Korean J. Zool. 34:426-431, 1991

Effect of Naloxone on the Estrogen-induced Prolactin Gene Expression and Secretion

Bhumsoo Kim, Kyungjin Kim, and Wan Kyoo Cho

Department of Molecular Biology and SRC for Cell Differentiation, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea

The present study examines the effect of naloxone, mu-opioid receptor antagonist, on prolactin (PRL) gene expression and secretion induced by estradiol (E) treatment *in vivo*. Adult rats were ovariectomized (OVX) and implanted with Silastic capsules containing either vehicle (oil) or E. Three days later, NAL (2 mg/kg BW) or saline were injected 30 min prior to sacrifice. To examine PRL secretion *in vitro*, the pituitaries were incubated in the superfusion system for 3 hrs. Superfusates were collected at 10 min intervals on ice and subjected to PRL radioimmunoassay. Endogenous release of PRL in OVX + E rats was significantly higher than that in OVX rats (mean ± SE; 24.5 ± 3.1 vs 14.5 ± 2.9 ng/10 min). A single injection of NAL clearly inhibited PRL release *in vitro* from pituitaries derived from OVX + E rats, but not from OVX group. PRL mRNA was determined by RNA-blot hybridization assay with nick-translated PRL cDNA. E stimulated PRL mRNA about 3 fold over that shown in OVX group. Treatment of NAL suppressed the E-stimulated PRL mRNA in OVX + E group, but not in OVX group. These data clearly showed that the NAL-induced inhibition of PRL secretion was well correlated with changes in PRL mRNA level and this inhibitory process appears to be mediated in E-dependent manner.

KEY WORDS: Prolactin, Coupling of gene expression and secretion, Estrogen, Naloxone

Prolactin (PRL) is one of polypeptide hormones synthesized and secreted from lactotrophs of anterior pituitary. PRL plays an important role in the development of breast, milk production and many other functions related to reproduction (Tollis, 1980). The regulation of PRL is rather complicated, since the feedback signal of steriod hormones and neural influences are dually involved (Leong *et al.*, 1983). PRL secretion is predominantly under inhibitory tone such as dopamine from the hypothalamus (Ben-Jonathan, 1985). Recent evidence also indicates that one of the neuropeptide (GAP) derived from GnRH precursor may be an inhibitory substance for PRL control, but it is not fully clarified yet (Nikolics *et al.*, 1985). Both estrogen (E) and thyrotropin-releasing hormone (TRH) are strong stimulators for PRL secretion (Leong *et al.*, 1983). E is able to stimulate PRL synthesis and PRL mRNA level in various experimental systems (Maurer, 1982; Maurer and Gorski, 1977).

Several lines of evidence suggest that endogenous opioid peptides (EOP) are involved in the regulation of PRL secretion (Meites *et al.*, 1979; Baumann and Rabil, 1990). In fact, the treatment of naloxone (NAL), a specific opioid antagonist, brought about decrease in the basal, proestrous surge and stress-induced PRL secretion (Ieiri *et al.*, 1980; Selmanoff and Gregerson, 1986). The NAL-induced inhibition of PRL secretion was certainly influenced by steroid *milieu*, but the precise role of steroids, in particular, E on PRL regulation

The present study was supported by grants from the Ministry of Education of Korea and Korea Science and Engineering Foundation.

by NAL remains to be elucidated. Moreover, no attempt has yet been made to elucidate the action of EOP in PRL gene expression in the anterior pituitary. The present study was, therefore, undertaken to determine the alteration of PRL mRNA and PRL release for the elucidation of the possible inhibitory role of EOP on the E-induced PRL gene expression and secretion.

Meterials and Methods

Materials

Naloxone, 17β-estradiol, bovine serum albumin (BSA), bacitracin, Nonidet P-40, formaldehyde and chloramine T were obtained from Sigma Chemical Co. (St. Louis, MO). Silastic capsules were purchased from Dow Corning (Midland, MI). *E. coli* polymerase I, DNase I and EcoRI were purchased from Bethesda Research Lab. (Gaithersburg, MD). Nitrocellulose filter paper (BA 85, 0.45 μm of pore size) was obtained from Schleicher and Schuell (Keene, NH). Formamide was obtained from BDH Chemicals (Atherstone, Warwickshire). ^{32}P-dATP was purchased from New England Nuclear (Boston, MA).

Animals and Experimental Treatments

Adult female Sprague Dawley rats, weighing 200-250 g, were obtained from Seoul National University Animal Breeding Center. They were housed under the controlled photoperiod condition (14 hr light and 10 hr dark, lights on 06:00 h). Food and water were available *ad libitum*. Rats were ovariectomized (OVX) for 10 days and received subcutaneous implants of Silastic capsule (15 mm in length; id 1.57 mm and od 3.18 mm) containing either sesame oil or 17β-estradiol (235 μg/ml in oil) for 3 days. NAL was dissolved in saline (0.9% NaCl) and 2 mg/kg BW was injected subcutaneously (2 mg/kg B. W.) to OVX + E rats. The four experimental groups were set: 1) OVX + oil + saline, 2) OVX + E + NAL, 3) OVX + E + saline and 4) OVX + E + NAL. Rats were sacrificed by decapitation between 08:30 h and 09:00 h.

In Vitro Superfusion System

The rat pituitaries were transferred to superfusion chamber following chopping into eight small pieces. The superfusion medium (Krebs-Ringer phosphate buffer, pH 7.4 with 0.1% BSA, 10 mM glucose and 0.02 mM bacitracin) was delivered to the superfusion chamber at a speed of 50-70 μl/min by a peristalitic pump with a consistent supply of air at 30°C. Following stabilization period (3 hr), fractions were collected at 10 min intervals on ice for 3 hrs. The superfusates were stored at −20°C until assayed by PRL radioimmunoassay (RIA).

PRL Radioimmunoassay

The concentration of PRL in superfusates was measured in duplicate by a double antibody RIA procedure (Ahn *et al.*, 1990). PRL RIA kit was supplied by the NIADDK and PRL level was expressed in terms of rPRL-RP-3 standards. rPRL-I-5 was iodinated by chloramine T method and ^{125}I-labelled PRL (6000-8000 cpm/tube) was used. Anti-rPRL-s-9 serum was used at the final dilution of 1:125000. The minimum detection level was about 0.02 ng/ml and inter- and intra-assay variations were approximately less than 12%.

RNA-blot Hybridization

The rat PRL cDNA in plasmid SP65 was a kind gift from Dr. R. A. Maurer (Univ. of Iowa, Iowa). Its size with the complete PRL coding sequence was about 0.8 kb (pPRL-2; Gubbins *et al.*, 1980). The plasmids were transformed to *E. coli* (HB 101 strain), amplified in LB medium and the transformations were subsequently lysed by alkali (Maniatis *et al.*, 1982). The PRL cDNA was cut by EcoRI and purified by electroelution. PRL cDNA (500-600 ng) was nick-translated using *E. coli* polymerase I and DNase I with ^{32}P-dATP (Davis *et al.*, 1986). The specific activity was $3-5 \times 10^7$ cpm/μg. The total cytoplasmic RNA was prepared by the method of White and Bancroft (1982). Briefly, the individual pituitaries were removed immediately and homogenized by a glass homogenizer (100 μl capacity) containing 50 μl of 10 mM Tris-HCl (pH 7.0), 1 mM EDTA and 1% Nonidet P-40 with 10 revolutions of pestle. The homogenate was transferred to a 1.5 ml sterile microcentrifuge tube and incubated for 1 min on ice. Following centrifugation at 12,000 g for 5

min, 50 μl of supernatant was transferred to a sterile tube containing 30 μl of 20 × SSC (1 × SSC; 0.15 M NaCl and 0.015 M trisodium citrate) plus 20 μl of 37% (v/v) formaldehyde. This mixture was incubated at 60°C for 20 min to denature RNA. For analysis of PRL mRNA level, Northern blot analysis and/or slot-blot hybridization were used. The detailed procedure was the same as that previously described (Ahn *et al.*, 1990).

Statistics

Data were analyzed by using an one-way analysis of variance (ANOVA) with $p < 0.05$ required for statistical significance. Fisher's least significance difference test was used for the *post-hoc* comparison.

Results and Discussion

Figs. 1 and 2 depict the time course of PRL secretion from the superfused rat pituitaries. The basal levels of PRL release in OVX + oil and OVX + E groups were stable, but our preliminary data showed that during the first 3 hrs (samples were not collected), PRL release *in vitro* was extremely high. Following the initial period, PRL release *in vitro* became rather plateau and this phenomenon appears due to removal of the strong inhibitory influences from the hypothalamus, as previously shown (Bentley and Wallis, 1986). The endogenous release of PRL in OVX rats was approximately 15 ng/ml. A single injection of NAL brought about decrease in PRL release, but the mean release of PRL was not modified. Estrogen implants to OVX rats significantly stimulated PRL release with high amplitude. The mean release of PRL was highly significant when compared to that observed in OVX rats. The E-simulated PRL release was markedly suppressed by a single injection of NAL (Fig. 3). When E was implanted to OVX rats, PRL mRNA increased more than 3-fold over that in OVX rats (Fig. 4). A single injection of NAL markedly inhibited PRL mRNA accumulation in OVX + E rats, but not in OVX rats. The present study clearly demonstrated that NAL was highly effective in suppressing the E-induced PRL release and PRL mRNA level in the rat pituitaries. And the change in PRL mRNA level

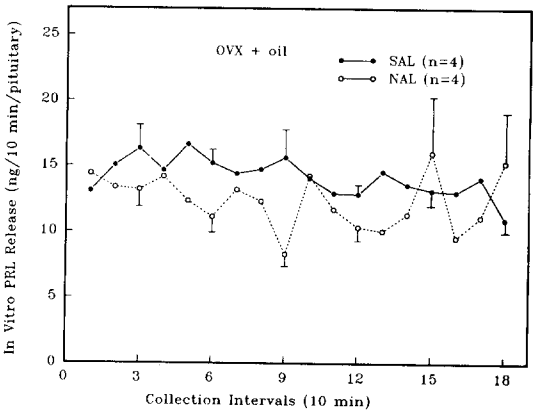

Fig. 1. PRL release *in vitro* from superfused pituitaries derived from ovariectomized (OVX) + oil treated rats. Animals received either saline (SAL) or naloxone (NAL, 2 mg/kg BW) 30 min before sacrifice. Each point represents the mean ± SE. Experiments were replicated 4 times.

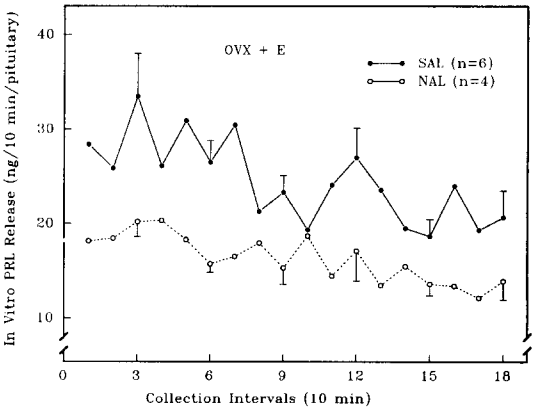

Fig. 2. PRL release *in vitro* from superfused pituitaries derived from ovariectomized and E (235 μg/ml) treated (OVX + E) rats. Either saline or naloxone (2 mg/kg BW) was injected 30 min prior to obtaining pituitary preparations. Each point represents the mean ± SE. Experiments were replicated 4-6 times.

was positively correlated with changes in PRL release *in vitro* (see Figs. 3 and 4), suggesting that the secretory activity of PRL may be a consequence of alteration of PRL mRNA.

The present finding that the NAL-induced inhibition of PRL release was E-dependent, indicates that the steroid *milieu* may be critical for the action of EOP. In fact, Blank *et al.*, (1980) showed that NAL was unable to stimulate PRL secretion in

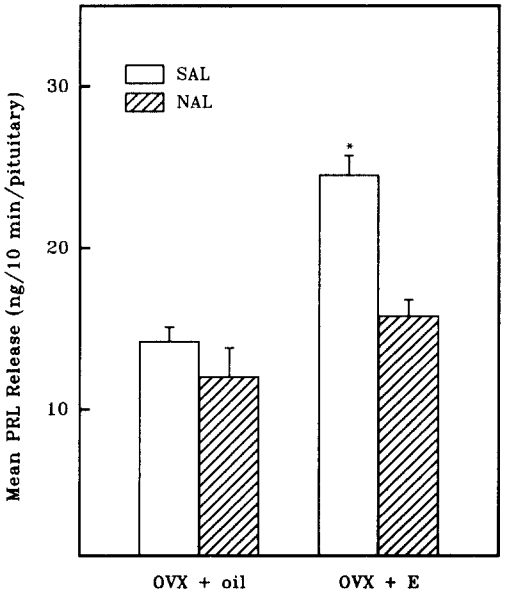

Fig. 3. Effect of naloxone on the estrogen-induced PRL release. Each bar represents the mean PRL release (± SE, n = 4 – 6) during 3 hr superfusion period. *OVX + E > OVX + SAL, OVX + E + NAL.

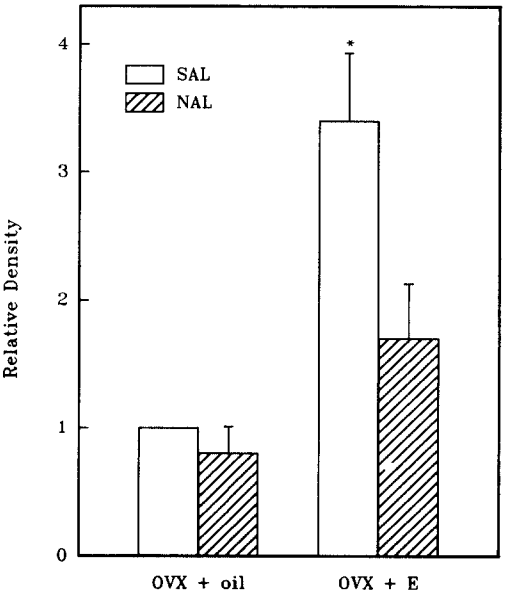

Fig. 4. Effect of naloxone on the estrogen-induced PRL mRNA accumulation. Animals received either saline (SAL) or naloxone (NAL, 2 mg/kg BW) 30 min before sacrifice. The total cytoplasmic RNA was isolated from the pituitary and PRL mRNA was determined by RNA-blot hybridization assay with the rat PRL cDNA. The density of PRL mRNA was scanned by desitometry and expressed as an arbitrary unit (the density of saline injected group of OVX rats was set as 1.0). Bars are the mean ± SE (n = 3).

immature male and female rats, which have apparently low levels of E. It has also been shown that E induces the significant changes in hypothalamic EOP, such as β-endorphin and met-enkephalin (Hong *et al.*, 1982; Petraglia *et al.*, 1982). The most relevant changes in hypothalamic EOP was ensued at the proestrous stage of the rat (Barden *et al.*, 1981; Kumar *et al.*, 1979). Similar changes also occurred during physiological conditions characterized by high levels of E (Lee *et al.*, 1980; Petraglia *et al.*, 1982; Wardlaw and Frantz, 1983). The mechanism by which the NAL inhibits PRL release as well as PRL mRNA level remains unknown. It is, however, plausible to assume that E initially affects the hypothalamic EOP tone, which in turn influences the responsiveness of lactotrophs. Indeed, Wilcox and Roberts (1985) clearly demonstrated that E can regulate POMC gene expression in the hypothalamus. It is also possible that E directly affect lactotrophs to regulate PRL gene expression. These two mechanisms seem to operate in a complementary manner. It has been well documented that E is able to stimulate PRL secretion and synthesis, as shown in primary culture of rat pituitary cells (Lieberman *et al.*, 1982) and GH cell lines (Amara *et al.*, 1987). The E-induced PRL mRNA appears to be achieved through the receptor-mediated mechanism involving PRL transcription (Maurer, 1982). The alteration in E receptor density appears to be well correlated with PRL gene expression (Schull and Gorski, 1984). It is of interest to note that the E-increased PRL mRNA was mediated by two distinct steps, indicating direct and/or indirect mechanisms (Shull and Gorski, 1985).

In this experimental protocol where NAL was subcutaneously injected to rats, it is rather difficult to determine whether NAL affects directly on the pituitary or indirectly through the hypothalamus. It is possible that NAL initially acts upon the hypothalamus to suppress EOP tone which in turn influences the function of pituitary. However, as discussed above, the other possibility that EOP exerts directly at the level of pituitary cannot be ruled out, since the opiate receptors evidently ex-

ist at the pituitary cells (Simantov and Levy, 1984). Moreover, our preliminary study using GH_3 pituitary tumor cells showed that NAL was effective in inhibiting the basal and the E-induced PRL mRNA levels (data not shown). Then, it seems likely that the EOP influence may exert dually. No matter where NAL acts upon, the present finding evidently indicates that the antagonism of EOP receptor, particular, mu-receptor, was effective in modulating the responsiveness of PRL to E. Finally, it is of interest to note a possible intracellular signalling in the NAL-induced PRL regulation. In the pituitary cells, there is no evidence that EOP modulates adenylate cyclase activity. It is, however, tempting to postulate that NAL may change the intracellular cAMP level which in turn influences PRL secretion as a consequence of PRL gene expression as shown in many other systems (Comb *et al.*, 1987; Montminy *et al.*, 1986). With respect to cAMP action on PRL gene expression, Maurer (1981) clearly demonstrated that a cAMP analogue can stimulate PRL transcription. Therefore, further exploration of intracellular mediator of EOP in the pituitary cells will provide a better insight into the molecular events underlying the NAL-induced inhibition of PRL gene expression.

References

Ahn, H., S. K. Yu, B. N. Cho, K. Kim, K. Ryu, and W. K. Cho, 1990. Alteration in prolactin messenger ribonucleic acid level during the rat estrous cycle: Effect of naloxone. *Korean J. Zool.* **33:** 183-190.

Amara, J. F., C. V. Itallie, and P. S. Dannies, 1987. Regulation of prolactin production and cell growth by estradiol: Differentiation in sensitivity to estradiol occurs at the level of messenger ribonucleic acid accumulation. *Endocrinology* **120:** 264-271.

Barden, N., Y. Merand, D. Rouleau, M. Garson, and A. Dupont, 1981. Changes in β-endorphin content of discrete hypothalamic nuclei during the estrous cycle of the rat. *Brain Res.* **204:** 441-445.

Baumann, M. H. and J. Rabil, 1990. Mu-selective opioid peptides stimulate prolactin release in lactating rats. *J. Neuroendocrinol.* **2:** 271-276.

Ben-Jonathan, N., 1985. Dopamine: A prolactin-inhibiting hormone. *Endocrine Rev.* **6:** 564-589.

Bentley, A. M. and M. Wallis, 1986. Effects of two enkephalin analogues, morphine sulfate, dopamine and naloxone on prolactin secretion from rat anterior pituitary gland *in vitro*. *J. Endocr.* **109:** 313-320.

Blank, M. S., A. Panerai, and H. G. Friesen, 1980. Effects of naloxone on luteinizing hormone and prolactin in serum of rats. *J. Endocr.* **85:** 307-315.

Comb, M., S. E. Hyman, and H. M. Goodman, 1987. Mechanisms of trans-synaptic regulation of gene expression. *TINS.* **10:** 473-478.

Davis, L. G., M. D. Dibner, and J. F. Battey, 1986. Methods in Molecular Biology. Elsevier Science Publishing Co. Inc., New York, N.Y. pp. 80-83.

Gubbins, E. J., R. A. Maurer, M. Lagrimini, C. R. Erwins, and J. E. Donelson, 1980. Structure of the rat prolactin gene. *J. Biol. Chem.* **255:** 8655-8662.

Hong, J-S., K. Yoshikawa, P. M. Hudson, and L. L. Uphouse, 1982. Regulation of pituitary and brain enkephalin systems by estrogen. *Life Sci.* **31:** 2181-2184.

Ieiri, T., H. T. Chen, G. A. Campell, and J. Meites, 1980. Effects of naloxone on the proestrous surge of prolactin and gonadotropin in the rat. *Endocrinology* **106:** 1568-1570.

Kumar, M. A. S., C. L. Chen, and T. F. Muther, 1979. Changes in the pituitary and hypothalamic content of methionine-enkephalin during the estrous cycle of rats. *Life Sci.* **25:** 1687-1696.

Lee, S., A. E. Panerai, D. Bellabara, and H. G. Friesen, 1980. Effects of endocrine modifications and pharmacological treatments on brain and pituitary concentration of β-endorphin. *Endocrinology* **107:** 245-248.

Leong, D. A., S. Frawley, and J. M. Neill, 1983. Neuroendocrine control of prolactin secretion. *Ann. Rev. Physiol.* **45:** 109-127.

Lieberman, M. E., R. A. Maurer, P. Claude, and J. Gorski, 1982. Prolactin synthesis in primary cultures of pituitary cells: Regulation by estradiol. *Mol. Cell Endocrinol.* **25:** 277-294.

Maniatis, T., E. F. Fritsch, and J. Sambrook, 1982. Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. pp. 88-91.

Maurer, R. A., 1981. Transcriptional regulation of the prolactin gene by ergocriptin and cyclic AMP. *Nature* **294:** 94-97.

Maurer, R. A., 1982. Estradiol regulates the transcription of the prolactin gene. *J. Biol. Chem.* **257:** 2133-2136.

Maurer, R. A. and J. Gorski, 1977. Effect of estradiol 17 β and pomozide on prolactin synthesis in male and female rats. *Endocrinology* **101:** 76-84.

Meites, J., J. F. Bruni, D. A. Van Vugt, and A. F. Smith, 1979. Relation of endogenous opioid peptide and morphine to neuroendocrine function. *Life Sci.* **24:** 1325-1336.

Montminy, M., K. Sevarino, J. Wagner, G. Mandel, and R. Goodman, 1986. Primary structure of the gene encoding rat preprosomatostatin. *Proc. Nat. Acad. Sci. U.S.A.* **83:** 6682-6686.

Nikolics, K., A. J. Mason, E. Szonyi, J. Ramachandran, and P. H. Seeburg, 1985. A prolactin-inhibiting factor within the precursor for human gonadotropin-releasing hormone. *Nature* **316:** 511-517.

Petraglia, F., A. Panalva, V. Locatelli, D. Cocchi, A. Panerai, A. E. Genazzani, and A E. Muller, 1982. Effects of gonadectomy and gonadal steroid replacement on pituitary and plasma endorphin levels in the rat. *Endocrinology* **111:** 1224-1229.

Selmanoff, M. and K. A. Gregerson, 1986. Suckling induced prolactin release is suppressed by naloxone and stimulated by β-endorphin. *Neuroendocrinology* **42:** 255-259.

Shull, J. D. and J. Gorski, 1984. Estrogen stimulates prolactin gene transcription by a mechanism independent of pituitary protein synthesis. *Endocrinology* **114:** 1550-1557.

Shull, J. D. and J. Gorski, 1985. Estrogen regulates the transcription of the rat prolactin gene *in vitro* through at least two independent mechanism. *Endocrinology* **116:** 2456-2462.

Simantov, R. and R. Levy, 1984. Opiate mechanisms: Characterization of selected neuroblastoma glioma cells with an enhanced response to opiate antagonists. *In* : Central and peripheral endorphins: Basic and clinical aspect. (Muller, E. E. and A. R. Genazzani eds.) Raven press, New York, N.Y. pp. 65-70.

Tollis, G., 1980. Prolactin: Physiology and Pathology. *In* : Neuroendocrinology. (Krieger, D. T. and J. C. Hughes eds.) PH Publishing Co. Inc., New York, N.Y. pp. 321-328.

Wardlaw, S. L. and A. G. Frantz, 1983. Brain β-endorphin during pregnancy, parturition and the postpartum period. *Endocrinology* **113:** 1664-1668.

White, B. A. and F. C. Bancroft, 1982. Cytoplasmic dot hybridization: Simple analysis of relative mRNA in multiple small cell or tissue samples. *J. Biol. Chem.* **257:** 8569-8572.

Wilcox, J. N. and J. L. Roberts, 1985. Estrogen decreases rat hypothalamic preopiomelancortin messenger ribonucleic acid levels. *endocrinology* **117:** 2392-2396.

(Accepted March 23, 1991)

Prolactin 유전자 발현과 분비에 미치는 naloxone의 영향

김범수 · 김경진 · 조완규(서울대학교 자연과학대학 분자생물학과)

본 연구에서는 에스트로젠(E) 처리에 의해서 유도된 prolactin(PRL) 호르몬의 유전자발현과 분비능에 미치는 mu-opioid receptor antagonist인 naloxone의 영향을 조사하였다. 성숙한 흰쥐의 난소를 제거한 후 oil 혹은 E(235 μg/ml)가 들어있는 캡슐을 경부피하에 삽입하고 3일 후에 naloxone(2 mg/kg)을 피하주사하였다. 적출된 뇌하수체는 *in vitro* superfusion system에서 3시간동안 배양하면서 10분간격으로 수거한 분획에서 PRL의 분비를 측정하였고, 뇌하수체에서의 PRL mRNA는 PRL cDNA를 사용한 RNA-blot hybridization에 의해서 조사하였다. 난소절제후 에스트로젠을 처리한 실험군(OVX + E)의 뇌하수체에서 분비되는 PRL은 난소절제후 oil을 처리한 대조군(OVX)에서 보다 유의하게 높았다(PRL 분비의 평균값 ± SE : 24.5 ± 3.1 vs 14.5 ± 2.9 ng/10 min). OVX + E 실험군에서 naloxone의 처리에 의해서 PRL 분비능은 크게 감소하였으나, 에스트로젠을 처리하지 않은 대조군에서는 큰 변화가 없었다. 난소절제후 에스트로젠을 처리한 실험군에서 PRL mRNA의 양이 대조군에 비해서 약 3배나 증가되었으며 이와같은 PRL mRNA의 증가는 naloxone처리에 의해서 강력히 억제되었다. 따라서 본 연구의 결과는 mu-opioid receptor antagonis가 에스트로젠 처리에 의해서 유도된 PRL의 유전자발현과 분비를 강력히 억제함을 보였으며, 이와같은 PRL의 유전자발현과 분비의 변화는 밀접하게 연관되어 있고 에스트로젠에 의존적임을 시사한다.