Oxytocin and tumor necrosis factor α stimulate expression of prostaglandin E₂ synthase and secretion of prostaglandin E₂ by luminal epithelial cells of the porcine endometrium during early pregnancy

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Abstract

Oxytocin (OXT) and tumor necrosis factor α (TNF) have been implicated in the control of luteolysis by stimulating endometrial secretion of luteolytic prostaglandin F₂α (PGF₂α). Nevertheless, OXT concentration in porcine uterine lumen increases markedly on days 11–12 of pregnancy, and TNF is expressed in endometrium during pregnancy. The objective of the study was to determine the effect of OXT and TNF on expression of the enzymes involved in PG synthesis: PG-endoperoxide synthase 2 (PTGS2), PGE₂ synthase (mPGES-1) and PGF synthase, and PGE₂ receptor (PTGER2), as well as on PG secretion by endometrial luminal epithelial cells (LECs) on days 11–12 of the estrous cycle and pregnancy. LECs isolated from gilts on days 11–12 of the estrous cycle (n=8) and pregnancy (n=7) were treated with OXT (100 nmol/l) and TNF (0.6 nmol/l) for 24 h. OXT increased PTGS2 mRNA and mPGES-1 protein contents, as well as PGE₂ secretion but only on days 11–12 of pregnancy. TNF stimulated PTGS2 and mPGES-1 mRNA, as well as mPGES-1 protein expression and PGE₂ release on days 11–12 of pregnancy and the estrous cycle. In addition, expressions of PTGER2 and PTGER4 were determined in corpus luteum (CL). Abundance of PTGER2 mRNA and PTGER4 protein in CL was upregulated on day 14 of pregnancy versus day 14 of the estrous cycle. This study indicates that TNF and OXT regulate PGE₂ synthesis in LECs during early pregnancy. PGE₂ secreted by LECs, after reaching ovaries, could have a luteoprotective effect through luteal PTGER2 and PTGER4, or may directly promote uterine function and conceptus development.

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Introduction

Embryo–endometrial crosstalk during early pregnancy involves an intricate network of cytokines, hormones, growth factors, adhesion molecules, and other biological molecules (reviewed in Waclawik et al. (2009a)). As endometrial prostaglandins F₂α (PGF₂α) and E₂ (PGE₂) exert opposite actions on the corpus luteum (CL), a tight control over their synthesis and secretion is critical either for the initiation of luteolysis or for the maintenance of pregnancy in domestic animals, including pigs (Bazer & Thatcher 1977, Davis & Blair 1993, Christenson et al. 1994, McCracken et al. 1999). In domestic ungulates, pulsatile release of uterine PGF₂α during the late luteal phase is responsible for inducing luteolysis (McCracken et al. 1999). In sheep, the major physiological stimulus for PGF₂α secretion is oxytocin (OXT; McCracken et al. 1999). Similarly, numerous reports indicate that OXT stimulates PGF₂α secretion from the porcine endometrium during the late luteal phase in vivo (Kieborz et al. 1991, Carnahan et al. 1996, Edgerton et al. 1996) and in vitro (Whiteaker et al. 1994, Tysseling et al. 1996, Ludwig et al. 1998, Uzumcu et al. 1998, Blitek & Ziecik 2005).

However, the role of OXT in the regulation of the estrous cycle is not as well defined in pigs as in ruminants. Systemic infusions of OXT antagonist between days 12 and 20 of the estrous cycle reduce the amplitude of PGF₂α metabolite (PGFM) pulses, but do not prevent luteolysis (Kotwica et al. 1999). In pigs, the neurohypophysis provides a greater amount of circulating OXT than does the CL during the luteal phase, and plasma OXT increases during luteolysis (Kotwica et al. 1990). However, the endometrium may be the primary source of OXT (Boulton et al. 1996, Vallet et al. 1998), since OXT mRNA is abundantly present in the porcine uterine endometrium (Boulton et al. 1996). Although, surprisingly, OXT concentration in the uterine lumen significantly increases on days 11–14 of pregnancy when compared with the corresponding days of the estrous cycle (Vallet et al. 1998), most in vitro studies...
concerning the effect of OXT on the porcine endometrial cells have been studied on the luteolytic period (Whiteaker et al. 1994, Tyseling et al. 1996, Uzumcu et al. 1998, Blitek & Ziecik 2005).

Luminal epithelial cells (LECs) of the endometrium possess the greatest number of OXT receptors and stromal cells possess the least (Boulton et al. 1995, Oponowicz et al. 2006). Moreover, LECs contain the greatest quantity of OXT peptide (Boulton et al. 1996). Therefore, it was suggested that OXT may act in an autocrine manner to stimulate PGF2α release from LECs during luteolysis (Hu et al. 2001). However, relatively little is known about the effect of OXT on the expression of enzymes involved in PG synthesis, and on the release of PGE2 by endometrial cells during early pregnancy not only in pigs but also in other domestic animals.

Tumor necrosis factor α (TNF) is another factor involved in the regulation of PG secretion by the endometrial cells whose role was documented in the estrous cycle in pigs (Blitek & Ziecik 2006). TNF, a cytokine originally identified as a product of activated macrophages, is now known to be expressed in placentas and embryos and the female reproductive tract, including ovaries, oviducts, uteri of humans and rodents (reviewed in Hunt (1993)), and the uterine endometrium of bonnet monkeys and pigs (Yu et al. 1998, Rosario et al. 2005). TNF receptors were detected in the bovine endometrium (Miyamoto et al. 2000). Interestingly, the endometrial expressions of TNF and its receptors in bonnet monkeys are significantly upregulated in the glandular epithelium in the preimplantation stage of pregnancy possibly contributing to the induction of local inflammatory reactions during implantation (Rosario et al. 2005). TNF has also been implicated in the control of uterine cell growth and differentiation during the estrous cycle (Hunt et al. 1996). Moreover, TNF stimulates PGF2α secretion in vitro from LECs of the porcine endometrium collected at luteolysis (Blitek & Ziecik 2006). Nevertheless, TNF mRNA is expressed in the porcine endometrium during early pregnancy (Yu et al. 1998), and limited information is available on its role during this period.

Elevated synthesis of PGE2 was observed in both the conceptus and endometrium between days 10 and 13 of pregnancy in pigs (Waclawik et al. 2006, Waclawik & Ziecik 2007). PGE2 could exert a luteoprotective effect (Akinlosoto et al. 1986, Christenson et al. 1994) probably mediated by luteal PGE2 receptors (PTGERS), PTGER2 and PTGER4, which act through a cAMP-signaling pathway (Hahlin et al. 1988, Richards et al. 1994, Narumiya et al. 1999, Boiti et al. 2000, Weems et al. 2006). Besides the effect on luteal function, PGE2 may act locally through endometrial PGE2 receptors, especially PTGER2 (Waclawik et al. 2009b). Expression of PTGER2 mRNA and protein in the porcine endometrium is upregulated during early pregnancy, on days 11–12. Moreover, this receptor was shown to be involved in a PGE2-positive feedback loop during the period of the maternal recognition of pregnancy (Waclawik et al. 2009b).

OXT and TNF have been implicated in the control of luteolysis by regulation of PGF2α secretion from porcine endometrial cells. However, limited information is available on the role of OXT and TNF in the endometrium during early pregnancy in pigs. Our hypothesis was that during early pregnancy, OXT, greatly secreted by luminal epithelium, and TNF of endometrial and immune cells origin regulate specifically PG synthesis and secretion, as well as PTGER2 expression in the porcine LECs. Therefore, the objective of the present studies was to determine the effect of OXT and TNF on the expression of the enzymes involved in PG synthesis: PG-endoperoxide synthase 2 (PTGS2), PGE2 synthase (mPGES-1) and PGF synthase (PGFS), and PTGER2, as well as on PG secretion by endometrial LECs derived from days 11 to 12 of the estrous cycle and pregnancy. Moreover, subsequent aim of the present study was to determine expression of PTGER2 and PTGER4 in porcine CL during the estrous cycle and pregnancy.

**Results**

**Effect of OXT and TNF on abundance of PTGS2, PGFS, mPGES-1, PTGER2, and PG secretion by LECs**

PTGS2 and mPGES-1 mRNA abundance in LECs was affected by treatment (P<0.002 and P=0.0005 respectively) but not by reproductive status (Figs 1 and 2A). However, content of mPGES-1 protein and PGE2 release by LECs was affected by both reproductive status (P=0.01 and P=0.04 respectively) and treatment (P<0.0001 and P=0.0008 respectively; Figs 2B and 3A).

OXT stimulated PTGS2 mRNA (P<0.05; Fig. 1) and mPGES-1 protein content (P<0.05; Fig. 2B), but only on days 11–12 of pregnancy. OXT-stimulated abundance of mPGES-1 protein in the cells derived from days 11 to
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The effect of oxytocin (OXT; 100 nmol/l) and tumor necrosis factor α (TNF; 0.6 nmol/l) treatment on expression of PGE2 synthase (mPGES-1) mRNA (A) and protein (B) in endometrial luminal epithelial cells derived from days 11 to 12 of the estrous cycle and pregnancy. The representative samples of western blots are shown in the upper panels. Data are expressed as the mean ± S.E.M. of ratios relative to β-actin. Abundance of mPGES-1 mRNA was affected by treatment (P=0.0005). Expression of mPGES-1 protein was affected by both reproductive status (P=0.01) and treatment (P<0.0001). Mean values with various superscripts differ: * denotes P<0.05; ** denotes P<0.01; *** denotes P<0.001 when compared with the control (C) value.

12 of pregnancy was greater (P<0.05) than OXT-stimulated expression of mPGES-1 protein in LECs from the corresponding days of the estrous cycle. OXT treatment resulted in approximately twofold increase in PGE2 secretion by LECs only on days 11–12 of pregnancy when compared with the basal PGE2 secretion on days 11–12 of pregnancy (P<0.05; Fig. 3A). OXT-stimulated release of PGE2 by LECs derived from days 11 to 12 of pregnancy was greater (P<0.05) than OXT-stimulated PGE2 secretion by LECs from the corresponding days of the estrous cycle. On days 11–12 of the estrous cycle, OXT had no effect either on expression of PTGS2 mRNA (Fig. 1), mPGES-1 mRNA, and protein content (Fig. 2) or on PGE2 secretion by LECs (Fig. 3A).

TNF elevated expression of PTGS2 (P<0.05; Fig. 1) and mPGES-1 mRNA (P<0.01; Fig. 2A), as well as mPGES-1 protein expression (P<0.001; Fig. 2B) in LECs on days 11–12 of pregnancy. Similarly, TNF stimulated expression of PTGS2 (P<0.05; Fig. 1) and mPGES-1 mRNA (P<0.05; Fig. 2A), as well as mPGES-1 protein expression (P<0.01; Fig. 2B) in LECs on days 11–12 of the estrous cycle. Moreover, TNF treatment resulted in stimulation of PGE2 release by LECs on days 11–12 of pregnancy and the estrous cycle (P<0.01 and P<0.01 respectively; Fig. 3A).

No significant effect of treatment or reproductive status×treatment interactions was detected for PGFS mRNA and protein abundance, release of PGF2α, and PTGER2 mRNA content in LECs (Figs 3–5). OXT and TNF had no effect either on PGFS mRNA and protein expression (Fig. 4) or on PGF2α secretion by LECs on days 11–12 of both the estrous cycle and pregnancy (Fig. 3B). No significant alternation in PTGER2 mRNA expressions by treatment with OXT or TNF was observed in the cells collected on days 11–12 of both the estrous cycle and the corresponding days of pregnancy (Fig. 5).

Basal expression of PTGER2 mRNA in LECs derived from days 11 to 12 of pregnancy was determined to be 0.00007±0.00001, whereas the expression in LECs collected from the corresponding days of the estrous cycle was 0.00005±0.00001.

PTGER2 and PTGER4 mRNA and protein abundance in the CL during the estrous cycle and pregnancy

A day×reproductive status interaction was detected for luteal PTGER2 mRNA (P=0.03) abundance (Fig. 6A). PTGER2 mRNA contents in the CLs were comparable on days from 9 to 12 in the estrous cycle and pregnancy.
However, on day 14 of pregnancy, PTGER2 mRNA abundance was greater when compared to day 14 of the estrous cycle (Fig. 6A). Expression of luteal PTGER2 protein was affected by day (P<0.0001), but not by reproductive status (Fig. 6C). The PTGER2 protein abundance was maximal on day 14 of the estrous cycle when compared to days 9, 11, and 12 of the estrous cycle (P<0.05). In pregnancy, PTGER2 protein expression was intermediate on day 9, least on day 11, and maximal on days 12 and 14 (P<0.05).

The abundance of PTGER4 mRNA was affected by day (P=0.048) but not by reproductive status (Fig. 6B). PTGER4 mRNA contents were similar across all studied days of the estrous cycle. However, during pregnancy, luteal PTGER4 mRNA expression gradually increased to reach maximal levels on day 14 of pregnancy.

A day×reproductive status interaction was detected for luteal PTGER4 protein abundance (P=0.004; Fig. 6D). PTGER4 protein contents increased from days 9 to 12 of the estrous cycle and then declined twofold on day 14 of the estrous cycle. However, there was maximal PTGER4 protein abundance on day 14 of pregnancy, significantly greater when compared to all studied days of pregnancy and day 14 of the estrous cycle (threefold greater).

**Figure 4** The effect of oxytocin (OXT; 100 nmol/l) and tumor necrosis factor α (TNF; 0.6 nmol/l) treatment on expression of PGF synthase (PGFS) mRNA (A) and protein (B) in endometrial luminal epithelial cells derived from days 11 to 12 of the estrous cycle and pregnancy. The representative samples of western blots are shown in the upper panels. There were no significant effects of treatment or reproductive status × treatment interaction detected for PGFS mRNA and protein. Data are expressed as the mean ± S.E.M. of ratios relative to β-actin. Mean values do not differ significantly when compared with the control value.

**Discussion**

Inhibition of PG synthesis before implantation causes pregnancy failure in many species, including pigs (Kraeling et al. 1985, Kennedy et al. 2007). A control over endometrial synthesis and secretion of luteotrophic PGE2 and luteolytic PGF2α is critical for maintenance of pregnancy (Bazer & Thatcher 1977, Davis & Blair 1993, Christenson et al. 1994, McCracken et al. 1999). Our recent research has indicated that the primary conceptus signal, estradiol-17β, and another conceptus-derived factor, PGE2, alter the expression of PG synthesis pathway enzymes in the porcine endometrium to favor PGE2 synthesis between days 10 and 13 of pregnancy (Waclawik et al. 2009b).

In the present study, the role of other potential modulators of PG synthesis and secretion by the endometrium was investigated. The results of the present research, for the first time, indicate an important role of TNF and OXT in the stimulation of PGE2 synthesis in cultured LECs of the porcine endometrium during early pregnancy (on days 11–12) in pigs. It is consistent with the previous observation that mPGES-1 expression is relatively high in the porcine endometrium between days 10 and 13 of pregnancy (Waclawik et al. 2006). Before implantation, the endometrium and conceptus synthesize elevated amounts of PGE2 that result in an increased content of this prostanoid in the uterine lumen and utero-ovarian circulation in pigs (Geisert et al. 1982, Christenson et al. 1994, Waclawik et al. 2006, Waclawik & Ziecik 2007). In contrast to PGF2α, PGE2 has a luteo-protective action (Akinlosotu et al. 1986, Christenson et al. 1994). Previous studies revealed that specific binding of [3H]PGE2 to luteal cells is greater on day 14 of pregnancy than on day 14 of the estrous cycle, but the number of PTGER2 was similar in pregnant and cyclic gilts (Feng & Almond 1999). However, luteal subtypes of PTGER have not been determined in the previous studies. PGE2 stimulates luteal progesterone secretion through a cAMP-mediated pathway in CL in different...
species (Hahlin et al. 1988, Richards et al. 1994, Boiti et al. 2000, Weems et al. 2006). Subtypes of PGE2 receptors, PTGER2 and PTGER4, are coupled to adenylyl cyclase and generate cAMP that in turn activates the protein kinase A signaling pathway (Narumiya et al. 1999). The present study revealed that there is a day × reproductive status interaction for luteal expression of PTGER2 mRNA and PTGER4 protein. Abundance of PTGER2 mRNA and PTGER4 protein was greater on day 14 of pregnancy when compared with day 14 of the estrous cycle. Therefore, it is likely that OXT stimulates PGE2 during pregnancy to promote CL maintenance. On the other hand, PGE2 may act directly to promote uterine function as well as conceptus development and migration in uterus (Pope et al. 1982, Geisert et al. 1990, Giguère et al. 2000). PGE2 is required for the local increase in endometrial vascular permeability and preparation for angiogenesis and implantation in rodents (Hamilton & Kennedy 1994, Kennedy et al. 2007). PGE2 may stimulate angiogenesis in the porcine endometrium through upregulating vascular endothelial growth factor synthesis and secretion by endometrial cells (Kaczmarek et al. 2008). Elevated amounts of PGE2 may exert a local effect in the porcine endometrium by PGE2 receptors, especially PTGER2 whose expression is altered in the pregnancy when compared to the estrous cycle (Waclawik et al. 2009b). However, OXT and TNF had no effect on mRNA abundance of this receptor on days 11–12 of both the estrous cycle and pregnancy.

There are numerous studies concerning the role of OXT in luteolysis, both in ruminants and pigs (Kiebzak et al. 1991, Kotwica et al. 1999, McCracken et al. 1999). Nevertheless, OXT receptors were found in the porcine endometrium not only during the estrous cycle (Whiteaker et al. 1994) but also in pregnancy (Okano et al. 1996, Ludwig et al. 1998). Moreover, OXT is secreted in large quantities into the uterine lumen, especially on days 11–12 of pregnancy (Vallet et al. 1998). In contrast to TNF, OXT increased PGE2 production through upregulation of PTGS2 mRNA and mPGES-1 protein in LECs only on days 11–12 of pregnancy, but not on the corresponding days of the estrous cycle. The current results clearly indicate that alteration of PG release cannot result from an artifact of contaminating stromal cells, because OXT-stimulated PGE2 secretion was increased only during pregnancy and OXT did not stimulate PGF2α secretion. The latter observation, in particular, rules out such possibility since endometrial stromal cells are responsive to OXT. Our studies indicate that endometrially secreted OXT may play a role in the luteal maintenance during early pregnancy. It is in agreement with the previous findings demonstrating that OXT is not luteolytic when administered locally into the uterine lumen as it is when administered systemically (Sample et al. 2000). The intra-uterine infusion of OXT during the late luteal phase in pigs even has an anti-luteolytic effect of reducing endocrine secretion of PGF2α (decreasing plasma concentrations of PGFM) and delaying the decline in progesterone that occurs during luteolysis (Sample et al. 2004). Our present results indicate an additional role of OXT during early pregnancy. It may increase PGE2 output from the endometrium on days 11–12 during the maternal recognition of pregnancy. Our results obtained with LECs are in agreement with the effect of OXT on PGE2 and PGF2α secretion by endometrial explants derived from days 12 to 14 of the estrous cycle and pregnancy (Franczak & Bogacki 2009). The OXT treatment of the endometrial explants collected from the intact uterine horn of pregnant gilts, but not cyclic gilts, resulted in an increase in PGE2 secretion.

In the present study, OXT did not have the effect either on PGFS mRNA and protein expression or on PGF2α release from LECs on days 11–12 of the estrous cycle or pregnancy. These results are consistent with the previous findings that LECs and endometrial tissue explants isolated from cyclic and pregnant gilts on day 12 are unresponsive to OXT in terms of PGF2α secretion (Ludwig et al. 1998, Uzumcu et al. 2000). Treatment with OXT...
during the early luteal phase (days 4–6) or mid-luteal phase (days 9–11) does not affect uterine secretion of PGF$_{2\alpha}$ in pigs (Kieborz et al. 1991, Carnahan et al. 1996). This is explained by the fact that the development of the endometrial response to OXT occurs between days 12 and 14 postestrus, OXT stimulates PGF$_{2\alpha}$ release during the late luteal phase, especially during the luteolytic period (Kieborz et al. 1991, Carnahan et al. 1996, Edgerton et al. 1996). Carnahan et al. (1996) showed that endometrial responsiveness to OXT had developed by day 14 of the estrous cycle, which was 1 day before progesterone began to decline. Kieborz et al. (1991) demonstrated that progesterone had declined by the time the endometrium was responsive to OXT. However, they did not examine this with sufficient frequency, and thus were not able to conclude whether progesterone declined before or after the endometrium became responsive to OXT. On the other hand, Franczkak et al. (2005) suggested that OXT is able to stimulate uterine PGF$_{2\alpha}$ secretion only after the beginning of the decrease in plasma progesterone concentration. Therefore, OXT may not be responsible for the initiation of luteolysis but is more likely involved in the control of pulsatile release of PGF$_{2\alpha}$, especially the height and frequency of the peaks of this hormone during luteolysis in pigs (Kotwica et al. 1999). Moreover, OXT and OXT receptors are present in high quantities in cultured LECs (Boulton et al. 1995, 1996). Therefore, it is possible that OXT acts in an autocrine and/or paracrine manner to increase the basal secretion of PGF$_{2\alpha}$ in LECs derived from the estrous cycle, while decreasing the responsiveness to exogenous OXT (Hu et al. 2001). On the other hand, this explanation probably does not apply to LECs derived from days 11 to 12 of pregnancy because, in the present study, LECs were responsive to OXT that resulted in increased PGE$_2$ secretion. Hu et al. (2001) did not study response of LECs to OXT during pregnancy.

The role of TNF in the process of luteolysis has been well established. TNF stimulates PGF$_{2\alpha}$ release from porcine LECs of the endometrium and the bovine endometrium at the initiation of luteolysis (Miyamoto et al. 2000, Skarzynski et al. 2003, Blitek & Zieck 2006). The present results, for the first time, indicate that TNF significantly stimulates PGE$_2$ synthesis and release through upregulation of PTGS2 mRNA as well as mPGES-1 mRNA and protein expression in porcine LECs collected from days 11 to 12 of the estrous cycle and pregnancy. Many studies concerning the effect of TNF on PG synthesis in the endometrium have been conducted on the bovine species. A similar effect of TNF on mPGES-1 expression and PGE$_2$ release was observed in the studies with bovine epithelial endometrial cell line (BEND), derived from day 14 of the estrous cycle (Parent & Fortier 2005). Interestingly, the research on primary cell culture of the bovine epithelial cells, although isolated from another period (i.e. early days of the estrous cycle), also indicates that TNF stimulates PGE$_2$ secretion (Parent et al. 2002). However, another group observed the effect of TNF on PGE$_2$ secretion only in the bovine stromal cells but not in the epithelial cells collected from various days of the estrous cycle (Murakami et al. 2001).

The source of TNF could be macrophages, which are present in endometrium during the estrous cycle and early pregnancy, as well as endometrial cells, embryo, and placenta (Hunt et al. 1996, Yu et al. 1998, Dalin et al. 2004). TNF has been implicated in the control of uterine cell growth and differentiation during the estrous cycle, whereas during early pregnancy it may contribute to local inflammatory reactions during implantation (Hunt et al. 1996, Rosario et al. 2005). Based on in vivo studies, the concept emerged that TNF at low concentrations plays an important role in luteolysis, especially with regard to the stimulation of PGF$_{2\alpha}$ production in uterus. On the other hand, TNF at increased concentrations prolongs the estrous cycle by inducing a survival pathway in the CL and by contributing to progesterone production in the cow (Skarzynski et al. 2003). Our results are consistent with the suggestion that TNF could have an indirect luteoprotective action by stimulating PGE$_2$ synthesis in the endometrium during early pregnancy (Murakami et al. 2001).

In conclusion, TNF and OXT are factors that not only influence secretion of PGF$_{2\alpha}$ by endometrial cells during luteolysis in pigs and ruminants, which has been demonstrated by previous studies, but also stimulate expression of mPGES-1 and secretion of luteotropic PGE$_2$ by LECs of the porcine endometrium during early pregnancy. The present results indicate an important role of TNF and OXT in the increase of PGE$_2$ synthesis and secretion by endometrial epithelial cells during the maternal recognition of pregnancy, before implantation process in pigs. We suggest that an additional mechanism of luteal maintenance could occur by the conceptus altering uterine response to OXT to promote the synthesis of luteoprotective PGE$_2$ by endometrial cells. After reaching ovaries, PGE$_2$ may act through luteal PTGER2 and PTGER4. On the other hand, increased endometrial synthesis of PGE$_2$ could have direct effect to promote uterine function and conceptus development.

Materials and Methods

Primary cell culture and treatment

The endometrial tissue was collected at exsanguinations on days 11–12 of the estrous cycle and pregnancy from crossbred gilts (Polish Landrace×Large White×Duroc) of known estrus or breeding date (day 0=first day of estrus) respectively. The stage of the estrous cycle was confirmed by macroscopic observation of the ovaries (follicles and corpora lutea) and uterus as described previously (Waclawik et al. 2006). Pregnancy was confirmed by the presence of conceptuses in the uterus. The concepti were flushed from uterine horns with 20 ml PBS (pH 7.4) at 37°C. The morphology of the flushed
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concepts for two gilts was filamentous, whereas for three gilts, there were both tubular and filamentous concepts for at the same time, and for two gilts, spherical and ovoid forms from 5 to 10 mm in length. Porcine endometrial LECs were isolated from the middle portion of the uterine horn using a procedure described earlier (Blitek & Ziecik 2004). The cell viability was > 90% as assessed by 0.5% (w/v) Trypan blue dye exclusion. The cells were seeded at a density of 2 x 10^6 on six-well plates in Medium 199 (Sigma–Aldrich) containing 2% BSA (w/v; ICN Biomedicals Inc., Costa Mesa, CA, USA), 10% newborn calf serum (w/v; Sigma–Aldrich), penicillin (100 IU/ml), and streptomycin (100 μg/ml), and cultured at 37 °C in a humidified atmosphere of 95% air/5% CO2. The purity of LECs culture was 85–90% as assessed by immunofluorescent staining for the presence or absence of vimentin and cytokeratin (Blitek & Ziecik 2004). The cells were cultured for 5–6 days before initiation of the experiment, until ~80–90% confluency. Afterwards, the LECs were treated for 24 h with fresh serum-free medium 199 alone (controls; C) or containing 100 nmol/l OXT (Sigma–Aldrich) or 0.6 nmol/l TNF (Sigma–Aldrich). Treatment doses of hormones were chosen based on the results of the previous studies (Uzumcu et al. 1998, Blitek & Ziecik 2006).

At the end of each treatment period, media were collected and stored at −40 °C until enzyme immunoassay (EIA) of PGE2 and PGF2α. The cells were then washed with PBS and lysed with RIPA buffer (50 mmol/l Tris–HCl; pH 7.4; 150 mmol/l NaCl; 1% Triton X-100 (v/v); 0.5% sodium deoxycholate (w/v); 0.1% SDS (w/v); 1 mmol/l EDTA; protease inhibitor cocktail), and total cellular protein content was measured (Bradford 1976), or cells were lysed with Fenozol buffer (A&A Biotechnology, Gdansk, Poland) for total RNA isolation.

All procedures involving animals were approved by the Local Research Ethics Committee and were conducted in accordance with the national guidelines for agricultural animal care.

**Tissue collection for PTGER study in the CL**

Peripubertal crossbred gilts of similar age (~5–5.5 months) were observed daily for onset of estrus. After exhibiting two natural estrous cycle gilts were assigned into two groups: pregnant and cyclic. Gilts assigned to the pregnant groups were artificially inseminated at 12 h after onset of estrus (day 0) and 24 h later. Gilts were slaughtered at a local abattoir on either day 9, 11, 12, or 14 of pregnancy or the estrous cycle (n=4–6 per group). The CLs were separated by blunt dissection from surrounding ovarian tissues, snap-frozen, and stored until further use (for RNA and protein extraction).

**Total RNA isolation**

Total RNA was extracted using the Total RNA Prep Plus kit (A&A Biotechnology) and treated with DNase I (Invitrogen Life Technology Inc.) according to the manufacturer's protocol to eliminate possible DNA contamination.

**Real-time PCR quantitation**

Real-time PCR was performed with the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using Power SYBR Green PCR master mix (Applied Biosystems) as described previously (Waclawik et al. 2006, 2009b). Briefly, total RNA was reverse transcribed using oligo(dT) primer and avian myeloblastosis virus reverse transcriptase (Promega). Real-time PCR (25 μl) included 12.5 μl Power SYBR Green PCR master mix, 0.5 mmol/l sense and antisense primers each, and reverse-transcribed cDNA (5 μl of diluted RT product). To evaluate mRNA levels, enzyme and PTGER2-specific primers were used (Table 1). For quantification, standard curves consisting of serial dilutions of the appropriate purified cDNA were included. Before amplification, an initial denaturation (15 min at 95 °C) step was used. The PCR programs for each gene, except for PTGER2 and PTGER4, were performed as follows: 38 cycles of denaturation (15 s at 95 °C), annealing (30 s at 52.5 °C for PGFS; or at 35 °C for PTGS2, mPGES-1, and β-actin), and elongation (60 s at 72 °C). The PCR program for PTGER2 and PTGER4 gene was performed as follows: 36 cycles of denaturation (15 s at 95 °C) and annealing (60 s at 60 °C). After PCR, melting curves were acquired by stepwise increases in the temperature from 50 to 95 °C to ensure that a single product was amplified in the reaction. Data obtained from the real-time PCR for PTGS2, mPGES-1, PGFS, PTGER2, and PTGER4 were normalized against β-actin. Control reactions in the absence of reverse transcriptase were performed to test for genomic DNA contamination.

**Table 1 Primers used for real-time PCR.**

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<th>Gene</th>
<th>Primer sequences</th>
<th>GenBank accession number</th>
<th>References</th>
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<tr>
<td>PTGS2</td>
<td>Sense: 5'-ATGATCTACCCCGCCCTCACAC-3'</td>
<td>AY028583</td>
<td>Waclawik &amp; Ziecik (2007)</td>
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<td>Antisense: 5'-AAAAAGCAGCTCCTGGTCAA-3'</td>
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<td>mPGES-1</td>
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<td>AY857634</td>
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<td></td>
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<td>AY863054</td>
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<td>β-Actin</td>
<td>Sense: 5'-ACATCAAGGAGAGTCTGCTC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-GACGGGCATGACCTTGATCCTCA-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Western blot analysis

Luteal tissues were homogenized on ice in buffer containing 50 mM Tris–HCl, pH 8.0; 150 mM NaCl, 1 mM EDTA, and supplemented with protease inhibitor cocktail (Sigma–Aldrich). Homogenates were then centrifuged for 15 min at 800 g at 4 °C and stored at −20 °C for further analysis. The protein concentration was determined (Bradford 1976).

Protein samples from cell lysates (25 μg) and luteal tissue homogenates (40 μg) were dissolved in SDS gel-loading buffer (50 mmol/l Tris–HCl, pH 6.8; 4% SDS, 20% glycerol, and 2% β-mercaptoethanol), heated at 95 °C for 4 min, and separated on 15% (for mPGES-1), 12% (for PGFS), or 10% (for PTGER2 and PTGER4) SDS-PAGE. Separated proteins were electroblotted onto 0.2 μm nitrocellulose membrane in transfer buffer (20 mmol/l Tris–HCl buffer, pH 8.2; 150 mmol/l glycerine, and 20% methanol). After blocking in 5% non-fat dry milk in Tris-buffered saline buffer (TBS-T, containing 0.1% Tween-20) for 1.5 h at 25.6 °C, the membranes were incubated overnight with 1:50 polyclonal anti-mPGES-1 antibodies (Cayman Chemical, Ann Arbor, MI, USA), or 1:2000 anti-lung-type PGFS antiseraum (kindly donated by Prof. Kikuko Watanabe), or 1:200 rabbit polyclonal antibodies against human EP2 (anti-PTGER2 antibody; Cayman Chemical), or 1:70 rabbit polyclonal antibodies against human EP4 (anti-PTGER4 antibody; Cayman Chemical) at 4 °C. Subsequently, the enzymes were detected by incubating the membrane with 1:20 000 dilution of secondary polyclonal anti-rabbit alkaline phosphatase-conjugated antibodies (Sigma–Aldrich) for 1.5 h at 25.6 °C. Immune complexes were visualized using alkaline phosphatase visualization procedure. Western blots were quantitated using Kodak 1D software (Eastman Kodak). Sample loading was standardized to expression of β-actin using specific antibodies (1:3000; Abcam, Cambridge, UK).

EIA of PGE$_2$ and PGF$_{2\alpha}$

Concentrations of PGE$_2$ in incubation medium were determined by a direct EIA (Blitek & Ziecik 2004). Cross-reactivities of the anti-PGE$_2$ antisemum (donated by Dr Seiji Ito, Kansai Medical University, Osaka, Japan) were as follows: PGE$_1$, 18%; PGA$_1$, 10%; PGA$_2$, 4.6%; PGB$_2$, 6.7%; PGD$_2$, 0.13%; PGF$_{2\alpha}$, 2.8%; PGJ$_2$, 14%, and 15-keto-PGE$_2$, 0.05%. Assay sensitivity was 0.19 ng/ml, and the intra- and inter-assay coefficients of variation were 9.7 and 13.6% respectively. Concentrations of PGF$_{2\alpha}$ were determined by a direct EIA test as described previously (Blitek & Ziecik 2004). Cross-reactivities of the anti-PGF$_{2\alpha}$ antisemum (Sigma–Aldrich) were as follows: PGF$_{1\alpha}$, 60%; PGE$_1$ and PGE$_2$, <0.1%; and PGA$_1$, PGA$_2$, PGB$_1$, and PGB$_2$, <0.01%. Assay sensitivity was 0.23 ng/ml, and the intra- and inter-assay coefficients of variation were 7.7 and 8.4% respectively. Levels of PGs were standardized on protein concentration per well (ng/mg protein) and expressed as fold increase relative to control.

Statistical analysis

Statistical analyses were conducted using two-way ANOVA followed by Bonferroni’s post test (GraphPad PRISM v. 5.02, GraphPad Software, San Diego, CA, USA). The statistical model used to evaluate changes of the enzymes involved in PG synthesis (PTGS2, mPGES-1, and PGFS) and PTGER2 as well as PG secretion by LECs included the effect of treatment (C, OXT, and TNF) and reproductive status (cyclic and pregnant) and the treatment×reproductive status interaction. The data are expressed as means±S.E.M. of values obtained in five to eight experiments (pigs), each performed in duplicate.

The statistical model used to evaluate changes in abundance of luteal PTGER2 and PTGER4 mRNA and protein included the effect of days (11, 12, and 14) and reproductive status (cyclic and pregnant) and the day×reproductive status interaction. All numerical data are presented as the mean ± S.E.M., and differences were considered as statistically significant when P<0.05.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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