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Summary — The effect of oxytocin on uterine estrogen and progesterone receptors (ER and PgR) was investigated *in vivo* in groups of immature female rats that were treated subcutaneously with oxytocin, 0.5 or 5 IU (1 and 10 μg) for 5 and 3 d, respectively. Receptor concentrations and affinities were estimated by Scatchard analysis, using radioactive hormones as ligands. Statistical analysis was performed by Student's *t* test and by ANOVA. Oxytocin did not alter the receptor affinity for either steroid hormone, but the lower dose significantly decreased the concentrations of receptors: ER = 486 ± 76 fmol/mg *vs* 346 ± 105 fmol/mg ($P < 0.001$) and PgR = 686 ± 237 fmol/mg *vs* 433 ± 236 fmol/mg ($P < 0.01$) (mean ± SD values for control and treated animals, respectively). There were no significant effects on the plasma 17β-E and Pg concentrations. In *in vitro* studies with mature rats, uterine specific binding of estradiol and progesterone in the presence or the absence of oxytocin showed no modification. Oxytocin could be a negative modulator of ER and PgR in the uterus, even though the mechanism of its action remains unknown. It could have potential implications on reproductive capacity and fertilization.

estrogen / progesterone / oxytocin / rat uterus / receptor

Résumé — Effet de l'ocytocine sur les récepteurs des œstrogènes et de progestérone dans l'utérus de ratte. *Dans le but d'évaluer l'influence de l'ocytocine sur les concentrations des récepteurs des œstrogènes (ER) et de progestérone (PgR), des rattes immatures ont reçu de l'ocytocine par voie sous-cutanée aux doses 0,5 UI et 5 UI (1 et 10 μg) pendant 5 et 3 j, respectivement. La concentration des récepteurs et l'affinité ont été mesurées par la méthode de Scatchard avec des hormones radioactives. L'analyse statistique a été pratiquée par le test «t» de Student et par ANOVA. L'affinité des récepteurs stéroïdes par les 2 hormones n'a pas été modifiée par le traitement avec l'ocytocine. En revanche, la plus faible dose d'ocytocine a induit une diminution significative de la concentration de récepteurs des œstrogènes et de progestérone: ER = 486 ± 76 fmol/mg* vs *346 ± 105 fmol/mg* (P *< 0,001) and PgR = 686 ± 237 fmol/mg* vs *433 ± 236 fmol/mg* (P *< 0,01), pour les animaux témoins*

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et traités, respectivement. Les taux plasmatiques d'œstradiol et de progestérone n'ont pas été modifiés. L'analyse des courbes de saturation a montré que la concentration des récepteurs et les constantes de dissociation, avec ou sans addition d'ocytocine, in vitro, *n'étaient pas statistiquement différents. L'ocytocine ne semble donc pas avoir un effet direct. L'ocytocine pourrait être un modulateur négatif des récepteurs stéroïdes dans l'utérus. Par conséquent, elle pourrait avoir des implications sur la capacité reproductive et la fertilité.*

œstrogènes / progestérone / ocytocine / utérus de ratte / récepteur

INTRODUCTION

Oxytocin (OT), a nonapeptide which is synthesized as part of a larger neurophysin molecule in the hypothalamus and secreted by the pituitary gland, has a well-characterized role in parturition and lactation (Caldeyro-Barcia and Sereno, 1961; Soloff and Pearlmutter, 1979). Recent observations have shown a wide variety of other effects on the central nervous system and endocrine organs (Richard *et al,* 1991). The ovaries synthesize OT in several species and there is a great deal of evidence relating ovarian OT to the luteolytic process (Fuchs, 1988).

A trophic role of exogenous OT on the growth of ovaries and uteri of immature rats has been reported (Al Janabi and Yousif, 1987). In contrast, exogenous OT and the related peptides, arginine-vasotocin (AVT) and arginine-vasopressin (AVP), suppress human chorionic gonadotrophin-stimulated uterine growth in immature mice (Wathes, 1984), perhaps through interactions with the estrogen receptor (ER) system.

Moreover, AVT, which differs structurally from OT by only one amino acid, inhibits the binding of tritiated estradiol (^{3}H-E) to the ER *in vitro* in the rat uterine cytosol (Vaughan *et al,* 1979).

ER and progesterone and oxytocin receptor (PgR, OTR) levels are under hormonal control, involving both hormones, estrogen (E) and progesterone (Pg) (Fuchs *et al,* 1983; Clark *et al,* 1985). It has been shown that OT down-regulates its own receptor *in vivo* (Flint and Sheldrick, 1985) and *in vitro* (Sheldrick and Flick-Smith, 1993), but the effect of OT on ER and PgR has not yet been investigated.

In the experiments described here, the influence of OT on rat uterine ER and PgR levels and *in vivo* and *in vitro* affinity is studied.

MATERIALS AND METHODS

Chemicals

^{3}H-17β-E ([2,4,6,7-^{3}H]-17β-estradiol, 86 Ci/mol) was obtained from CEA (Gif-sur-Yvette, France). ^{3}H-ORG 2058 (16α-ethyl-21-hydroxy-19-nor-[6,7,^{3}H]pregn-4-en-3,20-dione) (40 Ci/mmol) and unlabeled ORG 2058 were obtained from Amersham Int (Buckinghamshire, UK); 17β-estradiol, Pg, DES (diethylstylbestrol), and buffer reagents Tris, EDTA, DTT (dithiothreitol), Folin-Ciocalteu reagent and the other reagents for the protein determinations were obtained from Sigma Chemical Co (Saint Louis, MO, USA). Dextran T70 was purchased from Pharmacia (Uppsala, Sweden); the oxytocin (200 IU/ml) was from Sandoz (Basilea, Switzerland) and the scintillation liquid Ready Safe was from Beckman Inst (CA, USA).

Animals

Immature female rats (Wistar), 25–30 d old, weighing 42 ± 10 g, were used for the *in vivo* studies. They were housed in groups under controlled temperature, natural light and had a com-

mercial pellet diet and water *ad libitum*. These groups received subcutaneous injections of one of the following: 1) OT 0.5 IU (1 μg) in 0.1 ml of saline, daily for 5 d; 2) saline vehicle, the same as for group 1; 3) OT 5 IU (10 μg) in the same vehicle, twice a day for 3 d; 4) saline vehicle the same as for group 3; 5) 17β-E (10 μg in 0.1 ml of corn oil) daily for 3 d; 6) Pg (1 mg in 0.1 ml of corn oil) daily for 3 d; or 7) corn oil vehicle the same as for groups 5 and 6. The number of rats per group is indicated in table I.

The animals were killed by decapitation and their trunk blood was collected. The uteri were removed, stripped of fat and mesentery, weighed, pooled and frozen in dry-ice and stored at –80°C until the time of their assay. In each case, skeletal muscle from the leg was dissected and processed as a negative control. The ovaries were weighed and examined to determine the existence of ovulation points and corpora lutea. After the blood was collected, the serum was separated and stored at –20°C.

Twenty 4-month-old female adult rats (Wistar) weighing 185 ± 40 g were used for the *in vitro* studies. They were ovariectomized under pentobarbital (Tiopental, Abbott) anesthesia and maintained, in the same conditions as described above for 3 d. The animals were then killed by cervical dislocation and uteri were quickly dissected, pooled and frozen in dry-ice before storage at –80°C until the time of their assay.

Estradiol and progesterone receptor assay

The ER and PgR assays were performed using the biochemical method as previously described by Garofalo *et al* (1986, 1993). Briefly, the frozen tissues were homogenized in Tris 50 mM, EDTA 1.5 mM, DTT 0.5 mM, glycerol 10%, and natrium molybdate 16 mM at pH 7.4 (TEDG buffer), 1/10 wt/vol. The subcellular fractions were separated by centrifugation at 1 000 *g* for 15 min and then the supernatant fraction was centrifuged at 40 000 *g* for 90 min to yield the cytosol fraction. All these and subsequent procedures were carried out at 0–4°C. Aliquots of the cytosol fraction in duplicate were incubated with 5–6 increasing concentrations of ^{3}H-E (1.25–20 mM) or ^{3}H-ORG-2058 (3–100 nM) and identical samples were incubated with 100-fold molar excess of either unlabeled DES or unlabeled ORG-2058. After 18 h incubation, the free steroids were removed by the dextran-charcoal method and the radioactivity was measured by liquid scintillation count. Data relating to the specific binding were obtained by subtracting nonspecific binding from total binding. Scatchard analysis of the data was performed (Scatchard, 1949). The affinity or its reciprocal, the apparent dissociation constant K_d (nM), was obtained from the slope and the concentration of receptor sites, B_{max} (fmol/mg protein), was obtained from the intercept. The results were sub-

Table I. Effect of OT, 17β-E and Pg treatments on body, uterine and ovarian weights in immature rats (mean ± SD).

Treatment	n	*Body weight (g)*	*Uterine weight (mg/100 g)*	*Ovarian weight (mg/100 g)*
OT (5 IU)	25	40.0 ± 9.7	78.4 ± 28.0	64.7 ± 13.5
Controls	19	35.7 ± 10.5	77.2 ± 13.6	66.7 ± 8.2
OT (0.5 IU)	30	47.9 ± 7.0	82.6 ± 24.8	67.3 ± 6.3
Controls	24	48.3 ± 7.8	87.8 ± 10.2	66.5 ± 9.8
Pg (1 mg)	9	43.1 ± 17.0	143.4 ± 51.0	73.7 ± 20.0
17β-E (10 μg)	12	64.2 ± 10.3	266.7 ± 34.8	84.3 ± 22.8
Controls	12	42.7 ± 11.1	83.2 ± 13.1	67.1 ± 8.8

mitted to linear regression analysis; a linear correlation coefficient *(r)* $P < 0.05$ was considered significant.

Protein concentrations were determined by the method of Lowry *et al* (1951), using BSA as the standard.

Once the first groups of treated and control animals had been assayed, the dissociation constant values for each receptor were compared by analysis of variance (ANOVA) and no difference was detected. The number of binding sites was also calculated using saturating concentration and the results were compared with results obtained using the Scatchard plot. The differences between them were not statistically significant. Estradiol and Pg binding were then measured using a single saturating concentration in quadruplicate to determine both ER and PgR in the same experimental group of animals.

Oxytocin effect on the binding of steroid hormones in vitro

The circulating levels of OT could have been high at the time of tissue collection because of the daily pharmacological doses of this peptide. Competition assays were done to determine whether OT could directly influence the binding of steroid hormones *in vitro*. Assays were performed to determine the extent of steroid binding in the presence of OT 1, 10, 100, 1000 nM and 6.5 μM, or in the presence of its solvent, or in the absence of both (control). Parallel assays were done by preincubating the cytosol with OT for 1 h.

Estradiol and progesterone concentration determinations

Pg and E concentrations were measured in serum, by solid-phase ^{125}I radioimmunoassay, using Coat A Count Progesterone and Estradiol kits (Diagnostic Product Corporation, Los Angeles, CA, USA) with human serum-based standards. All samples were assayed in duplicate. Three control serums with low, intermediate and high concentrations (CON6, Immunoassay Trilevel Control, DPC) were also assayed. The sensitivity was 30 pmol/l for 17β-E and 0.16 nmol/l for Pg.

Calibration curves with supplied calibrators of 17β-E (70–1 320 pmol/l) and Pg (0.30–127.2 nmol/l) and external standards of 17β-E (300–3 700 pmol/l) and Pg (0.80–80.0 nmol/l) diluted in control rat serum (steroid deprived by dextran–charcoal adsorption) were performed in parallel assays to validate the results. Slopes for the logit–log curves were: for 17β-E, 2.0 and 2.1, and for Pg 1.7 and 1.7, for human-serum- and rat-serum-based standards, respectively. Superposition of curves performed with human and rat serum indicated that measurement of steroid hormone levels in rat serum using this method should be valid. Recovery of the external standard was 98 ± 8% for 17β-E and 98 ± 6% for Pg.

The agreement with control serums of high, intermediate and low concentrations was 92, 100 and 100%, respectively, for 17β-E, and 98, 100 and 100% for Pg. The intraassay and interassay variation coefficients were 5.3 and 7.1% for 17β-E and 7.2 and 7.9% for Pg.

Biological activity test for oxytocin

The biological activity of OT was tested *in vitro* by the contractile response of strips of lactating mouse mammary gland (Fielitz *et al*, 1970; Roca *et al*, 1972). The isometric tension developed by the strips was recorded using a polygraph and the effect of increasing concentrations of OT (5–5 000 mU/ml) preincubated in TEDG Buffer and in a physiological solution (Tyrode) as a control, were tested. This was done to verify whether the presence of DTT, a disulfide reducing agent, modified the peptide. The contractile responses of the strips to different OT concentrations were quantitatively maintained throughout the experiment, even though the minimal concentration of oxytocin in TEDG buffer was 5 mU/ml, the same order as in the receptor assays.

Statistical analysis

The data were analysed by Student's *t* test and ANOVA. For each experimental group, the data obtained for the treated animals were compared with their own controls by the Student's *t* for unpaired samples test. The apparent K_d values for each receptor were analysed by ANOVA.

RESULTS

Changes observed after oxytocin administration

Injection of OT did not cause significant variations in body weight or ovarian or uterine weights, expressed as mg/100 g of body weight in immature rats. A significant increase in uterine weight ($P < 0.001$) was observed in the control treatment with 17β-E (table I). Acceleration of vaginal canalization was also found in the 17β-E-treated animals, but not in the OT-treated groups. Direct examination of ovaries showed mature follicles, but neither ovulation points nor corpora lutea were observed.

Estrogen and progesterone receptors

The concentration of receptors and K_d in the different experimental groups treated with 5 or 0.5 IU oxytocin were measured and Scatchard analysis of the steroid-binding data showed a linear plot, indicating the presence of a single class of binding sites for both E and Pg in the control and treated groups. The apparent K_d values showed that each ligand had a high affinity for its binding site. The analysis of variance of the apparent K_d values showed no difference between treated and control groups (table II).

There was no evidence for the presence of multiple binding sites for ER and PgR in rat uteri. Relative concentrations of binding sites between samples using a single saturating labelled hormone concentration was estimated as valid and simultaneous determinations of both receptors could be performed in the same experimental group of animals.

Treatment with the highest dose of OT (5 IU) did not produce a significant effect on steroid receptor levels, but the lower dose (0.5 IU) produced a decrease in both ER and PgR levels, $P < 0.001$ and $P < 0.01$, respectively. Treatment with Pg decreased the level of ER and PgR, $P < 0.001$ and $P < 0.001$ *vs* respective controls (table III). Oxytocin was shown to down-regulate the number of ER and PgR in the rat uterus *in vivo,* although the extent of down-regulation was not as pronounced as with Pg. The reduction of the concentration of receptors in the animals treated with OT (0.5 IU) was 29% for ER and 37% for PgR; in Pg-treated animals the reduction was 59% for both receptors.

Table II. Apparent K_d values (nM) of ER and PgR in rats treated with OT.

OT dose (IU)	K_d *(nM)*			
	ER	(n)	*PgR*	(n)
0.5	0.8 ± 0.3	(8)	2.6 ± 0.2	(2)
0	0.8 ± 0.2	(4)	2.9 ± 1.9	(3)
5	0.5 ± 0.1	(2)	2.8 ± 0.7	(2)
0	0.8 ± 0.3	(2)	1.5 ± 0.6	(2)

Table III. Effect of OT and Pg treatments on ER and PgR contents in immature rat uteri (mean ± SD).

Treatment	*Receptor content (fmol/mg)*			
	ER	(n)	*PgR*	(n)
OT (0.5 IU)	346 ± 105	(17)	433 ± 236	(16)
Control	486 ± 76	(18)	686 ± 237	(14)
OT (5.0 IU)	436 ± 13	(6)	429 ± 45	(4)
Control	360 ± 72	(6)	352 ± 177	(8)
Pg (1 mg)	214 ± 146	(5)	315 ± 26	(5)
Control	527 ± 56	(5)	775 ± 57	(5)

Table IV. Effect of OT treatments on 17β-E and Pg plasma concentrations (mean ± SD)

OT treatment (n)		*17β-E (pmol/l)*	*Pg (nmol/l)*
0.5 IU	(30)	85.5 ± 22.4	24.2 ± 9.9
0	(24)	74.2 ± 5.1	12.4 ± 7.3
5 IU	(25)	80.0 ± 40.7	7.0 ± 6.4
0	(19)	49.2 ± 21.6	5.1 ± 4.8

Estradiol and progesterone concentrations

The plasma 17β-E and Pg levels showed no significant changes in OT-treated animals in comparison with controls (table IV).

Oxytocin effect on the binding of steroid hormones in vitro

The binding curves were similar when the cytosol was incubated in the presence or absence of OT or its solvent. There were no significant differences in the parameters of the high affinity binding for both hormones, the concentration of receptors and the apparent K_d according to the Scatchard plots of the same data. The extent of steroid binding was also the same, independently of whether the cytosol was preincubated with OT for 1 h.

DISCUSSION

In the present study, we found decreased uterine binding for both E and Pg in the experimental groups of animals treated with 0.5 IU OT. The affinity of ER and PgR in the uterus *in vivo* was similar to *in vitro* results and to previously reported studies, indicating the presence of the same receptor systems. Analysis of variance indicated that there was no OT effect on the affinity of both receptors. Thus, changes observed in E and Pg binding were due to changes in the receptor concentrations and not to changes in the affinity.

Although peptide and steroid hormones are structurally different and function through different mechanisms, AVT inhibits the binding of tritiated estradiol to the cytosol receptors *in vitro* as reported by Vaugham *et al* (1979). OT differs by only 1 amino acid from AVT and may influence the hormone binding properties of steroid receptors. Circulating levels of OT may have been high at the time of tissue collection because of the pharmacological doses of this peptide which were given for a period of 5 d. Therefore, the decreased number of ER and PgR could be a direct effect of OT inhibition on steroid binding to receptors, similar to that described by Vaughan *et al* (1979) *in vitro* for AVT. The lack of influence of OT on the binding characteristics of ER and PgR *in vitro*, apparent K_d and B_{max}, indicates that the decrease in binding was not due to a direct inhibition by circulating OT on the binding assay but to a real depletion of the uterine receptor contents. Furthermore, the receptor assay buffer contains DTT, a reagent to keep the sulfhydryl groups in a reduced state. This may reduce the OT disulfide bond and alter its biological activity. We therefore tested OT biological activity in the same conditions as in the receptor assay and no modifications were found.

We conclude that there was no direct OT effect on the binding of E and Pg to their receptors, similar to that described for AVT on ER (Vaughan *et al,* 1979).

Depletion of both receptors following treatment with 0.5 IU OT, provides evidence of its involvement in the regulation of ER and PgR contents in the rat uterus. Such an involvement may be systemic as well as local. Concentrations of ER, PgR and OTR are influenced by circulating levels of steroid hormones (Clark *et al,* 1985; Meyer *et al,*

1988). Macromolecular synthesis is stimulated by E, leading to the accumulation of ER, PgR and OTR in uterine target cells. On the other hand, Pg down-regulates these receptors through a Pg-induced reduction of ER concentrations.

In our study, 17β-E and Pg concentrations in peripheral blood obtained at the end of the experimental period were similar to those described for the basal range in mature female animals (Butcher *et al,* 1974); there were no significant differences between control and treated groups. OT has been reported to both stimulate and inhibit Pg production, or to have no effect (Kahn Dawood *et al,* 1989; Tan *et al,* 1982). Al Janabi and Yousif (1987) reported increased 17β-E concentrations with OT treatment, although their values were within the basal range. Our results were not consistent with the latter and indicated that there was no effect on the plasma concentrations of either hormones. The action of OT on ER and PgR concentrations could be exerted through a systemic mechanism mediated by the steroid hormones, but this is improbable owing to the absence of significant changes in circulating 17β-E and Pg concentrations.

Estrogens stimulate PgR production and uterine weight gain (Leavitt *et al,* 1978). The results of our experiments were not consistent with the postulated estrogenic action of OT (Al Janabi and Yousif, 1987), because neither uterotrophic nor PgR responses were observed.

Progesterone down-regulates both ER and PgR (Clark *et al,* 1985; Meyer *et al,* 1988). In agreement with this statement, our control animals treated with Pg showed a large decrease in the number of ER and PgR. OT would exert an effect similar to that of Pg in the down-regulation of ER and PgR. This is consistent with the previously reported inhibitory action of Pg and OT hormones on OTR (Sheldrick and Flick-Smith, 1993).

Uterine receptor populations for Pg and OT influence the timing of uterine prostaglandin (PG)$F_{2\alpha}$ secretion (Zollers *et al,* 1993) and the development of the positive feedback loop between $PGF_{2\alpha}$ and luteal OT, which completes the process of luteal regression (McCracken *et al,* 1984; Vallet *et al,* 1990). The down-regulation of steroid uterine receptors exerted by OT could have potential implications in the control of this loop and influence the reproductive capacity and fertilization. This is consistent with the reduction of the conception rates found in ewes treated with OT for immunization (Wathes *et al,* 1989). All this evidence strongly suggests a further and as yet unidentified reproductive function for OT.

In conclusion, concentrations of ER and PgR were lower in the OT-treated animals. Neither a direct *in vitro* action of OT nor a systemic regulation mediated by E and Pg was found for either sexual hormone receptor systems. The underlying mechanism responsible for OT-induced changes is unknown. Nevertheless, OT activates the phosphoinositol signal system through its membrane receptor and could be able to impair the steroid receptor phosphorylation and lower their binding capacity and/or their synthesis rate.

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