Novel Aspects of Cytokine Action in Porcine Uterus – Endometrial and Myometrial Production of Estrone (E₁) in the Presence of Interleukin 1β (IL1β), Interleukin 6 (IL6) and Tumor Necrosis Factor (TNFα) – in Vitro Study*

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In pigs estrone (E₁) and estradiol (E₂) produced by porcine embryos were shown to be signals for maternal recognition of pregnancy (BAZER & TCHATTER 1977). Previously we have demonstrated that tissues of porcine uterus can produce estrogens (FRANÇZAK 2008; FRANÇZAK & KOTWICA 2008; FRANÇZAK & KOTWICA 2010) and the endometrium together with the myometrium on days 14 to 16 of early pregnancy are an important source of estrone (E₁) (FRANÇZAK 2008; FRANÇZAK & KOTWICA 2008). E₁ was found to be a main contributor in endometrial and myometrial total steroid secretion (FRANÇZAK 2008). On days 14 to 16 of pregnancy the endometrium releases more E₁ than on days 14 to 16 of the estrous cycle, while the myometrium apparently equally partici-

ated in the total uterine basal E₁ secretion. Thus, in pigs during early pregnancy the endometrium and the myometrium, while during luteolysis only the myometrium, may mostly contribute to the basal total secretion of E₁ (FRANÇZAK 2008).

The uterus of pigs is known to be a source of estrogens, however the factors involved in the regulation of myometrial E₁ production remain unknown. In general, P₄ was shown to be a substrate for steroid synthesis in the porcine uterus (FRANÇZAK 2008; FRANÇZAK & KOTWICA 2008; FRANÇZAK & KOTWICA 2010), but the hormone did not stimulate E₁ secretion on days 14 to 16 of pregnancy (FRANÇZAK 2008). This phenomenon was not explained. Little is known about the hormones or other factors that influence uterine steroidogenesis.

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In the present study we have examined if interleukin 1β (IL1β), interleukin 6 (IL6) and tumor necrosis factor α (TNFa), which are produced by porcine embryos and the uterus, may be involved in the regulation of uterine E\textsubscript{1} production and release in vitro. Cytokines, acting through specific receptors, may regulate the secretory activity of the uterus in pigs (FRANCAK et al. 2010; 2012). Peri-implantation porcine embryos and the endometrium express IL1β (TUO et al. 1996; ROSS et al. 2003a,b) and IL6 (MATHILAGAN et al. 1992; ANEGON et al. 1994). Tumor necrosis factor α is also produced by the uterus of pregnant pigs (YU et al. 1998). In a recent study we documented that IL1β, IL6 and TNFa affect prostaglandin F\textsubscript{2α} (PGF\textsubscript{2α}) release and activate PGF\textsubscript{2α} metabolism in the endometrium to protect the corpus luteum (CL) in pregnant pigs (FRANCAK et al. 2012). IL1β induced synthesis and secretion of luteotropic prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) to overcome luteolysis in pregnant pigs (FRANCAK et al. 2010) and may be involved in intraluteal luteotropic regulation of CL functions in gravid and cyclic pigs (ZMIEWESKA et al. 2013). In the pig, the expression of IL1β at the site of embryo-maternal contact in the uterus is unique and species-specific (TUO et al. 1996). TNFa is expressed in embryos, ovaries, oviducts and uteri of human and rodents (HUNT 1993). In gravid pigs TNFa stimulates PGE\textsubscript{2} synthesis and secretion by luminal epithelial cells of the endometrium (WACLAWIK et al. 2010). It was found that during days 15 to 16 of pregnancy TNFa stimulates secretion of the prostaglandin F2α metabolite – 13,14-dihydro-15-keto PGF2α (PGFM) by the endometrium (FRANCAK et al. 2012). In cyclic pigs TNFa is involved in the regulation of PGF2α synthase (PGFS) mRNA expression (FRANCAK et al. 2012) and prostaglandin secretion (BLITEK & ZIECIK 2006). Because the ability of the uterine tissues to induce steroidogenesis has been confirmed, we hypothesized that IL1β, IL6 and TNFa may be involved in conditioning this process and may increase E\textsubscript{1} concentrations that enhance the effects of estrogen in the uterus.

The objective of this investigation was to test the hypothesis that endometrial and myometrial production of E\textsubscript{1} is regulated by cytokines. To address this issue, we have determined if the endometrium and the myometrium of pigs can produce E\textsubscript{1} in vitro in response to IL1β, IL6 and TNFa on days 10 to 11, i.e. before the time of maternal recognition of pregnancy, on days 12 to 13, i.e. during the time of maternal recognition of pregnancy and on days 15 to 16, i.e. during the time of corpus luteum maintenance, beginning of implantation and prevention of luteolysis. The effects observed in pregnant pigs were compared with effects on corresponding days of the estrous cycle.

**Material and Methods**

Animals and collection of endometrial and myometrial tissue

All experiments were approved by the Animal Ethics Committee, University of Warmia and Mazury in Olsztyn, Poland. Post-pubertal, six month old crossbred pigs (Large White × Polish Landrace), weighing 90-110 kg were used during early pregnancy or the estrous cycle. Gilts were observed for estrus behavior in the presence of an intact boar. The onset of the second estrus was designated as day 0 of the estrous cycle. Gilts assigned to the early pregnancy group were naturally bred on the second day of estrus. The animals on days 10 to 11 (n = 5), 12 to 13 (n = 5) and 15 to 16 (n = 5) of pregnancy, or days 10 to 11 (n = 5), 12 to 13 (n = 5) and 15 to 16 (n = 5) of the estrous cycle were slaughtered in a commercial slaughterhouse. Pregnancy in mated gilts was confirmed by the presence of embryos after flushing each uterine horn with 20 ml sterile saline. The stage of the estrous cycle was also confirmed by morphological changes of the ovaries and CL quality (AKINS & MORRISSETTE 1968). Uterine horns from early-pregnant or cyclic gilts were placed immediately in ice-cold PBS supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin and transported to the laboratory on ice.

Preparation of endometrial and myometrial slices

The middle part of the uterine horns collected from experimental gilts was opened longitudinally on the mesometrial surface and the endometrium and the myometrium were separated using a scalpel blade. The endometrium and the myometrium were sliced thinly (200-210 mg, 3 mm thick) and washed twice with PBS supplemented with antibiotics.

**In vitro** incubation of endometrial and myometrial slices

Individual fresh endometrial and myometrial slices were placed separately in culture vials containing 2 ml of Medium 199 (Sigma, Germany) supplemented with 0.1% BSA fraction V (ICN, USA) and 20 μg gentamycin (Sigma, Germany). These tissue cultures were pre-incubated in a water bath for 18 h at 37°C in an atmosphere of 95% O\textsubscript{2} and 5% CO\textsubscript{2}. After preincubation, endometrial and myometrial slices were incubated for 6 and 12 h in control medium or in medium supplemented with progesterone (P\textsubscript{s}, 10^{-7}M), IL1β (1 ng/ml and 10 ng/ml), IL6 (1 ng/ml and 10 ng/ml) or TNFa (10 ng/ml). P\textsubscript{s} was used as a control and substrate for...
E estrone (E$_1$) production. Cytokines were obtained from Bio-
mol, GmbH, Germany. The doses of cytokines
were selected according to earlier studies (FRAN-
CZAK et al. 2010, 2012). After incubation, culture
vials were placed in an ice bath, culture medium
was collected and frozen at -20$^\circ$C until E$_1$ assay
with radioimmunoassay (RIA).

Estrone (E$_1$) determination

Concentration of E$_1$ was determined by the RIA
method (CIERESZKO 1999). Cross-reactivity of
antisera against E$_1$ has been reported (SZAFRAN
SKA et al. 2002). The efficiency of extraction for
E$_1$ was 85.3 ± 0.07%. E$_1$ assay sensitivity was 1
pg/ml. The coefficient of correlation between the
added and recovered amount of E$_1$ concentrations
was 0.975. The intra- and interassay coefficients of
variation were 0.5% and 1.9%, respectively.

Statistical analysis

Mean concentrations of E$_1$ released in response
to cytokines were log-transformed to reduce het-
erogeneity of variance and were analyzed by
multi-way ANOVA with the dose of treatments,
time of incubation, reproductive status and days of
pregnancy or the estrous cycle as the main effects
followed by a LSD post-hoc test (Statistica, Stat-
Soft Inc, Tulsa, OK, USA).

Results

Basal endometrial and myometrial release of E$_1$
during days 10 to 11, 12 to 13 and 15 to 16 of
pregnancy and the estrous cycle

The results of the basal endometrial and myome-
trial production of E$_1$ are presented in Table 1. Dur-
ing pregnancy basal endometrial release of E$_1$ after
6 h of incubation did not differ among days 10 to
11, 12 to 13 and 15 to 16 (P>0.05). After 12 h of in-
cubation in vitro basal E$_1$ release was higher on
days 12 to 13 and 15 to 16 than on days 10 to 11 of
pregnancy (P<0.05). During the estrous cycle E$_1$
production by the endometrium was about two-
fold lower (P<0.05) on days 12 to 13 than that ob-
served during days 10 to 11 and 15 to 16, both after
6 and 12 h of incubation. Basal myometrial E$_1$
production did not differ among the studied days of
pregnancy (P>0.05) after 6 and 12 h of incubation and
did not differ among the studied days of the es-
trous cycle after 6 h of incubation. After 12 h of in-
cubation myometrial production of E$_1$ in cyclic
pigs was about two fold higher on days 15 to 16 of

| Table 1 |
|---|---|
| **Endometrium** | **The estrous cycle** |
| Days | Pregnancy | The estrous cycle |
| | 6 hours of incubation in vitro | 12 hours of incubation in vitro | 6 hours of incubation in vitro | 12 hours of incubation in vitro |
| 10 to 11 | 55.5 ± 9.6$^{ab}$ | 23.1 ± 2.8$^{ab}$ | 58.0 ± 8.0$^{ab}$ | 58.9 ± 14.3$^{ab}$ |
| 12 to 13 | 32.8 ± 7.1$^{ab}$ | 39.1 ± 7.6$^{ab}$ | 27.4 ± 6.7$^{ab}$ | 23.9 ± 4.9$^{ab}$ |
| 15 to 16 | 39.6 ± 5.2$^{ab}$ | 39.9 ± 11.3$^{ab}$ | 50.2 ± 8.1$^{ab}$ | 55.2 ± 18.8$^{ab}$ |

**Myometrium**

| Days | Pregnancy | The estrous cycle |
| | 6 hours of incubation in vitro | 12 hours of incubation in vitro | 6 hours of incubation in vitro | 12 hours of incubation in vitro |
| 10 to 11 | 40.5 ± 6.2$^{ab}$ | 44.9 ± 13.1$^{ab}$ | 55.0 ± 14.7$^{ab}$ | 40.9 ± 14.8$^{ab}$ |
| 12 to 13 | 33.7 ± 8.1$^{ab}$ | 40.4 ± 6.8$^{ab}$ | 41.7 ± 7.9$^{ab}$ | 40.2 ± 8.5$^{ab}$ |
| 15 to 16 | 37.7 ± 5.8$^{ab}$ | 48.0 ± 19.8$^{ab}$ | 46.3 ± 15.3$^{ab}$ | 76.5 ± 14.0$^{ab}$ |

$^{a,b}$ Different uppercase letters designate significant differences among the studied days of pregnancy or the estrous cycle within
the same time of incubation (data in columns).

$^{a,b}$ Different lowercase letters designate significant differences between corresponding days of pregnancy and the estrous cycle
after the same time of incubation (data in rows).
During the estrous cycle than on days 10 to 11 and 12 to 13 (P<0.05).

IL1β, IL6 and TNFα-induced endometrial E3 release in vitro during early pregnancy and the estrous cycle

IL1β, IL6 and TNFα increased E3 release in vitro (P<0.05) in endometrial explants harvested on days 10 to 11 from gravid pigs and incubated in vitro for 12 h as well as in endometrial explants harvested on days 12 to 13 of pregnancy and incubated for 6 h (Fig. 1). After 12 h in vitro incubation on days 12 to 13 of pregnancy only IL6 (1 ng/ml and 10 ng/ml) increased E3 release (P<0.05), while an effect of IL1β or TNFα was not observed (P>0.05). During days 15 to 16 of pregnancy the enhanced release of E3 was observed only in the presence of IL6 after 12 h of in vitro incubation (Fig. 1). Neither IL1β, nor TNFα affected endometrial E3 release during days 15 to 16 of pregnancy (P>0.05). In non-gravid pigs the stimulatory effect of IL1β, IL6 and TNFα on E3 release was observed only on days 12 to 13 of the estrous cycle, both after 6 and 12 h incubation (Fig. 2).

IL1β, IL6 and TNFα-induced myometrial E3 release in vitro during early pregnancy and the estrous cycle

On days 10 to 11 of pregnancy IL1β, IL6 and TNFα did not affect E3 release from myometrial explants (P>0.05) (Fig. 3). Myometrial E3 production was increased on days 12 to 13 of pregnancy in the presence of IL1β and IL6 after 6 and 12 h of in vitro incubation (Fig. 3, P<0.05). An effect of TNFα during days 12 to 13 of pregnancy was not observed (P>0.05). The cytokines did not affect E3...
release during days 15 to 16 of pregnancy (Fig. 3, P>0.05). In cyclic pigs (Fig. 4) only IL6 increased myometrial E/1 release after 6 h of in vitro culture on days 12 to 13 and 15 to 16 of the estrous cycle (Fig. 4). Neither IL1β, nor TNFα affected myometrial E/1 release in cyclic pigs (P>0.05, Fig. 4).

The effect of P4 on endometrial and myometrial E/1 release in vitro

In gravid pigs P4 (10⁻⁴ M) increased endometrial release of E1 on the studied days of pregnancy (P<0.05, Fig. 1) both after 6 and 12 h of incubation. In non-gravid pigs a stimulatory effect of P4 was observed after 6 h of incubation on days 12 to 13 and after 12 h of incubation on days 10 to 11, 12 to 13 and 15 to 16 of the estrous cycle (Fig. 2). Myometrial production of E1 in the presence of P4 was increased during all studied days of pregnancy and the estrous cycle (P<0.05) (Figs 3 & 4).

Discussion

The objective of this investigation was to determine if endometrial and myometrial production of E1 is up-regulated by cytokines. To address this issue, we used porcine endometrial and myometrial explants to study the effects of IL1β, IL6 and TNFα on E1 release. The effects of the cytokines were determined during three important periods of early pregnancy in pigs, e.g. on days 10 to 11, 12 to 13 and 15 to 16, and compared with the effects that occurred on the respective days of the estrous cycle. The tissues explants were treated with IL1β, IL6 and TNFα during 6 h or 12 h in vitro incubation. The main findings showed that in gravid pigs IL1β, IL6 and TNFα increased endometrial production of E1 on days 10 to 11 only after 12 h of in vitro incubation and after 6 h of in vitro incubation on days 12 to 13 of pregnancy. Moreover, after 12 h of incubation in vitro IL6 increased endometrial E1
production on days 12 to 13 and 15 to 16 of pregnancy. In non-gravid pigs IL1\(\beta\), IL6 and TNF\(\alpha\) increased E\(_1\) release in vitro from the endometrium only during days 12 to 13, both after 6 h and 12 h of exposure to cytokines. Myometrial production of E\(_1\) in vitro was specifically enhanced in the presence of IL1\(\beta\) and IL6 on days 12 to 13 of pregnancy. TNF\(\alpha\) did not affect myometrial E\(_1\) production in pregnant and cyclic pigs. Interestingly, in cyclic pigs IL6 increased myometrial E\(_1\) release only on days 12 to 13 and 15 to 16 after 6 h of incubation in vitro.

Previously it was established that E\(_1\) together with E\(_2\) produced by porcine embryos provide a signal for pregnancy maintenance and successful implantation (BAZER et al. 1977; HEAP et al. 1981; GEISERT et al. 1982). During early pregnancy in pigs the production of estrogens is the result of androgen conversion (BAZER & TCHATCHER 1977; RYAN 1982). Androstenedione (A\(_4\)), a principal circulating androgen in pigs (SIMPSON et al. 2001), is converted to E\(_1\) (RYAN 1982). Basal production of E\(_1\) indicates the aromatization of A\(_4\) to E\(_1\) in the porcine uterus both during early pregnancy and the estrous cycle.

We have determined that in gravid pigs on days 10 to 11 IL1\(\beta\), IL6 and TNF\(\alpha\) increased endometrial production of E\(_1\) after 12 h of in vitro stimulation, while on days 12 to 13 after 6 h of in vitro stimulation. IL6 increased E\(_1\) production on days 12 to 13 and 15 to 16 of pregnancy only after 12 h of incubation in vitro. Thus, the effect of the cytokines depends on the stage of pregnancy and the duration of exposure to cytokines. From days 12 to 16 of pregnancy E\(_1\) production in the endometrium is only IL6 dependent. Previously it was found that in postmenopausal women formation of estrogens in the adipose tissue and skin is controlled primary by IL6, IL1\(\beta\) and TNF\(\alpha\) (for a review see BULUN et al. 2002).
The present study showed for the first time that IL1β and IL6, not TNFα, acting exactly during maternal recognition of pregnancy, e.g. on day 12 to 13 of pregnancy caused the release of E1/B31 by myometrial explants cultured in vitro. This time is accompanied by the highest expression of IL1β and IL6 by porcine conceptuses (MATHIALAGAN et al. 1992; ANEGON et al. 1994; TUO et al. 1996; ROSS et al. 2003a, b). Interestingly, after this time, on days 15 to 16 of pregnancy, when the process of implantation begins, IL1β, IL6 and TNFα did not affect E1. This is indicative of a specific responsiveness of the myometrial tissue and the myometrial ability to produce E1 in the presence of cytokines. We have found that the response of pregnant endometrium was dependent on the time of IL1β, IL6 and TNFα exposure and it developed earlier than the myometrial response, e.g. on days 10 to 11 of pregnancy, after 6 h of in vitro incubation. On days 12 to 13 of pregnancy the endometrium after 6 h of in vitro incubation responded positively in the presence of all cytokines, while after 12 h of incubation increased concentration of E1 was observed only in the presence of IL6. Interestingly, on days 15 to 16 of pregnancy, endometrial E1 was induced only by IL6 after 12 h of in vitro incubation.

Comparing uterine responsiveness to cytokines in pregnant and cyclic pigs we have found that pregnant myometrium as well as pregnant and cyclic endometrium harvested on days 12 to 13 of pregnancy or the estrous cycle enhanced production of E1/B31 in response to IL1β and IL6. This period of pregnancy and the estrous cycle is accompanied by similar, high concentrations of P4/B34 and E1/B32 in peripheral blood plasma (FRANCZAK et al. 2010). We suggest that uterine responsiveness to cytokines may depend on the concentrations of endogenous P4.
It is important to note that factors affecting uterine (endometrial and myometrial) secretion may influence the efficiency of conceptus proliferation, growth and development as well as uterine activity and capacity (Vallet & Christenson 1996). Despite the fact that E1 is a less active estrogen than estradiol (Vallet & Christenson 1996), E1 can be converted to more potent and active estradiol. Moreover E1 can be converted to catecholestrogens (Chakraborty et al. 1989) which may have high biological activity (Rosenkrans et al. 1990). Thus, the level of E1 production by the uterus in general may be very important for modulation of endometrial protein secretion (Trott et al. 1992) and for proper communication between embryos and the endometrium (Roberts & Bazer 1988). It was found that treatment of pregnant gilts with E1 resulted in increased endometrial secretion of nondialyzable macromolecules in culture, reflecting total protein synthesis in the tissue (Vallet & Christenson 1996). It is important to note that the proper level of uterine estrogen synthesis and metabolism during early pregnancy may be a prerequisite for successful implantation. Previously, we have found in pregnant endometrium increased expression of genes encoding sulfotransferase 1E1 and sulfotransferase 2A1 (Franzczak et al. 2013). Sulfotransferases convert estrogens into non-active estrogen sulphates. Thus, the mechanism of E1 metabolism may allow quick adjustment of steroid concentration in the uterus.

In conclusion: 1) endometrial and myometrial explants of pigs release E1 in vitro in response to cytokines; 2) in pregnant pigs IL1β, IL6 and TNFα regulate endometrial production of E1 on days 10 to 11 and on days 12 to 13 in a time of culture-dependent manner; 3) on days 15 to 16 of pregnancy IL6 caused E1 release from the endometrium; 4) in cyclic pigs IL1β, IL6 and TNFα increase E1 release in vitro from the endometrium only during days 12 to 13 of the estrous cycle; 5) in the presence of IL1β and IL6 in gravid pigs myometrial production of E1 in vitro was enhanced specifically on days 12 to 13 of pregnancy.

References


