

The effect of progesterone on oxytocin-stimulated intracellular Ca^{2+} mobilisation and prostaglandin secretion in porcine endometrium

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Abstract

We have studied in the porcine endometrium the expression of oxytocin receptor (*OTR*) mRNA and the effect of progesterone (P_4) on oxytocin/oxytocin receptor (*OT/OTR*) function concerning intracellular Ca^{2+} mobilisation ($[\text{Ca}^{2+}]_i$), prostaglandin $\text{F}_2\alpha$ ($\text{PGF}_2\alpha$) and E_2 (PGE_2 ; PG) secretion. Tissue was taken from cyclic and early pregnant pigs (days 14-16). A higher expression of *OTR* mRNA ($P < 0.05$) was observed in the endometrium of cyclic than pregnant pigs. The stimulatory ($P < 0.05$) effect of *OT* (10^{-7} M) on $[\text{Ca}^{2+}]_i$ mobilisation was noticed within 15-60 s and 30-60 s in endometrial stromal cells of cyclic and pregnant pigs, respectively. In the presence of P_4 (10^{-5} M) basal and *OT*-stimulated $[\text{Ca}^{2+}]_i$ concentrations decreased in stromal cells during luteolysis and pregnancy. In stromal cells P_4 delayed mobilisation of $[\text{Ca}^{2+}]_i$ in response to *OT* by 15 s during luteolysis and had no effect during pregnancy. In cyclic and pregnant epithelial cells *OT* stimulated mobilisation of $[\text{Ca}^{2+}]_i$ in 45 s and 60 s, respectively. Oxytocin increased ($P < 0.05$) $\text{PGF}_2\alpha$ secretion during luteolysis and pregnancy and PGE_2 during luteolysis from endometrial slices. Progesterone did not inhibit this stimulatory effect. During luteolysis *OT* increased ($P < 0.05$) $\text{PGF}_2\alpha$ in epithelial and stromal cells and PGE_2 secretion in epithelial cells. In the presence of P_4 this effect of *OT* was reduced only in stromal cyclic cells (6 h culture). The presence of P_4 decreased the effect of *OT* on $[\text{Ca}^{2+}]_i$ mobilisation only in stromal cells. We found that, in most conditions, P_4 did not inhibit the *OT*-stimulated secretion of PG in the porcine endometrium.

Key words: pigs, oxytocin, prostaglandins, luteolysis, pregnancy

Introduction

Oxytocin regulates prostaglandin $\text{F}_2\alpha$ ($\text{PGF}_2\alpha$) and prostaglandin E_2 (PGE_2) secretion from porcine endometrium during luteolysis and early pregnancy (Whiteaker et al. 1995, Uzumcu et al. 2000, Blitek and

Ziecik 2004). This hormone acts through specific oxytocin receptors (*OTR*) present on the endometrial cells of cyclic and early pregnant pigs (Ludwig et al. 1998, Franczak et al. 2005). The concentration of *OTR* in porcine endometrium is controlled mainly by estradiol 17β and P_4 and thus depends on the phase of

the estrous cycle and the period of pregnancy (Okano et al. 1996, Franczak et al. 2002).

Oxytocin binds to OTR and activates the intracellular phosphatidylinositol pathway generating two second messengers: inositol triphosphate, which increases $[Ca^{2+}]_i$ concentration, and diacylglycerol (Whiteaker et al. 1995, Franczak et al. 2006). We have previously shown that OT-controlled prostaglandins (PG) secretion by porcine myometrial cells harvested during luteolysis and pregnancy is associated with mobilisation of $[Ca^{2+}]_i$ and that P_4 delays this effect of OT (Franczak et al. 2006). The role of P_4 in modifying the action of OT on $[Ca^{2+}]_i$ mobilisation in porcine endometrial cells is still unknown.

In various species OT action in target cells may be regulated by P_4 . This steroid modulated *OTR* gene expression in endometrial and myometrial cells in rats (Engström et al. 1999, Murata et al. 2000) and decreased OT-stimulated $PGF2\alpha$ secretion in bovine endometrial cells via genomic (Skarzynski et al. 1999) and nongenomic (Bogacki et al. 2002) action. The precise mechanisms of P_4 action on OT-controlled effects in porcine endometrium remain unclear.

In the present study we have examined: 1) *OTR* mRNA expression in porcine endometrium harvested during luteolysis (days 14-16 of the estrous cycle) and early pregnancy (days 14-16), 2) the role of P_4 in modulation of OT action on endometrial $[Ca^{2+}]_i$ mobilisation and PG secretion by endometrial slices and isolated endometrial cells cultured *in vitro*.

Materials and Methods

Animals and collection of the blood samples and endometrial tissue. All experiments were approved by the Animal Ethics Committee, University of Warmia and Mazury in Olsztyn. Pubertal crossbred gilts (Large White × Polish Landrace) weighing 90-110 kg were used during the natural estrous cycle (days 14-16) and early pregnancy (days 14-16). Blood samples and uteri from the studied pigs were taken immediately after slaughter. Isolated uteri were placed in ice-cold PBS supplemented with 10 IU/ml penicillin (Polfa, Poland) and 100 µg/ml streptomycin (Polfa, Poland) and transported to the laboratory. Plasma samples were collected and stored at -20°C until P_4 determination. Sections of the middle part of the porcine uterine horns were opened longitudinally on the mesometrial surface and the endometrium was separated from the myometrium. Fragments of the endometrium of cyclic and pregnant pigs were frozen in liquid nitrogen and stored at -70°C until total RNA isolation to study the expression of *OTR* mRNA (experiment 1). Separated endometrial slices were used to study the effect of OT on PG secretion in the presence or absence of P_4 (experiment 3). The remaining

endometrium was digested to obtain isolated epithelial and stromal cells used to study the effect of OT and P_4 on $[Ca^{2+}]_i$ mobilisation (experiment 2) and PG secretion (experiment 4).

Expression of *OTR* mRNA. Total RNA extraction from the endometrium was performed using Qiagen RNasy kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Expression of *OTR* mRNA in porcine endometrium during luteolysis ($n=4$) and early pregnancy ($n=4$) was investigated with Real-Time PCR. The primers for amplification of the *OTR* gene fragment used in this study were described previously (Gorbulev et al. 1993, Ludwig et al. 1998). Results were normalized using the housekeeping gene, glyceraldehydes-3-phosphate-dehydrogenase (GAPDH; Bogacka et al. 2006). Real-Time PCR was performed using a kit for one-step RT-PCR (Roche, Mannheim, Germany) and fast Start DNA Master SYBR Green I kit (Roche, Switzerland). A standard Light Cycler program was run with a 57°C annealing temperature for *OTR* and 59°C for GAPDH.

Isolation and culture of endometrial cells. The epithelial and stromal cells were isolated with enzymatic dispersion of the endometrium (Zhang et al. 1991). The epithelial cells were obtained by digestion with use of 0.48% dispase (Gibco, Germany). After separation of epithelial cells, the remaining tissue was treated successively with 0.25% trypsin (Sigma, Germany) and 0.06% collagenase I (Sigma, Germany) to isolate the stromal cells. Cellular viability ($>98\%$) was estimated with a 0.4% Trypan Blue dye exclusion test. Viable epithelial and stromal cells were cultured separately, in 24-well plates (Nunc, Germany) at 37°C in a humidified atmosphere of 5% CO_2 in air, in medium M-199 (Sigma, Germany) supplemented with 2% BSA fraction V (ICN, USA) and 10% FCS (Gibco, Germany). The culture medium was changed every 24 h until the cells were confluent (3-4 days after plating).

Endometrial $[Ca^{2+}]_i$ mobilisation. Measurement of $[Ca^{2+}]_i$ mobilisation in epithelial and stromal cells was performed according to the method of Grynkiewicz et al. (1985). Endometrial cells ($5 \times 10^4/\text{ml}$) of the cyclic ($n=5$) and early pregnant ($n=5$) pigs were loaded with the cell-permeable form of fluorescent Ca^{2+} indicator Fura-2AM (5 µg/ml) at 37°C for 40 min in an atmosphere of 95% air and 5% CO_2 . Cells were then washed in M-199 with 0.1% BSA and pre-incubated with or without P_4 (10^{-5} M; Serva, Germany) for 20 min at 37°C followed by 10 min at room temperature. The intensity of $[Ca^{2+}]_i$ -dependent fluorescence was measured in individual cells every 5 s from 15 s before and through 60 s after treatment with OT (10^{-7} M; Sigma, Germany) using a fluorescent inverted microscope (Olympus IX70) supplied with camera (Ikegami color SL3) and MicroImage 4.0

image analysis software (Olympus Optical Co, Hamburg, Germany).

Prostaglandin secretion from endometrial slices. Slices of porcine endometrium isolated during luteolysis ($n=7$) and early pregnancy ($n=8$) were washed twice in PBS and used to study the influence of OT alone or with P_4 on $PGF2\alpha$ or $PGE2$ release. Individual slices (200–210 mg, 3 mm thick) were placed in culture vials containing 2 ml M-199 medium supplemented with 0.1% BSA and 20 $\mu\text{g/ml}$ gentamycin (KRKA, Slovenia). Slices were pre-incubated for 24 h at 37°C in an atmosphere of 95% O_2 and 5% CO_2 . After pre-incubation, the medium was changed to fresh and slices of the endometrium were treated with control medium, OT (10^{-7} M), P_4 (10^{-5} M) or P_4 +OT for the next 6 h. After incubation, the medium was collected and frozen at -20°C until $PGF2\alpha$ and $PGE2$ assay.

Prostaglandin secretion by endometrial cells. Isolated epithelial and stromal cells ($3 \times 10^5/\text{ml}$) were cultured in M-199 medium with 0.1% BSA and 20 $\mu\text{g/ml}$ gentamycin until they reached confluence. The cells from cyclic ($n=3$ to 6) and early pregnant ($n=3$ to 5) uteri were treated for 6 h and 12 h with control medium, OT (10^{-7} M), P_4 (10^{-5} M) or P_4 +OT. After incubation, the medium was collected and frozen at -20°C until analysis of $PGF2\alpha$ and $PGE2$ concentrations. The selected doses of OT (10^{-7} M) and P_4 (10^{-5} M) were used in previous studies for stimulation of PG secretion and for inhibition of OT-induced PG release by uterine tissues, respectively (Whiteaker et al. 1995, Uzumcu et al. 2000, Franczak et al. 2006).

Progesterone and prostaglandin determination. Progesterone plasma level was determined with RIA according to Ciereszko et al. (1998). The sensitivity of P_4 assay was 0.1 ng/ml and the intra-assay coefficient was 6.9%. Concentrations of $PGF2\alpha$ and $PGE2$ in the medium were determined with EIA as described by Uenoyama et al. (1997) and Skarzynski and Okuda (2000), respectively. The sensitivity of the $PGF2\alpha$ and $PGE2$ assays was 0.1 and 0.8 ng/ml, respectively. The inter- and intra-assay coefficients were 16.2% and 6.1% for $PGF2\alpha$ and 13.5% and 4.1% for $PGE2$, respectively.

Statistical analysis. All data were expressed as mean \pm SEM. Plasma P_4 concentration and the expression of mRNA encoding endometrial *OTR* during luteolysis and early pregnancy were analysed with Student's *t*-test (Statistica, StatSoft, Tulsa, OK, USA). The total increase of $[Ca^{2+}]_i$ concentration in the cells in the presence of OT was measured by calculating the area under the $[Ca^{2+}]_i$ mobilisation curve (AUC; relative units). The baseline for $[Ca^{2+}]_i$ was defined on the basis of AUC determination from the period before OT treatment. Differences between control and OT-stimulated AUC were determined with ANOVA

analysis for repeated measurements followed by LSD post hoc test. The effect of P_4 on OT-induced AUC was calculated with two-way Anova followed by LSD post hoc test. Prostaglandin $F2\alpha$ and E2 concentrations in the medium after endometrial slices and endometrial cell cultures were log-transformed and submitted to Anova for repeated measurements followed by LSD post hoc test (Statistica, Statsoft, Tulsa, OK, USA).

Results

Experiment 1. Plasma P_4 level was lower ($P<0.05$) in sows during luteolysis ($n=4$) compared with early pregnant pigs ($n=4$) (Fig. 1A). The relative transcript abundance of *OTR* gene was higher ($P<0.05$) during luteolysis than during pregnancy (Fig. 1B).

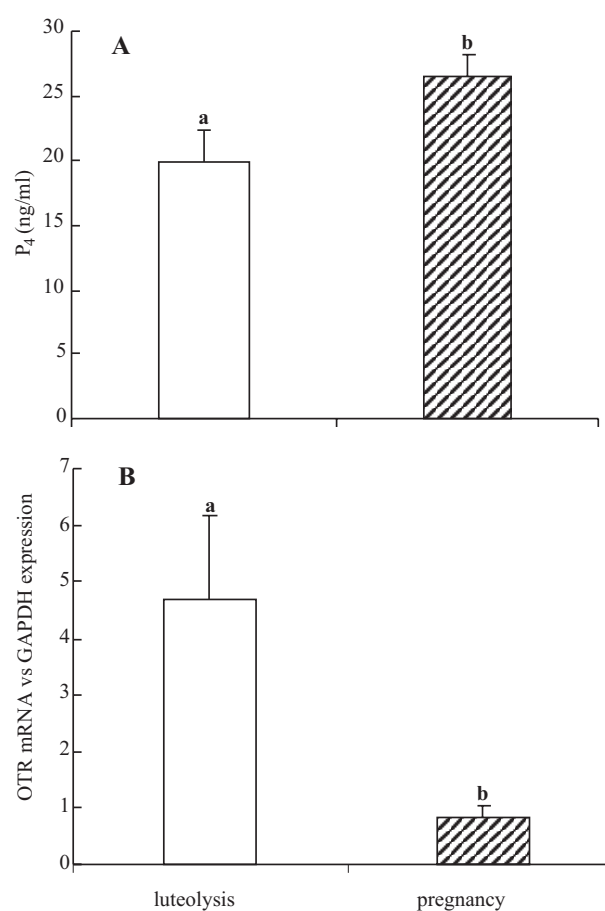


Fig. 1. A. Progesterone (P_4) plasma concentration (ng/ml) in pigs during luteolysis (days 14–16 of the estrous cycle; $n=4$) and early pregnancy (days 14–16; $n=4$). B. Quantitative expression of oxytocin receptor (*OTR*) gene in the endometrium of pigs during luteolysis (days 14–16 of the estrous cycle; $n=4$) and early pregnancy (days 14–16; $n=4$). ^{a,b} – different superscripts designate significant differences ($P<0.05$).

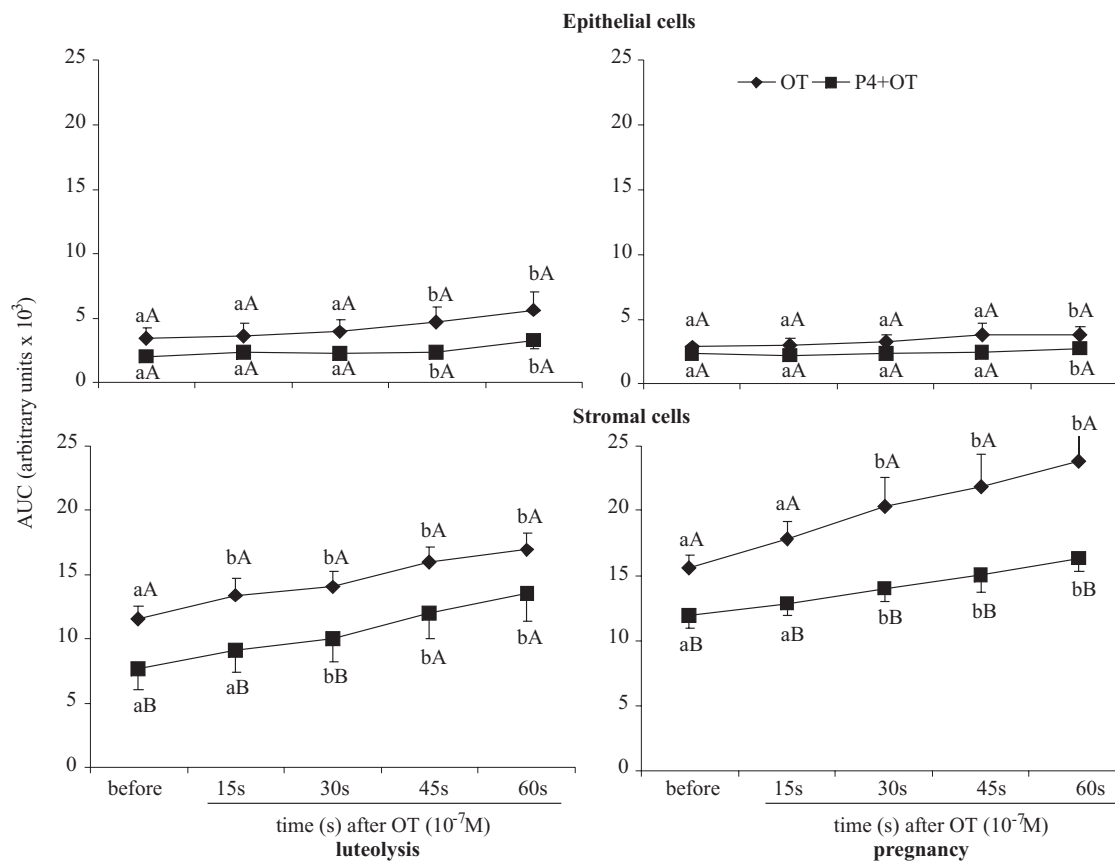


Fig. 2. Mean \pm SEM concentrations of $[Ca^{2+}]_i$ (measured as an area under the $[Ca^{2+}]_i$ curve of 15 s ranges) in epithelial and stromal cells isolated from endometrium of pigs during luteolysis (days 14-16 of the estrous cycle; $n=5$) and early pregnancy (days 14-16; $n=5$). The cells were treated with OT alone (10^{-7} M) or with OT after their incubation for 30 min with progesterone (P_4 10^{-5} M). ^{a,b} – $P<0.05$ – OT induced vs. basal (before OT) $[Ca^{2+}]_i$ AUC, ^{A,B} – $P<0.05$ – OT vs. P_4+OT $[Ca^{2+}]_i$ AUC.

Experiment 2. Oxytocin increased $[Ca^{2+}]_i$ mobilisation in epithelial and stromal cells (Fig. 2) from porcine endometrium harvested during both luteolysis ($n=5$) and early pregnancy ($n=5$). After OT treatment an increased ($P<0.05$) of $[Ca^{2+}]_i$ concentration was observed in epithelial cells within 45 s and 60 s during luteolysis and pregnancy, respectively. In stromal cells an OT-induced increase ($P<0.05$) of $[Ca^{2+}]_i$ was observed within 15 s and 30 s during luteolysis and pregnancy, respectively. Pre-treatment of the cells with P_4 did not change basal and OT-induced $[Ca^{2+}]_i$ mobilisation in epithelial cells (Fig. 2). In stromal cells P_4 pre-treatment decreased ($P<0.05$) basal and OT-induced $[Ca^{2+}]_i$ accumulation after the first 30 s of measurement in cyclic pigs and within 60 s after OT treatment in pregnant pigs. During luteolysis P_4 delayed OT-induced mobilisation of $[Ca^{2+}]_i$ by 15 s and did not change the time of $[Ca^{2+}]_i$ mobilisation in these cells during pregnancy.

Experiment 3. Oxytocin stimulated ($P<0.05$) secretion of $PGF2\alpha$ during both luteolysis ($n=7$) and early pregnancy ($n=8$) and $PGE2$ secretion during luteolysis from endometrial slices (Fig. 3). Progesterone

had no effect on $PGF2\alpha$ and $PGE2$ release in response to OT by endometrial slices.

Experiment 4. Separately cultured epithelial and stromal cells isolated from porcine endometrium harvested during luteolysis ($n=3$ to 6) and pregnancy ($n=3$ to 5) released $PGF2\alpha$ (Fig. 4) and $PGE2$ (Fig. 5). Basal secretion of $PGF2\alpha$ from epithelial cells cultured within 12 h was higher in cyclic than in pregnant pigs (Fig. 4). Basal release of $PGE2$ from epithelial cells was higher ($P<0.05$) during early pregnancy than during luteolysis after 6 h in vitro culture (Fig. 5). In stromal cells basal secretion of $PGF2\alpha$ and $PGE2$ did not differ ($P>0.05$) between luteolysis and early pregnancy. Oxytocin (10^{-7} M) increased ($P<0.05$) $PGF2\alpha$ release (Fig. 4) after 6 and 12 h of culture from both epithelial and stromal cells harvested during luteolysis. In pregnant pigs the stimulatory effect of OT on $PGF2\alpha$ release from endometrial cells was not observed ($P>0.05$). The stimulatory effect of OT on $PGE2$ release was observed only in epithelial cells harvested during luteolysis. Progesterone alone increased $PGE2$ secretion from cyclic epithelial cells

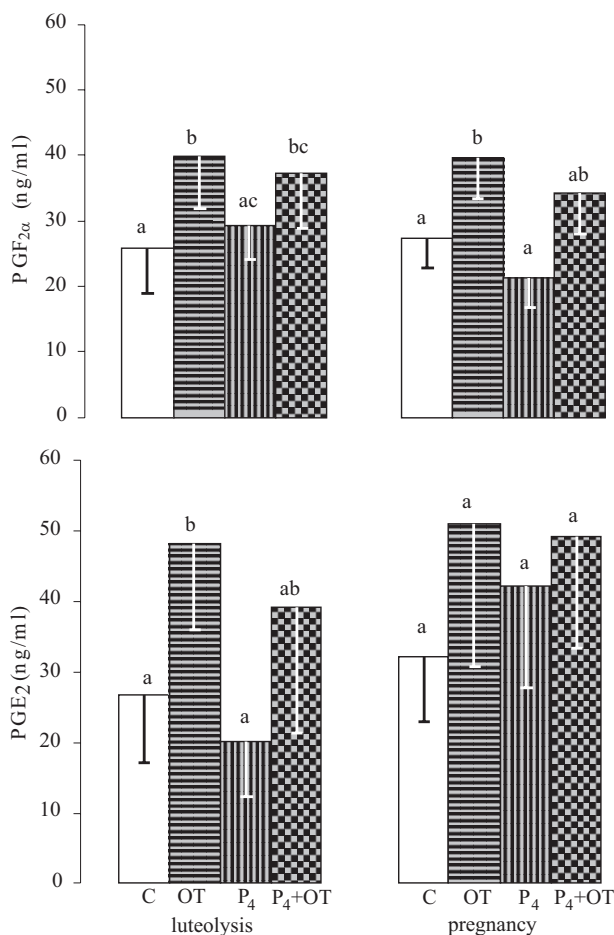


Fig. 3. The effect of oxytocin (OT 10^{-7} M), progesterone (P_4 10^{-5} M) and P_4 +OT on PGF2 α and PGE2 release (mean \pm SEM) by endometrial slices isolated from uteri of pigs during luteolysis (days 14-16 of the estrous cycle; $n=7$) and early pregnancy (days 14-16; $n=8$). Slices of endometrium were preincubated (24 h) and then incubated (6 h) at 37°C in an atmosphere of 95% O $_2$ and 5% CO $_2$ in control medium (C) or in the presence of OT, P_4 and P_4 +OT treatments. ^{a,b,c} – bars with different superscripts are significantly different ($P<0.05$).

cultured in vitro within 6 h (Fig. 5). Oxytocin-stimulated secretion of PGF2 α was inhibited ($P<0.05$) in the presence of P_4 (10^{-5} M) only in stromal cells after 6 h in vitro culture during luteolysis (Fig. 4).

Discussion

In the current study we document that the level of *OTR* mRNA expression in porcine endometrium depends on the female reproductive status. During luteolysis under low plasma P_4 level, expression of the gene encoding *OTR* in porcine endometrium was higher compared with early pregnancy when the P_4 plasma level was increased. Comparable expression of the *OTR* gene in porcine endometrium was previously demonstrated using in situ hybridization (Oponowicz

et al. 2006). The status of *OTR* expression at the gene (the current study) and protein level (Franczak et al. 2005) indicates that the porcine endometrium should be more responsive to OT during luteolysis than early pregnancy. Although in the present study the expression of *OTR* mRNA in pregnant endometrium was lower when compared to that observed during luteolysis, we have documented that the action of OT in the endometrium was not completely reduced and the tissue is OT-sensitive both during early pregnancy and luteolysis.

Our current data reveal a stimulatory effect of OT on $[Ca^{2+}]_i$ concentration in both cyclic and pregnant endometrial cells. Similar effects of OT on $[Ca^{2+}]_i$ accumulation were confirmed in cyclic porcine myometrium (Franczak et al. 2006) and in porcine and bovine endometrium (Braileanu et al. 1999, Duras et al. 2005). We have determined that in stromal cells of gravid pigs the mobilisation of $[Ca^{2+}]_i$ in response to OT was delayed in comparison with these cells of non-gravid endometrium. The effect of OT observed in the present study may be caused by lower expression of *OTR* and/or high P_4 plasma concentration at the time of early pregnancy.

In this study we observed that P_4 pre-treatment of cyclic and pregnant stromal cells decreased basal and OT-induced $[Ca^{2+}]_i$ concentrations. In studies by Gehrig-Burger et al. (2010) P_4 depleted intracellular calcium stores. We suppose that this mechanism could be attributed to the inhibitory effect of P_4 on OT action in $[Ca^{2+}]_i$ accumulation in stromal cells observed in this study. Such an effect of P_4 was not determined in epithelial cells. It is known that the effect of this steroid on epithelial cell functions may vary between experiments due to in vitro culture conditions (Glasser and Mulholland 1993, Asselin et al. 1996, Srinivasan et al. 2006), female reproductive status (Blitek and Ziecik 2004, Srinivasan et al. 2006) or species differences (Glasser and Mulholland 1993, Jamshidi et al. 2007).

In our study OT increased secretion of luteolytic PGF2 α and luteotropic PGE2 from endometrial slices isolated during luteolysis (a period accompanied by decreasing plasma P_4 concentrations) and secretion of PGF2 α during early pregnancy (a period of increased plasma P_4 concentrations). Thus, in porcine endometrium OT can act as a stimulator of both pro-luteolytic and pro-luteotropic actions. Both the luteolytic and luteotropic action of OT was previously noticed locally in porcine corpus luteum (Wuttke et al. 1995). This potentially dual effect of OT may be mediated via paracrine interactions among cells consisted in endometrial slices, variable contribution of epithelial and stromal cells in total mass of the endometrium, and by tissue sensitivity to OT (Ludwig et al. 1998, Uzumcu et al. 2000, Blackwell et al. 2003). It was found that the sensitivity of the endometrium to OT

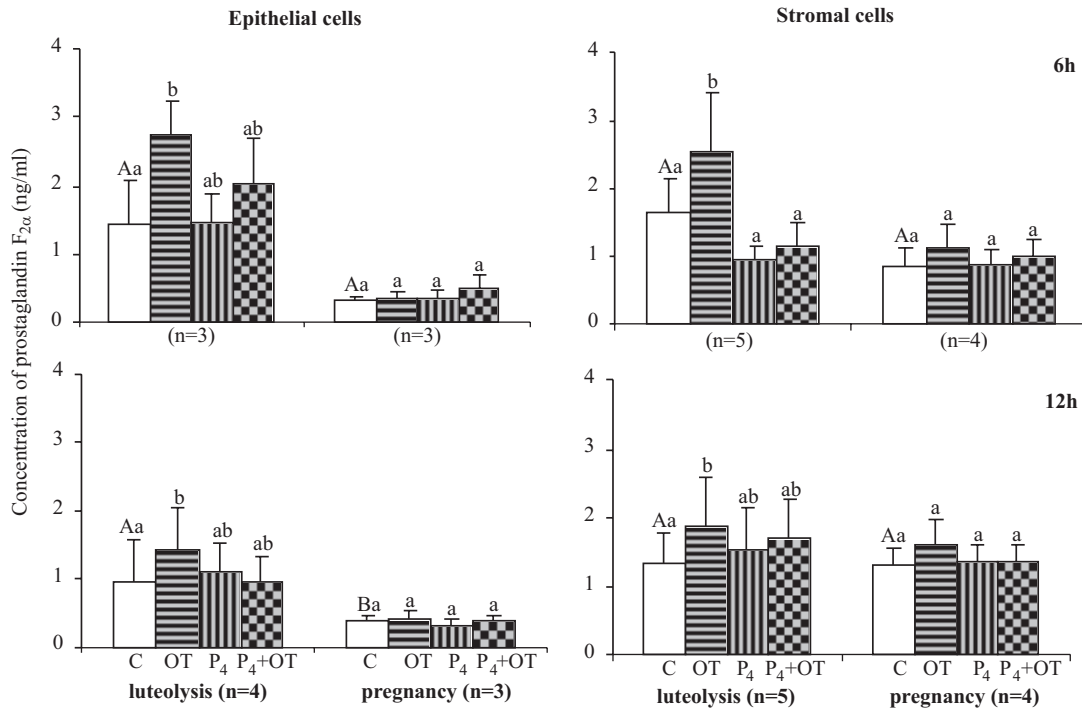


Fig. 4. The effect of oxytocin (OT 10^{-7} M), progesterone (P_4 10^{-5} M) and P_4 +OT on PGF $_{2\alpha}$ release (mean \pm SEM) by epithelial and stromal cells isolated from endometrium of cyclic (days 14-16 of the estrous cycle) and early pregnant (days 14-16) pigs. The cells (3×10^5 /ml) were pre-incubated (3-4 days) until they reached confluence and then incubated for 6 h or 12 h at 37°C in an atmosphere of 5% CO $_2$ in air, in control medium (C) or in presence of OT, P_4 or P_4 +OT treatments.

^{A,B} – bars with different uppercase letters are significantly different (C during luteolysis vs. pregnancy, $P < 0.05$); ^{a,b} – bars with different letters are significantly different ($P < 0.05$).

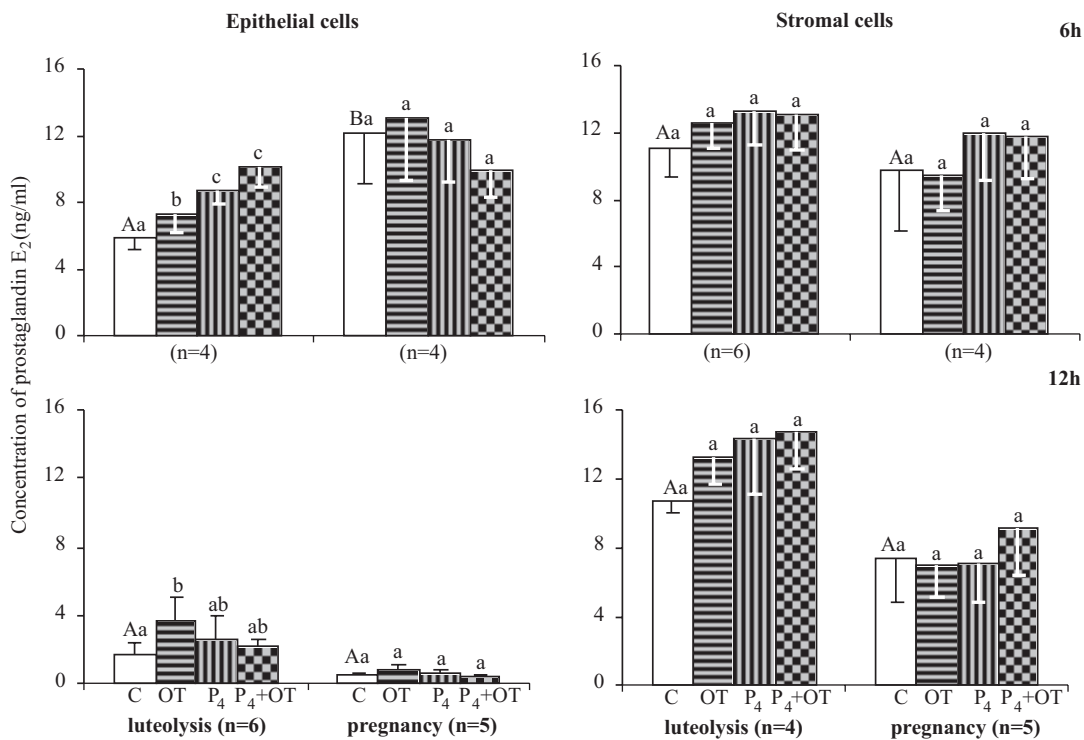


Fig. 5. The effect of oxytocin (OT 10^{-7} M), progesterone (P_4 10^{-5} M) and P_4 +OT on PGE $_2$ release (mean \pm SEM) from epithelial and stromal cells isolated from endometrium of cyclic (days 14-16 of the estrous cycle) and early pregnant (days 14-16) pigs. The cells (3×10^5 /ml) were pre-incubated (3-4 days) until they reached confluence and then incubated for 6 h and 12 h at 37°C in an atmosphere of 5% CO $_2$ in air, in control medium (C) or in presence of OT, P_4 or P_4 +OT treatments.

^{A,B} – bars with different uppercase letters are significantly different (C during luteolysis vs. pregnancy, $P < 0.05$); ^{a,b} – bars with different letters are significantly different ($P < 0.05$).

may be regulated by steroids locally produced in the porcine uterus (Franczak 2008, Franczak and Kotwica 2008).

In the present study we observed that OT significantly increased PGF2 α secretion from isolated epithelial and stromal cells only during luteolysis. Thus, it seems that OT promotes mainly pro-luteolytic effects during corpus luteum regression in both types of separated endometrial cells and in endometrial slices. However, in response to OT, PGE2 was released only from cyclic epithelial cells, suggesting higher sensitivity of these cells to OT when compared to stromal cells. It has been shown that epithelial cells express a higher level of *OTR* mRNA (Oponowicz et al. 2006).

It is interesting that responsiveness to OT-induced mobilisation of [Ca²⁺]_i in epithelial cells does not correspond to their endocrine response to OT measured as PG secretion. This phenomenon could be explained by the different size and amounts of epithelial and stromal cells in the endometrium (Uzumcu et al. 2000). Thus, [Ca²⁺]_i concentration measured by Fura-2AM fluorescence between individual epithelial and stromal cells and PG secretion can not be comparable. It also suggests other factors (e.g. uterine steroids) involved in the modulation of signalling from *OTR*.

During luteolysis the stimulatory effect of OT on PGF2 α and PGE2 production was observed in both endometrial explants as well as in isolated epithelial and stromal cells. However, a lack of such consistence between slices and isolated cells appeared during pregnancy. This indicates that communication between epithelial and stromal cells maintained in porcine endometrial tissue is important for PG secretion in response to OT. We suppose that the different signals of early pregnancy which operate on endometrial cells may interfere with PG production (Bazer et al. 2009).

In summary: 1) *OTR* mRNA expression in porcine endometrium depends on reproductive status of the female; 2) progesterone decreases basal and OT-stimulated [Ca²⁺]_i accumulation in endometrial stromal cells isolated during luteolysis and early pregnancy (days 14-16); 3) in most conditions, P₄ did not inhibit the OT-stimulated secretion of PG in the porcine endometrium.

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Author contributions. G Kotwica, A Oponowicz, B Kurowicka, A Franczak contributed equally to this study and should be considered as co-first authors; J Bogacka took a part in experiment 1.

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