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Oxytocin receptors in the porcine endometrium during the estrous cycle and early pregnancy

Akira Okano [a,*], Kiyoshi Okuda [b], Masashi Takahashi [a], Dieter Schams [c]

[a] *Department of Animal Reproduction, National Institute of Animal Industry, Tsukuba Norindanchi, Ibaraki 305, Japan*

[b] *Division of Animal Science and Technology, Faculty of Agriculture, Okayama University, Okayama 700, Japan*

[c] *Institute of Physiology, Technical University of Munich, 8050 Freising-Weihenstephan , Germany*

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Abstract

Oxytocin (OT) receptors in the porcine endometrium were investigated at four stages of the estrous cycle (Days (D) 0, 5, 10 and 15, $n = 3$), and at two stages of early pregnancy (D5 and D15 after mating, $n = 3$) by a radioreceptor assay using ^{125}I-labeled OT antagonist [d(CH_2)$_5$,Tyr(Me)^2,Thr^4,Tyr-NH_2^9]-vasotocin. Binding specificity was demonstrated by displacement with four peptides related to oxytocin ([Arg^7]-vasopressin, [Thr^4,Gly^7]-OT, OVT, OT) and two peptides unrelated to oxytocin (luteinizing hormone-releasing hormone, [Ile^3]-pressinoic acid (tocinoic acid)). The dissociation constant (K_d) of endometrial OT receptors on D0 (0.59 ± 0.10 nM) was similar to those on D10 and D15 (D10, 0.75 ± 0.21; D15, 0.60 ± 0.14 nM; mean ± SEM). In the early luteal stage (D5), K_d (2.41 ± 0.24 nM) was higher than on D0, D10 and D15 ($P < 0.01$). In early pregnancy, K_d values were 3.25 ± 0.29 nM on D5 and 2.44 ± 0.44 nM on D15. Binding site concentration (B_{max}) on D0 (910.0 ± 25.1 fmol mg^{-1} protein) was significantly higher than on D5 and D10 (D5, 322.5 ± 71.7; D10, 147.5 ± 25.8 fmol mg^{-1} protein; $P < 0.01$) of the estrous cycle and D5 and D15 (D5, 302.5 ± 82.6; D15, 315.0 ± 20.1 fmol mg^{-1} protein; $P < 0.01$) of early pregnancy. In the two stages of early pregnancy, B_{max} values were constant and similar to that on D5 of the early luteal stage.

Our results reveal the existence of specific OT binding sites in the porcine endometrium during the estrous cycle and early pregnancy. Furthermore, the fluctuation in the binding of OT to the

* Corresponding author. Tel.: 0298-38-8637; fax: 0298-38-8606; e-mail: aokano@niai.affrc.go.jp.

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endometrium during the different stages of the estrous cycle suggests that OT plays an important role in regulating the estrous cycle of the pig as seen in other animals.

Keywords: Pig—uterus; Oxytocin receptors; Endometrium; Estrous cycle; Pregnancy

1. Introduction

It is known that prostaglandin (PG) F2α secreted from the uterus plays an important role in luteolysis in sows as in ruminants (Bazer et al., 1984; Bazer, 1989; Bazer, 1992). However, the endocrine mechanisms regulating the secretion of PGF2α from porcine uterus at the time of luteal regression have not been clearly delineated. In sheep and cows, the concentration of uterine oxytocin (OT) receptors varies during the estrous cycle (Roberts et al., 1976; Sheldrick and Flint, 1985) and is correlated with the amount of PGF2α released in response to OT (McCracken et al., 1984). The establishment of pregnancy in sheep and cows is associated with a reduction of OT effect on PGF2α release from the uterus (Roberts et al., 1976). Administration of exogenous OT to sows in the late phase of the estrous cycle induced a prompt increase in uterine secretion of PGF2α (Kieborz et al., 1991), and the endometrium of pigs responds in vitro to OT with increased secretion of PGF2α (Gross et al., 1988). Further, this action of OT is possibly mediated via specific OT receptors. Although there has been a report on the binding sites of OT in porcine myometrium in late pregnancy (Soloff and Swartz, 1974), this information may not be applicable to the endometrium of the estrous cycle. Recently a report of OT receptors in porcine endometrium has been presented (Whiteaker et al., 1995). The elucidation of OT receptors in the porcine endometrium is essential for better understanding of the mechanism regulating the estrous cycle and establishment of pregnancy. Therefore, the present study was conducted to quantify and characterize specific OT binding sites in porcine endometrium during the estrous cycle and early pregnancy.

2. Materials and methods

2.1. Tissue preparation

Uteri from crossbred sows (Landrace × Large White × Duroc) were obtained immediately after slaughter at four stages of the estrous cycle (Days (D) 0, 5, 10 and 15; $n = 3$) and of early pregnancy (D5 and D15 after mating; $n = 3$). Once removed, the uterus was placed on ice and transported to the laboratory within 10 min. The days selected for sample collection represent estrus (D0), an initial period of luteinization (D5), the mid luteal phase (D10) and the period when corpora lutea begin to regress (D15). To confirm pregnancy in the mated pigs, we flushed their uterine horns with phosphate-buffered saline (PBS) and observed the presence of embryos or elongated conceptuses in the flushed PBS. Endometrial tissues were separated from myometrium in ice cooled conditions. The separated tissues were frozen and stored at −80°C until

processed for receptor assay. Tissues were rinsed in 10 mM Tris–HCl buffer containing 1.5 mM EDTA and 0.5 mM dithiothreitol (DTT) (pH 7.4), and homogenized with five 5-s pulses at full speed in the same buffer using Polytron homogenizer (Brinkman, Westbury, NY, USA). The inclusion of EDTA ensures the complete dissociation of any endogenous receptor-bound OT (Fuchs et al., 1983; Sheldrick and Flint, 1985). Homogenized endometrial tissue was centrifuged at $1000 \times g$ for 15 min. The supernatant was centrifuged further at $120\,000 \times g$ for 30 min and the pellet resuspended and recentrifuged three times in 50 mM Tris–HCl (pH 7.6) to wash away EDTA and DTT. The pellet was resuspended in the same buffer and the protein concentration of the membrane preparation was measured by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

2.2. Radioreceptor assay

The OT antagonist [d(CH_2)$_5$,Tyr(Me)^2,Thr^4,Tyr-NH_2^9]-vasotocin (OVT), (Peninsula Laboratories, Belmont, CA, USA) was iodinated by a modified lactoperoxidase method (Schams et al., 1979). Labeled ^{125}I-OVT was separated from unlabeled OVT by a CM-Sephadex C-50 (Pharmacia, Uppsala, Sweden) using 0.5 mM ammonium acetate buffer (pH 4.0). Binding assays were similar to those of Serina et al. (1989). Briefly, total specific binding was measured by the binding assay, which consisted of 0.1 ml of membrane suspension (1 mg protein ml^{-1}), 0.05 ml ^{125}I-OVT (400 000 cpm ml^{-1}), and 0.1 ml assay buffer (50 mM Tris–HCl, 5 mM $MgCl_2$, 0.1% BSA at pH 7.6) with or without an excess amount of unlabeled OT (100 nM; Peninsula Laboratories). The samples were incubated at 24°C for 2 h and then bound ^{125}I-OVT was precipitated by the addition of 0.3 ml bovine γ-globulin (4 mg ml^{-1}) and 1.5 ml of 22% (w/v) polyethylene glycol followed by centrifugation at $1500 \times g$ for 30 min and counted for 2 min in Packard Model 500 Auto γ-counter (Packard, Downers Grove, IL, USA). The counts in the tubes with excess OT (i.e. the non-specific binding) were subtracted from the counts in the tubes without added OT to obtain specific counts bound. To determine the ligand specificity of the receptor, competitive binding assays were performed with related peptides ([Arg^8]-vasopressin (AVP), [Thr^4,Gly^7]-OT, OVT, OT) and with unrelated peptides (luteinizing hormone-releasing hormone (LHRH) and [Ile^3]-pressinoic acid (tocinoic acid)) (all purchased from Peninsula Laboratories). To obtain receptor concentration and the affinity (K_d) for OT binding, samples of membrane were incubated with serial concentrations of unlabeled OT (0–23 700 pg per tube).

2.3. Statistical analysis

Data obtained from binding of ^{125}I-OVT and OT to porcine endometrium membrane were analyzed using the LIGAND program (Munson and Rodbard, 1980) using nonlinear iterative curve-fitting procedures (McPherson, 1985). The initial parameters were calculated by Scatchard analysis (Scatchard, 1949) and were then iteratively refined until the weighted sum of squares was minimized. The goodness of fit for the selected model was assayed by the 'runs test': different models (one- or two-site models) were compared using F-test statistics to determine whether a change in the model resulted in

a significant reduction of the weighted sum of squares. The criteria for rejection or accepting a particular model were based on the calculated probability (Munson and Rodbard, 1980). Specific binding of ^{125}I-OVT to membrane preparation from different stages of the estrous cycle and early pregnancy were compared among groups of data by analysis of variance. Data are presented as mean ± SEM. Tukey's multiple comparison was used to determine the significance of differences among means of each group when significant F-values were obtained.

3. Results

3.1. Radioreceptor assay conditions

The conditions for the radioreceptor assay on porcine endometrium as described above were validated. Specific binding of ^{125}I-OVT to uterine membrane increased with increasing protein amount from 3.1 to 100 mg protein per tube (Fig. 1(A)). For this reason, subsequent assays were performed with 100 μg protein per tube. Specific binding reached maximum values between 2 and 4 h at 24°C (Fig. 1(B)). Addition of $MgCl_2$ to an assay buffer at a concentration of 5 mM gave better binding specificity for OT than $MnCl_2$ at the same concentration. The potency of unlabeled OT and OVT in displacing the ^{125}I-labeled ligand was similar in the presence of the Mg^{2+} or Mn^{2+} ions (Fig. 2). In contrast, the displacement curves for AVP were different: the relative affinity of AVP was considerably lower in the presence of $MgCl_2$ than in the presence of $MnCl_2$. Therefore, only $MgCl_2$ was used in the following experiments.

3.2. Binding characteristics

Fig. 3 shows the displacement curves of ^{125}I-OVT with four peptides related to OT and two peptides unrelated to OT on the endometrium from D0. Two displacement

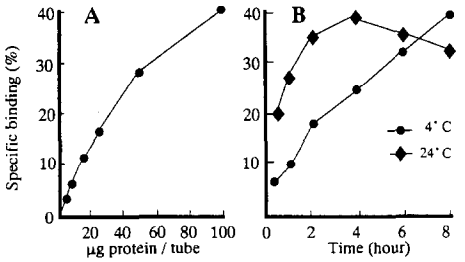

Fig. 1. Binding of ^{125}I-OVT to membrane protein of porcine endometrial membrane obtained during the estrous stage (D0). The difference in the binding of ^{125}I-OVT in the presence and absence of 100 nM OT was used to calculate the specific binding, expressed as a percentage of total radioactivity added. (A) Specific binding of ^{125}I-OVT to membrane protein increased with increasing amount of membrane protein from 3.1 to 100 mg protein per tube. Binding curve fitted for the polynomial regression ($y = 0.34 + 0.79x - 5.99x^2 + 2.15x^3$, $r^2 = 1.00$). (B) Relationships between incubation time and temperature (4 or 24°C). Maximum specific binding was reached between 2 and 4 h at 24°C.

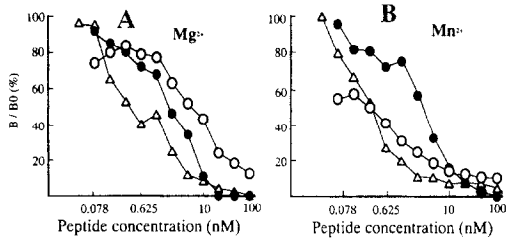

Fig. 2. Competitive binding of ^{125}I-OVT and unlabeled OT (△), OVT ([d(CH$_2$)$_5$,Tyr(Me)^2,Thr^4,Tyr-NH$_2^9$]-vasotocin, ●) and AVP ([Arg^8]-vasopressin, ○) in the presence of Mg^{2+} (A) or Mn^{2+} (B) to porcine endometrial membrane from estrous stage (D0). The displacement curves for AVP were different: the relative affinity of AVP was considerably lower in the presence of $MgCl_2$ than in the presence of $MnCl_2$.

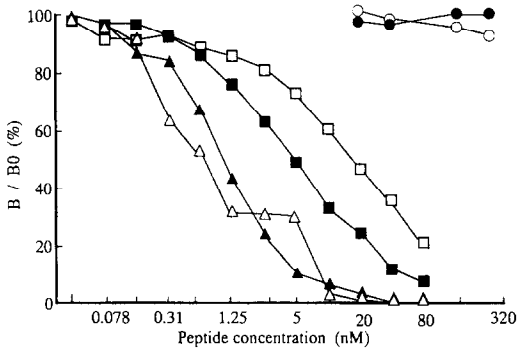

Fig. 3. Competitive binding of ^{125}I-OVT and unlabeled neurohypophysial hormones as well as some of their synthetic analogues to porcine endometrial cell membrane from the estrous stage. ▲, oxytocin (OT); △, [d(CH$_2$)$_5$,Tyr(Me)^2,Thr^4,Tyr-NH$_2^9$]-vasotocin (OVT); ■, [Arg^8]-vasopressin (AVP); □, Tyr^4,Gly^7-OT; ●, luteinizing hormone-releasing hormone (LHRH); ○, [Ile^3]-pressinoic acid (tocinoic acid).

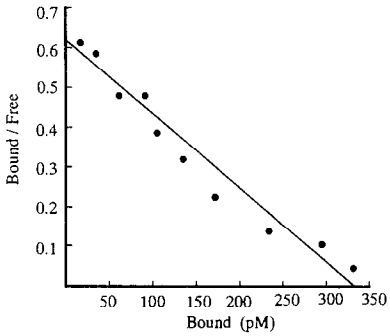

Fig. 4. Representative Scatchard plot for the competitive binding of ^{125}I-OVT and unlabeled OT to porcine endometrial membrane from the estrous stage (D0), linear and fitted for the one-site model by the 'runs test'.

curves of ^{125}I-OVT with OVT and OT were similar in the range from 0.039 to 80 nM. However, the binding was specific for OT and related peptides, 10- to 20-fold greater concentrations of AVP and [Tyr^4,Gly^7]-OT than of OT were required to displace 50%

Table 1
K_d values for OT receptors in porcine endometrial membrane preparations ($n = 3$, mean ± SEM)

Values	K_d (nM)				P
	Day 0 (estrus)	Day 5	Day 10	Day 15	
Non-pregnant	0.59 ± 0.10	2.41 ± 0.24*	0.75 ± 0.21	0.60 ± 0.14	< 0.01
Pregnant	–	3.25 ± 0.29	–	2.44 ± 0.44 *	NS
P	–	NS	–	< 0.01	

* $P < 0.01$; NS, non significance. K_d values from different stages were compared within non-pregnant or pregnant pigs, and values from same stages (Days 5 and 15) of non-pregnant pigs were also compared.

of the ^{125}I-OVT binding. Unrelated peptides such as LHRH and tocinoic acid did not show any displacement.

Scatchard analysis showed that K_d and binding site concentration (B_{max}) values of the sample from D0 for OT were 0.59 nM and 910.0 fmol mg^{-1} protein, respectively. Fig. 4 shows a representative Scatchard plot of OT binding to endometrium from estrous pigs. Scatchard analyses of the binding data (^{125}I-OVT/OT) at all stages of the estrous cycle and early pregnancy gave linear plots and were fitted for the one-site model by the 'runs test'.

3.3. Specific binding at different stages of the estrous cycle and early pregnancy

In further experiments, investigation of the assay and characterization of the binding affinity was extended to a membrane preparation from the endometrium on D5, D10 and D15 of the estrous cycle and D5 and D15 of early pregnancy.

Table 1 presents a comparison of K_d values for receptors on membrane protein prepared from endometrium at four different stages of the estrous cycle and two stages of early pregnancy. The K_d value of the endometrial OT receptor on D0 was similar to that on D10 and D15. In the early luteal stage, K_d on D5 was significantly higher than on D0, D10 and D15 ($P < 0.01$). In the early pregnant stage, K_d on D5 was similar to

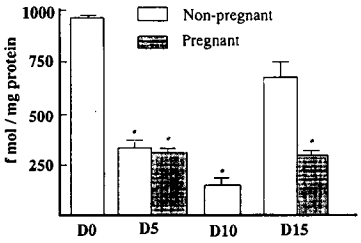

Fig. 5. Concentrations of [^{125}I]-OVT binding sites in porcine endometrial membrane preparations from pigs at some stages of the estrous cycle (shaded bars) and early pregnancy (shaded bars) (fmol mg^{-1} protein; $n = 3$ pigs on each day). Values are means ± SEM. Values for the days of the estrous cycle and early pregnancy were compared by Tukey's multiple comparison test. Asterisks indicate significant difference ($P < 0.01$). [^{125}I]-OVT binding capacity was greater ($P < 0.01$) on D0 and D15 of the estrous cycle than on other days of the estrous cycle and early pregnancy.

that on D15. The highest binding capacity was seen on the membrane protein from pigs of the estrous stage (D0). Results for OT receptors in the porcine endometrium are shown in Fig. 5. B_{max} on D0 (910.0 ± 25.1 fmol mg^{-1} protein) was not significantly different from that on D15 (697.5 ± 77.0 fmol mg^{-1} protein), but was significantly higher than that on D5 (322.5 ± 71.7 fmol mg^{-1} protein) and D10 (147.5 ± 25.8 fmol mg^{-1} protein). B_{max} values in the early pregnancy stage (D5, 302.5 ± 82.6; D15, 315.0 ± 20.1 fmol mg^{-1} protein) were significantly lower than on D0 and D15 of the estrous cycle ($P < 0.01$). In samples taken during early pregnancy, K_d and B_{max} for OT binding were similar, and were the same on D5 in the early luteal stage.

4. Discussion

In this study we used an iodinated OT antagonist (^{125}I-d[$(CH_2)_5$,Tyr(Me)^2, Thr^4, Tyr-NH_2^9]-OVT) as radiolabeled ligand for radioreceptor assay. This OVT is a highly selective OT receptor ligand which can be labeled to a high specific activity without losing its biological activity (Elands et al., 1987; Okuda et al., 1992). ^{125}I-OVT showed a higher radioactivity and selectivity for OT binding sites than ^{3}H-OT (Elands et al., 1987). Binding of ^{125}I-OVT to membrane protein was closely related to incubation conditions such as protein amount, temperature (4 and 24°C) and time. Presence of a heavy metal ion (Mg^{2+}) in the assay buffer improved the binding specificity of ^{125}I-OVT. This radiolabeled ligand was displaced by radioinert OT or OVT in low concentrations, and by considerably greater concentrations of radioinert AVP or [Thr^4,Gly^7]-OT. In contrast, ^{125}I-OVT was not displaced by high concentrations of LHRH or tocinoic acid, peptides which are unrelated to OT. Soloff and Swartz (1974) studied the OT receptor in porcine myometrium during the late stage of gestation, using ^{3}H-OT as radioactive ligand. The results of the experiments reported here have provided evidence for the existence of OT binding sites in the porcine endometrium. The K_d value reported here for the single site in the endometrium in the estrous stage ($K_d = 0.59 \pm 0.10$ nM) was lower than that given for the myometrium of pigs in the late stage of gestation ($K_d = 1.5$ nM, Soloff and Swartz, 1974) and for the endometrium on D15 in the late luteal stage ($K_d = 1.59$ nM, Whiteaker et al., 1994). Soloff and Fields (1989) reported that OT bound to bovine endometrial membrane obtained during various stages of the estrous cycle with K_d values ranging from 1.3 to 25.7 nM. Conversely, K_d values from our study for porcine endometrium in some stages of estrous cycle and early pregnancy had a small range of binding affinities, which depended on the stage of cycle and pregnancy. Changes in K_d during the estrous cycle and early pregnancy suggest that the endometrial response to OT may be regulated at the receptor level and by circulating OT concentrations (Soloff and Fields, 1989). OT receptor concentration on D0 was higher than on D5 and D10. However, the concentration on D15 may indicate a tendency to increase the number of OT receptors as the next estrus approached. Accordingly, our data on OT binding characteristics using ^{125}I-OVT in porcine endometrial tissues would be comparable to that of the ovine and the bovine uterus (Roberts et al., 1976; Sheldrick and Flint, 1985; Meyer et al., 1988; Soloff and Fields, 1989; Serina et al., 1989; Fuchs et al., 1990).

Changes in binding affinity and receptor concentration at different stages of the estrous cycle suggest that OT plays an important role in regulating the estrous cycle by means of stimulating PGF2α synthesis in pigs (Kieborz et al., 1991; Whiteaker et al., 1994) and in ruminants (Roberts et al., 1976; Sheldrick and Flint, 1985; Fuchs et al., 1990). It has been well demonstrated that the porcine endometrium in vitro responds to OT by secreting PGF2α (Gross et al., 1988). In the present study, OT binding affinities on D5 of the estrous cycle and early pregnancy were lower than in other stages of the estrous cycle, but OT receptor concentrations on D0 and D15 of the estrous cycle were higher than on D5, D10 and early pregnancy. These results suggested that high affinity and concentration of endometrial OT receptors from luteolysis to estrous would interrelate with the luteal regression by means of stimulating PGF2α synthesis in the endometrium. Pulsatile secretion of PGF2α, which is released from the ovine uterus during the latter part of the luteal stage, ensures the progress of luteolysis and the final demise of the corpus luteum in the ewe (McCracken et al., 1984) and in the pig (Moeljono et al., 1977). Although pulsatile PGF2α secretion was detected in cyclic pigs, it did not occur in pregnant pigs (Moeljono et al., 1977). Jenner et al. (1991) suggested that uterine oxytocin receptor concentrations are important in the process of luteolysis in the cow, and the prevention of the rise in concentration of endometrial OT receptors is important in maternal recognition and the maintenance of pregnancy. Sheldrick and Flint (1985) demonstrated that OT receptor concentrations in pregnant ewes are low at the expected time of luteolysis and that this also occurs in cows. It has been suggested that maintaining low concentrations of OT receptors in pregnancy may be a way in which uterine responsiveness to OT is reduced, in order to ensure a decreased uterine secretion of PGF2α at this time (Sheldrick and Flint, 1985; Fuchs et al., 1990). LaFrance and Goff (1985) reported that the release of PGF2α in response to OT in pregnant heifers in vivo is suppressed on days 17, 18, and 19 in comparison with non-pregnant heifers.

In the present study, low concentrations of OT receptors in the endometrium of early pregnant pigs were in agreement with the above mentioned findings in the cow and ewe. Since binding affinities and OT receptor concentrations in the endometrium on D5 and D15 of early pregnancy were constant, it is possible that signals from the conceptuses in early pregnant pigs suppress an increase in the OT receptor concentration in the endometrium as seen in the cow (Fuchs et al., 1990). We may conclude that OT plays an important role in luteolysis and the estrous cycle by synthesizing PGF2α, via OT receptors, in the porcine endometrium, as has also been reported for the cow and ewe. In addition, suppressed OT receptors in the endometrium of early pregnant pigs are an important component of the mechanism of the recognition of pregnancy in the pig.

Acknowledgements

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