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Changes of Receptor mRNAs for Oxytocin and Estrogen during the Estrous Cycle in Rat Uterus

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ABSTRACT. Oxytocin receptor (OTR) mRNA levels increase dramatically near term and is potently stimulated by estrogen because increased OTR mRNA levels result from estrogen treatment in ovariectomized rat uterus. In this study, OTR, estrogen receptor (ER) α and ERβ mRNA levels in the rat uterus during the estrous cycle were examined by quantitative RT-PCR. OTR mRNA levels during the estrous cycle began to increase on diestrus ($P<0.05$, vs value on estrus), reached maximal increase both in the morning (1000–1130 hr) and afternoon (1600–1630 hr) on proestrus ($P<0.01$, vs metestrus, diestrus and estrus) and then declined on estrus. In contrast ERα mRNA levels began to decrease on diestrus, reached statistical significance both in the morning and the afternoon on proestrus ($P<0.01$, vs metestrus, diestrus and estrus) and returned to the value of metestrus on estrus. ERβ mRNA levels were low in the morning and the afternoon on proestrus ($P<0.01$, vs metestrus and estrus) and also returned to metestrus values on estrus. Treatments with estrogen for 3 days significantly decreased both ERα and ERβ mRNA levels. It can be concluded from these results that during the estrous cycle, OTR mRNA levels in rat uterus predominantly increase at proestrus with a decrease in ERα and ERβ mRNA levels, which is probably due to the increased estrogen levels in circulation before ovulation.

KEY WORDS: estrogen receptor, estrous cycle, oxytocin receptor, rat.

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Oxytocin (OT) was initially isolated as a neurohypophysial hormone which stimulates the contraction of the myometrium and myoepithelium to facilitate parturition and milk ejection respectively, and is considered to act on various reproductive functions in the ovary, the uterus or the brain. In the uterus, the near-term myometrium is extremely sensitive to oxytocin and this increased uterine responsiveness to oxytocin occurs in parallel with an increase in the number of uterine oxytocin binding sites in rats [6, 40], humans [7], rabbits [22, 23] and cows [9]. Corresponding increases in uterine oxytocin receptor (OTR) mRNA expression in late pregnancy and parturition have been reported in cows [12], rats [20, 21, 31], humans [17] and sheep [50, 52].

Estrogen stimulates the number of uterine oxytocin binding sites [6, 38, 41] and OTR mRNA expression in ovariectomized (OVX) virgin rats [20, 21]. However, injection of estrogen does not stimulate oxytocin receptor mRNA expression in late pregnant rats or in progesterone-primed OVX virgin rats, but is effective only after ovariectomy and removal of progesterone, respectively [27]. These results suggest that, in addition to the increase in serum estrogen level near term in rats, regulation of the uterine responsiveness to estrogen is important for understanding the role of estrogen in the uterus. The actions of estrogen are mediated through transcription-regulating intracellular estrogen receptors [1, 30]. Two types of estrogen receptor (ER), ERα and ERβ have been cloned from human uterus [11] and rat prostate [19] respectively.

During the estrous cycle, alterations in the number of oxytocin binding sites have also been reported in cows [8, 13, 25, 33, 39], sheep [46, 49] and rats [20], and in OTR mRNA expression in humans [44], sheep [42, 43] and rats [20]. In ruminants, oxytocin secreted from the corpora lutea is considered to be involved in the luteolysis by stimulating prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) release from the uterus. In rats the regulation of the luteolysis is different from the ruminants because the involvement of ovarian oxytocin for the luteolysis has not been reported, although oxytocin injection increased the $PGF_{2\alpha}$ levels in the uterus [3]. On the other hand, some evidences that oxytocin affect the regulation of preovulatory secretions of luteinizing hormone (LH) [14, 32] and prolactin (PRL) from the pituitary in rats [34] suggest that oxytocin plays important roles in reproductive function on proestrus.

Considering the stimulatory effect of estrogen on OTR induction, it is likely that uterine OTR mRNA levels predominantly increase at proestrus, but the changes in OTR mRNA levels during proestrus is low compared with that during labor [20]. These results lead to the possibility that oxytocin may exert some effect on proestrus in the uterus, however its regulation may be different from that during labor. In this study, to investigate possible differences between the regulation of OTR mRNA expression during the estrous cycle and labor, OTR mRNA levels during the estrous cycles (especially on proestrus) in the rat uterus were examined by competitive RT-PCR. Also, because the uterine responsiveness to estrogen is important for the regulation of OTR, the expression of the ERs (ERα and ERβ mRNA) were also measured and compared with the OTR mRNA levels.

MATERIALS AND METHODS

Animals: Adult female Wistar rats (body weight 180–220 g) were obtained from Japan SLC (Hamamatsu, Japan) and

were kept in an environmentally-controlled room (temperature 23 ± 3°C; lights on 0600–1800 hr) with free access to tap water and pelleted rat food (NMF; Oriental Yeast Co., Ltd., Tokyo, Japan). The estrous cycle was monitored by vaginal smears taken each morning (0900–1000 hr). The rats were killed at 1000–1130 hr on metestrus, diestrus (following day of metestrus), proestrus and estrus and at 1600–1630 hr on proestrus. Ovariectomized virgin rats were given an s.c. injection of sesame oil (0.5 m*l*), 17β-estradiol-3-benzoate (Sigma, St Louis, U.S.A.; 10 μg in 0.2 m*l* sesame oil) and 17β-estradiol-3-benzoate and progesterone (Nacalai Tesque Inc., Kyoto, Japan; 5 mg in 0.5 m*l* sesame oil) at 1100–1130 hr for 3 days, respectively [21]. Samples were obtained from rats at 1100–1130 hr on the following day of the last treatment. The uteri were collected and frozen at –70°C until RNA extraction. Animal care, maintenance and surgery were approved by Animal Care and Use Committee and were conducted for according to Guidelines for Animal Experiment, Fukui Medical University.

Competitive PCR analysis: Complementary DNA syntheses were performed as described previously [21, 27]. Briefly, uterine tissue (50–100 mg) was homogenized with TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). Total RNA samples were prepared according to the acid guanidinium thiocyanate-phenol-chloroform extraction method and treated with RNase-free DNaseI (Invitrogen) to exclude genomic DNA. The quantity of total RNA was assessed with a spectrophotometer at a wave length of 260 nm. Total RNA (1 μg) of samples were reverse transcribed with 200 U reverse transcriptase (TOYOBO, Osaka, Japan) and 10 pmol 6-mer random primer.

Construction of normal and mutant DNAs of ERα, ERβ and β-actin: The oligonucleotide primers for OTR, ERα, ERβ and β-actin are as follows: OTR, 5'-CGATTGCTGGGCGGTCTT-3' and 5'-CCGCCGCTGCCGTCTTGA-3' [21]; ERα, 5'-TGACCAACCTGGCAG ACAGG-3' and 5'-GCCTTTGTTACTCATGTGCC -3' [18]; ERβ, 5'-GCTGTGATGAACTACAGTGTTCCC-3' and 5'-TGGACTAGTAACAGGGCTGGCACA-3' [36]; β-actin, 5'-CATGTCAGTGGACAGATGCT-3' and 5'-TAACT TCAGACATCATTTCCGG-3' [26]. The PCR products sizes of OTR, ERα, ERβ and β-actin were 161 bp, 527 bp, 267 bp and 542 bp, respectively. Each PCR product was subcloned into pGEMT easy vector (Promega, Madison, WI, U.S.A.). Mutated DNAs, used as competitors for each assay, were prepared by inserting 106 bp DNA fragment for OTR as previously described [21] or deleting 30–40% nucleotides in length from normal DNA. Deletion of normal DNA in ERα and ERβ were done by amplifying each plasmid with lower primer and mutant upper primer. Mutant primers were designed to amplify shorter DNA than primary (normal) DNA amplified by RT-PCR and were linked with normal upper primer at 5' end (ERα, 5'-TGACCAACCTGGCAGACAGG-AAGGCATGGT-3'; ERβ, 5'-GCTGTGATGAACTACAGTGTTCCC-CATTGCCAAT-3'). Mutant DNAs ERα and ERβ amplified with mutant upper primer and lower primer were subcloned into pGEMT vectors. For deletion in β-actin, the inserted vector was digested with Eco0190I and AvaI, reacted with T4 DNA polymerase (Invitrogen) to make the both restriction sides blunt and ligated with T4 ligase (Invitrogen). Normal and mutant DNAs were amplified with M13 primer or gene specific primers and purified with rapid PCR Clean-up system (Invitrogen). Concentrations of amplified DNAs were assessed with a spectrophotometer at a wave length of 260 nm. Normal and mutant DNAs were used as standard DNA and competitor respectively.

Competitive PCR analysis: All the samples and the standards in each experiment were assayed in a single run. Constant amounts of competitor DNA for OTR, ERα, ERβ (0.1–2 fM) and β-actin (5.5 pM) were added to all samples. The PCR amplification was carried out in PCR buffer [50 mM KCl, 2 mM $MgCl_2$ and 20 mM Tris-HCl, pH 8.4], with 0.2 μmol of gene-specific upper and lower primers and 1 U Taq polymerase (Applied Biosystems) in a total volume of 20 μl for 30–50 cycles as previously described in OTR reaction [21] and β-actin [26] or consisting of denaturation of 94°C for 1 min, annealing at 58°C (ERα) or 68°C (ERβ) for 1 min and extension at 72°C for 1 min after the initial denaturation at 94°C for 10 min. Amplification was completed with an additional extension step at 72°C for 10 min. The PCR products were separated on 2% agarose gel and stained with ethidium bromide (0.5 μg/m*l*). Fluorescence density was analyzed using NIH Image software. The ratio of mRNA-derived DNA (normal DNA) to competitor-derived DNA (mutant DNA) of each sample was obtained and calibrated from a standard curve as the amount of standard normal DNA. Each value for ERα and ERβ mRNA was standardized by dividing by the value for β-actin in the same sample. Data are expressed as the relative amounts (%) by dividing the value of each sample with the mean value of the corresponding control group.

Statistical analysis: The data are expressed as the mean ± SEM and evaluated statistically using one-way ANOVA followed by Student-Newman-Keuls test or Scheffè test.

RESULTS

OTR mRNA levels during estrous cycle began to increase on diestrus ($P<0.05$, vs value on estrus), attained maximal values in the morning and afternoon on proestrus ($P<0.01$, vs metestrus, diestrus and estrus) and then declined on estrus (Fig. 1). In contrast, ERα mRNA levels began to decrease on diestrus, reached statistical significance in the morning and afternoon on proestrus ($P<0.01$, vs metestrus, diestrus and estrus) and returned to metestrus values at estrus (Fig. 2). The levels of ERβ mRNA were low in the morning and afternoon on proestrus ($P<0.01$, vs metestrus and estrus) and reached metestrus values at estrus (Fig. 2).

Three days of estrogen treatment significantly decreased both ERα and ERα mRNA levels (Fig. 3). The inhibition in ERα was 60% and in ERβ most samples were undetectable (Fig. 3).

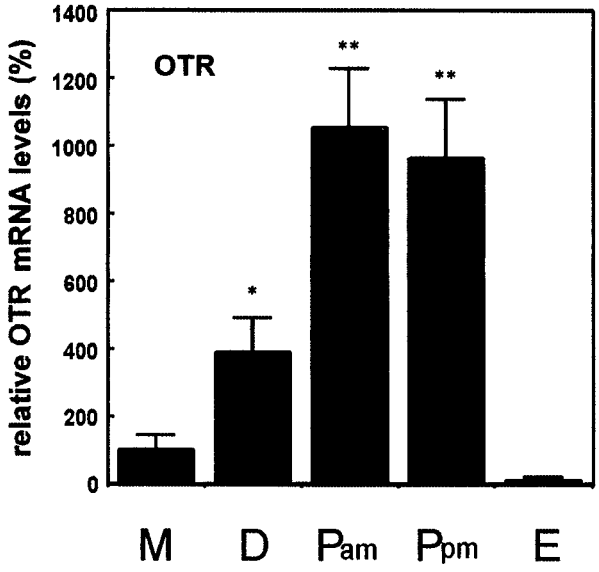

Fig. 1. Changes in the relative OTR mRNA levels in the uterus during the estrous cycle. Samples were obtained from rats at 1000–1030 hr on metestrus (M), diestrus (D), proestrus (Pam), and estrus (E) and at 1600–1630 hr on proestrus (Ppm). Data are expressed as the mean ± SEM (n=5). The value on metestrus was defined as 100%. *, P<0.05, *vs.* E; **, P<0.01 *vs.* M, D and E (one-way ANOVA followed by Student-Newman-Keuls test).

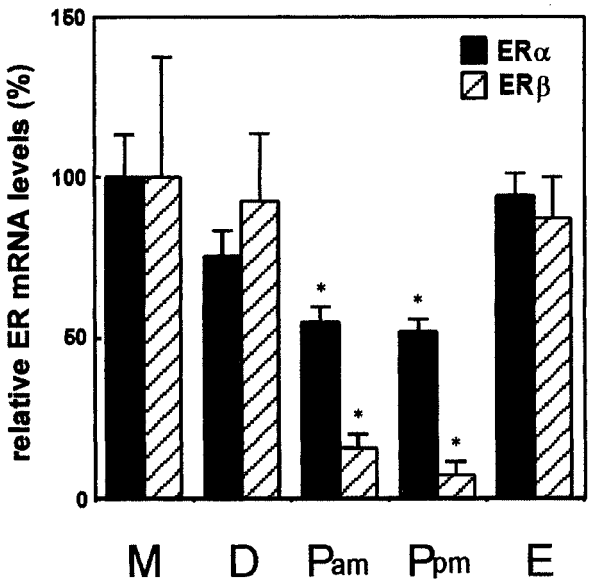

Fig. 2. Changes in the relative ERα and ERβ mRNA levels in the uterus during the estrous cycle. Samples were obtained from rats at 1000–1030 hr on metestrus (M), diestrus (D), proestrus (Pam), and estrus (E) and at 1600-1630 hr on proestrus (Ppm). Data are expressed as the mean ± SEM (n=5). The value on metestrus was defined as 100%. *, P<0.05, *vs* M and E (one-way ANOVA followed by Student-Newman-Keuls test for ERα or Scheffè test for ERβ).

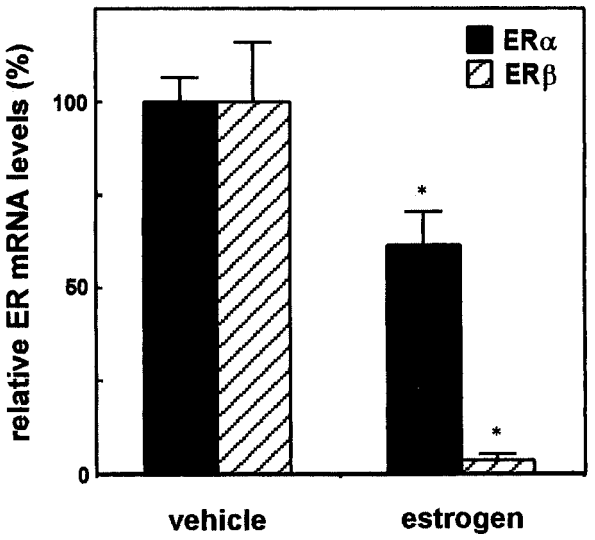

Fig. 3. Effects of estrogen on the relative ERα and ERβ mRNA levels in the uterus of ovariectomized rats. Control and estrogen-treated groups were given an s.c. injection of sesame oil (0.5 m*l*) or 17β-estradiol-3-benzoate (10 μg in 0.2 m*l* sesame oil) at 1100–1130 hr for 3 days, respectively. Samples were obtained from rats at 1100–1130 hr on the following day of the last treatment. Data are expressed as the mean ± SEM (n=8). The value of control group was defined as 100%. *: P<0.05, *vs* control group (one-way ANOVA followed by Student-Newman-Keuls test for ERα or Scheffè test for ERβ).

DISCUSSION

This study demonstrated that during the estrous cycle OTR mRNA levels in the rat uterus began to increase from diestrus, reached and maintained maximal values during the morning and afternoon of proestrus, respectively. In earlier studies, the increased OTR mRNA level in $PGF_{2\alpha}$-induced labor was suppressed by an anti-estrogenic compound, tamoxifen [27] and estrogen treatment increased OTR mRNA levels about 5–6 folds in ovariectomized virgin rats [20, 21]. These observations raise the possibility that the increase in OTR mRNA levels arises, at least in part, in response to the increased plasma estrogen secreted from ovarian follicles before ovulation.

The role of oxytocin in uterine function on proestrus remains to be determined. But one of the possibilities is the involvement of luteolytic effect of oxytocin. Hysterectomy has been reported to prolong the duration of luteal phase [10] and decrease $PGF_{2\alpha}$ levels in the corpus luteum of pseudopregnant rats [29]. Also, oxytocin injection increases $PGF_{2\alpha}$ levels in the uterus [3]. Therefore, an effect of oxytocin on the uterus may be involved in the regulation of corpora lutea regression after ovulation during estrous cyclicity. Another possibility is an involvement in the regulation of LH and PRL secretions. However, the effect of oxytocin on preovulatory LH and PRL secretions have been reported to be mediated at hypothalamic and pituitary levels

respectively [14, 35]. Hysterectomy lowered serum LH concentrations in the pseudopregnant and the ovariectomized rats [4], increased suckling-induced PRL secretion and caused the disappearance of the diurnal PRL surges in lactating rats [15], without affecting the preovulatory LH [4] and PRL surge. Taken together with the high OTR mRNA levels observed in the afternoon of proestrus, oxytocin might be involved in the regulation of LH and/or PRL secretion through the uterus after their preovulatory surges.

In ruminants, oxytocin binding and OTR mRNA expression in the uterus increased around preovulatory phase in the endometrium and myometrium [8, 13, 25, 39, 42, 43, 49]. The endometrial oxytocin receptor is believed to play an important role in the regulation of luteolysis through stimulation of $PGF_{2\alpha}$ release from the uterus [5]. But the appearance of OTR mRNA and oxytocin binding on deep glands, caruncular stroma and myometrium of the ovine uterus were observed at estrus when plasma progesterone concentrations have already decreased [42]. Thus, the role of oxytocin appears to involve more than the luteolysis around ovulation. In rats, OTR is expressed in the myometrium and not in the endometrium [20]. Therefore, elucidating the mechanism regulating OTR expression around ovulation may help to reveal other functions of oxytocin other than luteolysis in other animals including ruminants.

In this study, the increment of OTR mRNA levels during the estrous cycle was as potent as that observed during labor in rat uterus [21]. Larcher *et al.* [20] reported only about 2-fold increase in OTR mRNA levels in the morning of proestrus compared with that on metestrus using Northern blot analysis, although oxytocin binding increased more than 10-fold in the rat uterus. It is difficult to explain the disparity in the increase between the observations of the present study and those earlier results. In our experiment, however, we assayed two lines of samples in a single run. One line was composed of the undiluted cDNA samples and the other was 10-times diluted samples, because all samples could not be detected in a same dilution. Therefore, the present results indicated at least that OTR mRNA which can be amplified with the primer set (used in this study) increases about 10-fold on proestrus compared with the value on metestrus.

This study also demonstrated a decrease in ERα and ERβ mRNA levels on proestrus in rat uterus. Treatments with estrogen decreased both ERα and ERβ mRNA levels in ovariectomized virgin rat uterus. Therefore the decrease in ERα and ERβ mRNA levels observed in proestrus appears to be due to the down-regulation of estrogen. However, in late pregnancy and during labor OTR mRNA levels increased from day 21 of pregnancy and maintained high levels through day 22 and during labor [27] accompanied by an increase in ERα mRNA levels and the positive correlation exists between the two [28]. Considering the stimulatory and the inhibitory effects of estrogen on OTR and ERs mRNA expressions respectively in OVX virgin rats, these results suggest that different mechanisms are involved in ER regulation between during the estrous cycle and labor. A possible explanation for the significance of this difference is that during the estrous cycle the effect of estrogen on OTR induction might be necessary for a short period and immediately terminated by the negative-feedback action of estrogen through the inhibition of ER induction, while during labor the longer OTR induction is required and maintained probably by the cancellation of negative-feedback circuit.

In this case the effects of estrogen on ER regulation appear to be different under several physiological conditions. Indeed, the mechanisms underlying regulation of ERα and ERβ by estrogen are still controversial. Treatment with 17β-estradiol increased both ERα and ERβ mRNA levels in ovariectomized rat uterus [47] and 17β-estradiol increased ERα mRNA levels in the sheep uterus [24]. On the other hand ovariectomy increased ERα mRNA levels and 17β-estradiol treatment reduced its increase in ovariectomized rat uterus [37] and 17β-estradiol down-regulated ERα mRNA levels in human breast cancer cell line, MCF-7 cells [2]. In cultured rat decidual cells, 17β-estradiol had no effect on ERα mRNA expression but increased ERβ mRNA expression in a dose dependent manner [45]. While 48 hr estrogen treatment up-regulates ERβ expression in human uterine muscle cells, estrogen followed by the phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate, down-regulated ERα expression and up-regulated ERβ expression [51]. In addition, estrogen caused a greater inhibitory effect on ERβ mRNA levels than on ERα. The regulation of ERβ might depend mainly on the transcriptional step and under the estrogenic control more than ERα.

Using *in situ* hybridization Katsuda *et al.* [16] reported that ERα mRNA level in the luminal and glandular epithelial cells of rat uterus were high during proestrus and was lacking in the glandular epithelial cells at estrus. Wang *et al.* [48] reported that rat uterine ERα mRNA level at proestrus is significantly higher than that during metestrus and no significant change was observed in ERβ mRNA levels during the estrous cycle by solution hybridization method. The present results obtained by competitive RT-PCR were quite different form others. Therefore further studies might be necessary to evaluate how mechanisms regulating ERs mRNA expression reflect these differences.

In summary, this study demonstrates that OTR mRNA levels predominantly increase during proestrus with associated decrease in ERα and ERβ mRNA levels and this reciprocal change between OTR and ERs mRNA levels is probably due to the increased estrogen levels in circulation before ovulation.

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