Peptidylarginine Deiminase mRNA Contents - To see whether or not the pituitary peptidylarginine deiminase content changes during the the estrous cycle, we collected pituitaries from female rats staged at the four defined phases of the estrous cycle. Three rats were used for each phases. Total RNAs extracted from individual pituitaries were serially diluted, dotted to a nitrocellulose sheet, and hybridized with peptidylarginine deiminase cDNA. Diestrous and proestrous RNAs showed strong hybridization signals (Fig. 2a). The signal intensities of individual samples in the same cycle phases showed close similarity. The diestrous RNA yielded readily detectable spots even at the minimum input (0.04 μg). The intensities of the proestrous RNAs were about half as much as those of the diestrous RNAs. In contrast, estrous RNAs

showed markedly decreased hybridization signals. Only faint spots were visible even at the maximum input (5 μg). Metestrous RNAs yielded weak but significant signals. Hybridization with chicken β-actin to the deprobed series of spots visualized no significant changes during the estrous cycle (Fig. 2b). This suggests that the pituitary peptidylarginine deiminase mRNA changes specifically during the estrous cycle.

Detection of peptidylarginine deiminase mRNA in other tissues - We examined estrous cycle dependence of peptidylarginine deiminase contents in total RNA fractions derived from several other tissues. RNA samples derived from the same animal groups were pooled, and applied to nitrocellulose sheets in serially decreasing amounts for dot blot analyses. Cerebellum, spinal cord, and submaxillary gland RNAs showed no significant differences in hybridization intensities between different estrous cycle phases (Fig. 3a-c). In contrast, the hybridization intensities of uterine RNAs showed variations resembling those of the pituitary RNAs (Fig. 3d).

DISCUSSION

We have shown previously that the pituitary peptidylarginine deiminase content in the male rat remains negligibly small from 3 weeks to 6 months of age while that in the female rat increases with maturation showing a characteristic variation around the estrous cycle (10). It is high at proestrus and estrus, and low at metestrus and diestrus. The enzyme contents at the former two phases are 2.5- to 3-times higher than those in the latter two phases. The sex difference in the pituitary peptidylarginine deiminase mRNA content shown above can account for the sex difference in the enzyme content. The variation pattern of the mRNA content during the estrous cycle reported here shows a striking contrast to the variation pattern of the enzyme content. The mRNA content is impoverished at estrus. It starts increasing at metestrus reaching the maximum level at diestrus. It remains high till proestrous afternoon followed by a rapid decline to negligible levels by estrous afternoon. Moreover, the mRNA content shows much greater variation than the enzyme protein content. These suggest complex mechanisms of the pituitary enzyme regulation involving transcription, translation, as well as mRNA stability. In fact, peptidylarginine deiminase cDNA contains a putative degradation signal sequence -AUUUA- (14) in nucleotides 3073-3077 in the 3'-untranslated region (8).

We previously suggested estrogen dependence of pituitary peptidylarginine deiminase by its depletion after ovariectomy and its substantial restoration by 17β-estradiol injections (10). The serum estrogen level in cycling female rats shows a characteristic variation (15). It starts increasing in metestrous evening. The rate of increase is gradually accelerated from diestrous afternoon until reaching the maximum concentrations at around noon of proestrus followed by rapid decrease thereafter. The increased enzyme contents at proestrus and estrus can be correlated with the preceding increase in the serum estrogen. However, the sharp increase in the mRNA content from metestrus to diestrus shown above occurs about a day before the serum estrogen reaches the maximum concentrations. This can not be accounted for by simple transcriptional regulation unless low levels of serum estrogen cause maximum activation of peptidylarginine deiminase gene transcription. The rapid decline in the mRNA content between proestrus and estrus may reflect the concurrent decline in the serum estrogen. Ovalbumin (16), conalbumin (17), and vitellogenin (18) mRNAs in avian oviduct an d liver have been shown to be degraded extremely rapidly in the absence of estrogen. It has also been shown that estrogen stabilizes Xenopus liver vitellogenin mRNA (19). Exact mechanisms of estrogen action on the pituitary peptidylarginine deiminase expression remain to be investigated.

Among the other tissues we examined, only uterine peptidylarginine deiminase mRNA showed estrous cycle dependent variation similar to that observed in the pituitary. Estrous cycle depended changes of the enzyme content in mouse uterus have also been reported (7). Less pronounced variations have been reported for ovary and uterine proenkepharin mRNAs (20), supraoptic nuclear oxytocin mRNA (21), adrenal renin mRNA (22), and pituitary prolactin mRNA (23). Further investigation on the regulation of peptidylarginine deiminase will provide us valuable information concerning the mechanism of estrous-cycle dependent gene expression as well as its physiological role in prolactin producing cells.

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