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Assessment of VEGF-receptor system expression in the porcine endometrial stromal cells in response to insulin-like growth factor-I, relaxin, oxytocin and prostaglandin E2

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**Abstract**

Several factors participate in regulation of growth and development as well as angiogenesis of the uterus during pregnancy, and hence little is known about the role of hormonal regulation of vascular endothelial growth factor (VEGF)-receptor system expression. This study has examined the effect of insulin-like growth factor-I (IGF-I), relaxin (RLX), oxytocin (OT) and prostaglandin (PG) E₂, on VEGF secretion and VEGF-receptor system mRNA expression in the porcine endometrial stromal cells. IGF-I and RLX were identified as the most effective inducers of VEGF secretion and mRNA expression. Although PGE₂ stimulated VEGF secretion and VEGF164 mRNA expression, OT inhibited both secretion and mRNA expression of VEGF. When tested for VEGF receptors (R), all factors failed to affect their mRNA expression. Media conditioned by stromal cells collected after IGF-I and RLX treatment significantly increased endothelial cell proliferation and this effect was blocked by soluble VEGFR-1. These data suggest that during early pregnancy IGF-I, RLX and PGE₂ can affect VEGF expression in the endometrium and therefore may support uterine and embryo development, implantation and pregnancy.
1. Introduction

Periimplantation is a critical period during early pregnancy, when synchronization between developing embryo and maternal uterus must occur to ensure proper initiation of embryo attachment and implantation. More specifically, the embryo must reach the blastocyst stage and gain ‘implantation competence’; meanwhile, the uterus must reach the receptive phase (Dey et al., 2004). In pigs, as in other species, the embryo-maternal crosstalk is essential to gain such synchronization. This dialogue is regulated by several biological molecules, including growth factors, cytokines, prostaglandins and hormones (for a review see Ziecik et al., 2006).

Early pregnancy is associated with morphological and functional changes in the uterus, accompanied by vascular remodeling. One of the first reactions on the embryo-derived estrogenic signal is increased uterine blood supply. In pigs, increased uterine arterial blood flow was observed on days 11-13 of pregnancy (Ford and Christenson, 1979; Ford et al., 1982) and is thought to support prostaglandin production in the endometrium and corpus luteum (CL) functions. Moreover, early stages of embryo implantation are characterized by increased endometrial vascular permeability in many species, including primates (Enders et al., 1983), rodents (Psychosios, 1973), sheep (Boshier, 1970) and pigs (Keys et al., 1986; Keys and King, 1988). In general, increased permeability is thought to play an essential role in the induction and direction of tissue growth and remodeling wherever it occurs (Dvorak et al., 1995). Thus it may be a basic requirement for the rapid growth and differentiation of endometrium to reach the receptive phase. In addition, increased permeability facilitates the angiogenesis associated with the maternal component of placenta formation (Dvorak et al., 1995).

The most potent inducer of angiogenesis as well as microvascular permeability yet identified is vascular endothelial growth factor (VEGF). The various forms of VEGF are
generated by alternative splicing of single gene. Four different isoforms of VEGF were initially reported in humans VEGF121, VEGF165, VEGF189, VEGF206 (Ferrara et al., 1991; Tischer et al., 1991), however, to date several less frequent and tissue specific splice variants have also been reported, for example VEGF145 and VEGF183 (Anthony et al., 1994; Lei et al., 1998). VEGF activity is mainly mediated by two high affinity tyrosine kinase receptors (R); VEGFR-1 (fms-like tyrosine kinase, Flt-1) and VEGFR-2 (fetal liver kinase-1/kinase insert domain-containing receptor, Flk-1/KDR; Ferarra et al., 2003).

It is believed that VEGF play important roles during implantation. Experiments in rodents have indicated that VEGF participates in vascular events before implantation, regulating periimplantation vascular permeability (Rabbani and Rogers, 2001). Rockwell et al. (2002) showed that VEGF is the major mediator of estrogen-induced uterine microvascular permeability and is absolutely essential for implantation in mice. In pigs, vessel permeability appeared in conjunction with blastocyst elongation at day 12 (Keys et al., 1986). Recently, increased VEGF164 protein expression was found in porcine endometrium on days 9-15 of pregnancy (Kaczmarek et al., 2008), suggesting its possible participation in implantation-associated vascular events.

The endometrial growth and remodeling require the coordinated expression of many genes, and for many of these the estrogen receptor (ER) is an essential component of transcriptional complex. In pigs, during maternal recognition of pregnancy estrogenic signal of blastocyst occurs between days 10-12 of gestation (Geisert et al., 1982) and regulates expression of numerous genes by binding to and activating ER. Among the genes identified as targets for regulation by the estradiol/ER complex in the uterus is that encoding VEGF (Hyder et al., 1997) and insulin-like growth factor-I (IGF-I; Klotz et al., 2002), both increased in the uterus during early pregnancy in the pig (Letcher et al., 1989; Kaczmarek et al., 2008). Furthermore, relaxin (RLX), like estrogens, promotes the growth of the uterus and other
estrogen target tissues, since they share the ability to activate ER (Pillai et al., 1999). Although, serum RLX levels during early pregnancy in pigs are maintained low (Anderson et al., 1983), its mRNA and protein are present in the endometrium between days 10-20 of gestation (Knox et al., 1994).

It has been suggested that, besides estrogens, uterine synthesis of prostaglandins (PG) is a critical element of successful implantation in many domestic animals, including pigs (for a review see Ziecik et al., 2006). A dramatic increase in the PGE₂/PGF₂α ratio is observed in the uterine lumen and utero-ovarian vein of pregnant animals on days 11-12 (Davis and Blair, 1993; Christenson et al., 1994), suggesting that PGE₂ can overcome the luteolytic effect of PGF₂α and maintain the function of CL. Furthermore, it was suggested that maintenance of pregnancy in pigs is also mediated by oxytocin (OT), a potent inducer of uterine PGF₂α secretion (Carnahan et al., 1996; Edgerton et al., 1996), since a low concentration of OT receptors (OTR) in the endometrium of early pregnant pigs was found (Okano et al., 1996). However, several other results suggest that the OT-receptor system may not be mandatory in the establishment of pregnancy in pigs (for a review see Ziecik et al., 2006).

Knowledge about the regulators of endometrial VEGF-receptor system expression is rather limited. Therefore, to better identify the role of VEGF and its receptors in the endometrium during the periimplanation period, we investigated whether VEGF-receptor system expression in porcine endometrial stromal cells could be affected by IGF-I, RLX, OT and PGE₂.

2. Materials and methods

2.1 Materials

Chemicals were obtained from Sigma (St Louis, MO, USA), unless otherwise indicated. The recombinant bovine VEGF164 was a kind gift from Dr. D. Gospodarowicz
2.2 Primary cell culture and treatment

The endometrial tissue was collected on days 10-12 of pregnancy from 17 crossbred gilts of known breeding date. Pregnancy was confirmed by the presence of at least four conceptuses in the uterine horns. The concepti were flushed from uterine horns with 20 ml of phosphate-buffered saline (PBS; pH 7.4) at 37°C. Porcine endometrial stromal cells were isolated from the middle portion of the uterine horn using a procedure described earlier (Blitek and Ziecik, 2004). The cell viability was higher than 90% as assessed by 0.5% trypan blue dye exclusion. Cells were cultured in Medium 199 containing 2% BSA (ICN, Biomedicals, Inc., Costa Mesa, CA, USA), 10% NCS and 20 µg/ml gentamycin at 37°C in a humidified atmosphere of 95% air/5% CO₂. After 24 h of seeding (2.5x10⁵ for secretion studies or 1.5x10⁶ for gene expression studies) on culture plates (24- or 6-well plates), stromal cells were washed gently with PBS to remove contaminating epithelial cells. Afterwards, cells were additionally cultured for 24 h to allow complete cell adhesion before initiation of the experiment, until approximately 80-90% confluency. The purity of stromal cell culture was 95-98% as assessed by immunofluorescent staining for the presence or absence of vimentin and cytokeratin (Blitek and Ziecik, 2004).

Forty eight hours after plating, the medium was changed for fresh Medium 199 containing 0 (no treatment, control); 25-100 ng/ml RLX; 10-100 ng/ml IGF-I; 1-100 nM OT or 0.001-1 µM PGE₂ (Cayman Chemical, MI, USA). For dose and time-dependent studies, liquid medium was collected after 6, 12, 24 or 48 h of treatment and stored at -40°C. Cells were then washed with PBS, lysed with 100 mmol/l NaOH and total cellular protein content was measured (Bradford, 1976). For gene expression studies, treatments were performed for
24 h, cells were washed with PBS and total RNA was extracted. All experiments were performed in duplicates.

2.3 Immunofluorescence for VEGF

For immunofluorescent staining, isolated porcine endometrial stromal cells were grown on sterile plastic coverslips (Nunc, Rochester, NY, USA) inserted into 24-well plates. After 24 h of culture stromal cells were washed gently with PBS to remove contaminating cells and further cultured for 24 h to 90% confluency. Then, cells were washed gently with PBS, fixed in 4% paraformaldehyde in PBS for 10 min at room temperature and rinsed three times for 10 min with PBS. After 1.5 h at room temperature in blocking solution (1% BSA, 10% NDS in PBS), cells were incubated overnight at 4°C with antiserum for VEGF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1:100. In control staining, primary antibodies were omitted. Next day, cells were washed three times for 10 min with PBS and secondary antibodies (CY3- anti-rabbit; Jackson ImmunoResearch, West Grove, PA, USA) at a dilution of 1:300 were added for 2 h at room temperature. At the end of incubation coverslips were rinsed with PBS, mounted on slides with a mixture of glycerol and PBS (1:1), viewed with Olympus microscope (Olympus BX 40; Olympus Optical Co Ltd, Tokyo, Japan) equipped with a filter set for CY3 and photographed with a Olympus Digital Camera.

2.4 Radioimmunoassay (RIA) for VEGF

Concentrations of VEGF in media were measured by RIA according to the method described earlier (Berisha et al., 2000). Briefly, the recombinant bovine VEGF164 was used for preparation of rabbit antiserum and iodination. The incubation buffer for RIA was 3 M NaCl containing 1 % BSA, 0.1% Triton X-100 (pH 7.5). The antiserum was used at a final dilution of 1:400 000. Separation of bound and free VEGF was completed using the double
antibody technique and 6% polyethylene glycol (Serva, Heidelberg, Germany). The intra-
assay variations were below 6% and the inter-assay variations below 14%. The ED 50 of the
assay was 0.6 ng/ml. Dilution of samples containing VEGF from blood plasma, follicular
fluid and tissue extracts ran parallel to the standard curve. The average recovery of exogenous
VEGF was 93-95%.

2.5 Reverse transcription and Real-Time PCR

Total RNA was prepared using Total RNA Prep Plus kit (A&A Biotechnology, Gdansk, Poland) according to the manufacturer’s instructions. Constant amounts of 2 µg of
total RNA were treated with DNase I (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) as described in supplier’s protocol. The RT reaction was carried out according to the
protocol detailed previously (Kaczmarek et al., 2007). RT products were diluted fourfold in sterile water and stored at -20°C until Real time PCR amplification.

Real time PCR was performed with an ABI Prism 7300 sequence detection system
using SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) as
described elsewhere (Kaczmarek et al., 2007, Kowalczyk et al., 2008). Table 1 shows the
oligonucleotide primers used for PCR amplification of VEGF, VEGF164, VEGF120, VEGFR-
1, VEGFR-2 and β-actin genes and expected product sizes. Serial dilutions of the appropriate
DNA were used as standard curves. The following general PCR conditions were used:
denaturation for 10 min at 95°C, followed by 35 cycles of 15 sec at 95°C and 1 min at 59°C
(for VEGFR-1) or 60°C (for VEGF, VEGF 164, VEGF 120, VEGFR-2, and β-actin). Melting
curves were obtained after each PCR reaction. The reactions were also run either on blank-
only buffer samples or in absence of the reverse transcriptase. The specificity of RT/PCR
products was confirmed by gel electrophoresis and sequencing. Data obtained from the Real
time PCR for VEGF, VEGF164, VEGF120, VEGFR-1 and VEGFR-2 were normalized on the basis of β-actin mRNA content.

2.6 Endothelial cell proliferation assay

A colorimetric assay was used for quantification of cell proliferation, based on chemical reduction of growth indicator alamarBlue™ (Serotec Ltd., Oxford, UK). Human umbilical vein endothelial cells (HUVEC) at passage one to four were allowed to adhere and spread on 96-well dishes for 18 h (1x10^4 cells/well) in 10% NCS MCDB 131 supplemented with antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin). Cells were first pretreated with or without soluble VEGFR-1 or VEGFR-2 (100 ng/ml; sVEGFR-1, sVEGFR-2; Abcam, Cambridge, UK) in MCDB 131 for 40 min. Afterwards, 35% of conditioned medium collected during VEGF secretion studies (24 or 48 h treatment with IGF or RLX, selected in preliminary experiments) in 2% NCS MCDB 131, alone or in combination with 100 ng/ml sVEGFR-1 or sVEGFR-2 was added to each well in duplicates and incubated for 3 days. VEGF (10 ng/ml; PeproTech EC Ltd., London, UK) was used as positive control. Conditioned media were then replaced by fresh MCDB 131 containing 10% of alamarBlue™ reagent and incubated for 7 h. Absorbance was measured at 570 and 595 nm and cell proliferation equal to reduction of alamarBlue™ reagent was calculated according to the supplier’s manual. The plating density for HUVEC and length of incubation time with alamarBlue™ was determined in preliminary experiments, as suggested by the supplier.

2.7 Statistical analysis

The data are expressed as mean ± S.E.M. of values obtained in three to seven experiments (pigs), each performed in duplicate. Levels of VEGF were standardized on protein concentration per well (pg/µg protein). Statistical analyses were conducted using one-
way ANOVA for repeated measurements followed by Dunnets’s or Bonferroni’s multiple comparison post hoc tests (GraphPad PRISM v. 4.0, GraphPad Software, Inc., San Diego, CA). Differences were considered to be statistically significant at $P < 0.05$.

3. Results

3.1 Immunofluorescent localization of VEGF in stromal cell culture

Immunostaining for VEGF was detectable in stromal cells, with characteristic morphology, after 48 h of culture and was localized in the cytosol (Fig. 1A, B). Negative controls were consistently free from staining (Fig. 1C).

3.2 Effect of IGF-I and RLX on VEGF secretion

Both, IGF-I and RLX stimulated VEGF secretion from endometrial stromal cells in dose- and time-dependent manner (Fig. 2). Basal secretion of VEGF (pg/µg protein) increased from $2.5 \pm 0.4$ (12 h) to $5.3 \pm 0.6$ (48 h). IGF-I-stimulated secretion of VEGF was detected during all studied time-periods (12-48 h). Although, after 12 h only the highest dose of IGF-I was effective ($100 \text{ ng/ml}; P < 0.05$), longer incubation resulted in a significant enhance of VEGF secretion from stromal cells at all tested doses ($P < 0.01$). The most effective action of IGF-I was found after 48 h of incubation, when VEGF concentration in medium increased 1.8-fold for doses 10 and 100 ng/ml ($8.1 \pm 0.5$ and $8.2 \pm 0.6$ vs $4.6 \pm 0.4$, respectively; $P < 0.01$). Effect of RLX on VEGF release was observed only after 24 and 48 h of incubation, when two or all used doses significantly stimulated secretion of VEGF, respectively ($P < 0.01$). Long-term exposure (48 h) of stromal cells to 100 ng/ml RLX led to maximal 1.8-fold increase of VEGF release ($11.0 \pm 1.7$ vs $6.0 \pm 1.1$; $P < 0.01$).
3.3 Effect of OT and PGE$_2$ on VEGF secretion

Release of VEGF from cultured stromal cells was affected by both dose of OT or PGE$_2$ and time of incubation (Fig. 3). Basal secretion of VEGF (pg/µg protein) increased from $1.9 \pm 0.4$ (6 h) to $4.6 \pm 0.2$ (48 h). Although, OT seemed to inhibit VEGF secretion starting from 6 h of incubation, significant effect was detected only after 24 and 48 h, when 100 nM OT was used ($3.4 \pm 0.6$ vs $4.0 \pm 0.7$, $P < 0.05$ and $3.4 \pm 0.7$ vs $4.9 \pm 0.2$, $P < 0.01$; respectively). An augmentation of VEGF secretion by PGE$_2$ in stromal cells was detected as early as 6 h, but exclusively for 1 µM PGE$_2$ (1.4-fold, $P < 0.05$). However, the most effective action of PGE$_2$ was observed after 48 h of incubation, when amounts of VEGF released into medium were induced approximately 1.3-fold for doses 0.1 and 1 µM ($5.4 \pm 0.5$ and $5.8 \pm 0.3$ vs $4.4 \pm 0.3$, $P < 0.05$, $P < 0.01$; respectively).

3.4 Effect of IGF-I and RLX on VEGF gene expression

The dose-dependent effect of IGF-I and RLX (for both 0-100 ng/ml) on VEGF, VEGF164 and VEGF120 mRNA expression in stromal cells after 24 h of treatment is shown on Figure 4. The expression of VEGF, VEGF164 and VEGF120 mRNA was affected only by 50 and 100 ng/ml IGF-I ($P < 0.05$). The maximal, almost 2-fold increase of VEGF, VEGF164 and VEGF120 mRNA expression was observed when the highest dose of IGF-I was used (100 ng/ml; $P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively). Although VEGF and VEGF164 expression was induced by 50 and 100 ng/ml RLX ($P < 0.05$ and $P < 0.01$, respectively), VEGF120 expression was significantly increased when the highest dose was used ($P < 0.05$). The maximal stimulation of VEGF, VEGF164 and VEGF120 mRNA expression was found in cells stimulated with 100 ng/ml RLX (1.6-, 1.8- and 1.5-fold; $P < 0.01$, $P < 0.01$, $P < 0.05$, respectively).
3.5 Effect of OT and PGE$_2$ on VEGF gene expression

Figure 5 shows the dose-dependent effect of OT (0-100 nM) and PGE$_2$ (0-1 µM) on VEGF, VEGF164 and VEGF120 mRNA expression in endometrial stromal cells after 24 h of incubation. In OT-treated cells tendency to decreased expression of VEGF, VEGF164 and VEGF120 mRNA was observed, however significant inhibition was only detected for dose 100 nM ($P < 0.05$). Although, stimulation of VEGF, VEGF164 mRNA expression by PGE$_2$ was dose-dependent, VEGF120 levels were maintained constant. The maximal induction (1.8-fold) was obtained with 1 µM PGE$_2$ for VEGF and 0.1 and 1 µM PGE$_2$ for VEGF164 ($P < 0.05$).

3.6 Effect of IGF-I, RLX, OT and PGE$_2$ on VEGFRs gene expression

Table 2 shows the effect of IGF-I (0-100 ng/ml), RLX (0-100 ng/ml), OT (0-100 nM) and PGE$_2$ (0-1 µM) on VEGFR-1 and VEGFR-2 expression in endometrial stromal cells after 24 h of incubation. Both receptors mRNA expression in cells incubated with various concentrations of IGF-I, RLX, OT or PGE$_2$ was not affected by the treatment.

3.7 Endothelial cell proliferation assay

The ability of media conditioned by porcine endometrial stromal cells after IGF-I or RLX treatment to affect HUVEC proliferation is shown on Figure 6. Both IGF-I and RLX conditioned media increased HUVEC proliferation ($P < 0.001$ and $P < 0.01$; respectively). The effect was comparable to the level of proliferation observed for the positive control (45.6 ± 8.3 and 40.4 ± 6.3 vs 45.7 ± 7.2, respectively). sVEGFR-1 effectively neutralized the effect of IGF-I and RLX conditioned media on endothelial cell proliferation (45.6 ± 8.3 vs 38.0 ± 5.7; $P < 0.05$ and 40.4 ± 6.3 vs 31.4 ± 5.2; $P < 0.01$, respectively), whereas sVEGFR-2 had no significant effect on HUVEC proliferation stimulated by IGF-I and RLX conditioned media.
Both soluble receptors had no affect on cell proliferation in controls, however sVEGFR-2 slightly increased HUVEC proliferation (31.2 ± 4.7 vs 36.2 ± 6.4; \( P > 0.05 \)).

4. Discussion

Several studies, using different animal models, have shown that periimplantation period is critical for embryo implantation, pregnancy success as well as the future growth and developmental potential of embryos. Since VEGF regulates periimplantation vascular permeability in rodents (Rabbani and Rogers, 2001) and female mice treated with VEGF antiserum on day 4 after mating (the normal day of implantation) fail to deliver any pups (Rockwell et al., 2002) it is tempting to believe that VEGF is one of the factors of uterine periimplantation environment being crucial for pregnancy success. Recent results have indicated upregulation of VEGF164 protein in endometrium in the periimplantation period in pigs that agrees with patterns of estradiol secretion by the conceptus (Kaczmarek et al., 2008). Thus, we have investigated whether factors creating a unique microenvironment for successful embryo implantation in pigs may affect VEGF-receptor system expression in the porcine endometrium.

Our study demonstrated, for the first time, the effect of IGF-I, RLX, OT and PGE\(_2\) on VEGF secretion and VEGF-receptor system mRNA expression in porcine endometrial stromal cells from tissues collected during early gestation (days 10-12). Stromal cell cultures were selected for in vitro experiments, since this cell population is present in the uterus in a much higher number than luminal epithelial cells (Blackwell et al., 2003) and thus they are expected to contribute to the synthesis of the major portion of biological molecules produced by the endometrium. For example, Northern blot analysis revealed that porcine stromal cells are the main source of IGF-I (Rodriguez-Sallaberry et al., 2001) and in vitro experiments showed that stroma population collected on days 11-13 of pregnancy may produce more
PGE$_2$ than PGF$_{2\alpha}$ (Zhang and Davis, 1991). The close proximity of stromal cells to the uterine vascular bed also predestinates them to be the main source of factors regulating vascularization and vessel permeability, such as VEGF. Recently, we have demonstrated that uterine stroma may be an important source of VEGF during early pregnancy in pigs (Kaczmarek et al, 2008). Furthermore, results of the present immunofluorescent staining showed that after 48 h-long culture stromal cells were not deprived of VEGF expression.

During the periimplantation period, the porcine uterine endometrium and conceptuses exhibit dramatic biochemical, structural and morphological changes (Geisert et al, 1982). Prior to complete attachment to the endometrial epithelium, blastocysts are dependent upon hormones and nutrients secreted by the uterine endometrium. The presence of the IGF system in porcine endometrium as well as blastocyst suggests important paracrine/autocrine modes of IGF-I action in the uterine environment. In addition to the positive action on uterine growth in early pregnancy, uterine IGF-I is thought to be a critical component for the implantation process (for a review see Simmen et al., 1995). The endometrial IGF-I concentration between days 8-14 of pregnancy is maintained in constant levels (21.1 ± 1.9 ng/g tissue), but its content in the uterine lumen increases and peaks at day 12 of pregnancy (approximately 2.5 𝜇g of total recoverable IGF-I; Letcher at al., 1989). IGF-I has been proposed to increase estrogen synthesis through stimulation of porcine conceptus aromatase gene expression (Green et al., 1995). Although IGF-I was shown to influence VEGF expression in several cell types under physiological conditions, endometrial expression of this factor was studied only in human adenocarcinoma cells (Bermont et al., 2000). Therefore, the present results are the first available data showing IGF-I as the potent stimulator of VEGF mRNA expression as well as VEGF secretion in the normal endometrium, during early pregnancy in the mammalian female. It seems that IGF-I, together with estrogens of embryonic origin, may be
the main factor involved in regulation of VEGF, but not VEGFR, synthesis in endometrial stroma during periimplantation period in pigs.

It is well established that the CL is the main source of circulating RLX throughout pregnancy in several species, including pigs (for a review see Sherwood, 2004). Although serum RLX levels rise gradually from about 0.15 ng/ml on day 6 to about 10 ng/ml on day 110 of pregnancy (Anderson et al., 1983; Eldridge-White et al., 1989), it seems unlikely that this hormone exerts important effects during early gestation in pigs. However, it is tempting to believe that small amounts of RLX produced locally by the endometrium may support implantation through autocrine/paracrine mechanisms. Interestingly, RLX immunoactivity and mRNA was localized between days 10-20 of pregnancy in pigs (Knox et al., 1994). On the other hand, in humans, having a placentation type different to the porcine, circulating RLX is highest during the first trimester (Stewart et al., 1990). Nevertheless, clinical observation makes it seem unlikely that circulating RLX is an important factor during implantation in humans. Further experiments demonstrating local synthesis of RLX in human endometrial cells and its positive effect on VEGF expression (Unemori et al., 1999; Palejwala et al., 2002) led to the hypothesis that this hormone supports uterine vascular events needed for implantation and pregnancy maintenance in humans. Similarly, we showed that porcine stromal cells secrete increased amounts of VEGF in response to RLX treatment. The present results suggest that, also in pigs, endometrial RLX may be involved in regulation of vasculature function within the endometrium during implantation through the control of VEGF expression. However, definitive studies are needed to verify that hypothesis.

The uterotropic actions of exogenous RLX are pronounced in ovariectomized gilts receiving estrogen as well as progesterone (Hall et al., 1992). The important synergism is thought to exist between conceptus-derived estrogen and uterine-derived RLX that stimulate uterine growth during the early stages of conceptus attachment and placentation (Knox et al.,
1994). Since RLX was more effective in stimulation of VEGF164 than VEGF120 mRNA expression, it seems likely that previously established specific pattern of VEGF164 expression during early pregnancy in the pig (Kaczmarek et al., 2008) is a result of the synergistic action of estrogen of embryonic origin and endometrial RLX.

In addition to direct action through specific receptor and initiation of a signal transduction cascade, RLX may exert its biological effects indirectly by influencing production of growth factors and/or their availability. The involvement of the uterine IGF system in the local action of RLX was demonstrated by coincidence of increased uterine weight and protein synthesis with a significant enhancement of uterine luminal content of IGFs and IGF-binding proteins (IGFBP), but not IGF-I-receptor expression in the uterus (Ohleth et al., 1997). Thus, induction of mRNA expression and secretion of VEGF in cultured porcine endometrial stromal cells by RLX may be additionally mediated via stimulation of IGF-I expression. Collectively, the evidence for the local production of RLX by the pig endometrium around the time of implantation (Knox et al., 1994) together with the RLX-induced increase in IGF-I secretion by the endometrium (Ohleth et al., 1997) and presently observed stimulation of VEGF expression in stromal cells by both, IGF-I and RLX support an important role of these factors in preparation of a suitable environment for pregnancy success in the pig.

Moreover, PGs are thought to be pivotal for establishment of pregnancy in the pig, since inhibition of its synthesis results in pregnancy failure (Kraeling et al., 1985). On days 11-12 of gestation, embryo-derived estrogens provide a signal for maternal recognition of pregnancy and increase PGE$_2$/PGF$_{2a}$ ratio due to augmented production of luteoprotective PGE$_2$ (Davis and Blair, 1993; Christenson et al., 1994). In addition, during implantation prostaglandins are thought to participate in important vascular events: modulation of blood flow and increase of vascular permeability (Ford et al., 1982; Keys et al., 1986; Keys and
King, 1988). PGE\(_2\) has been shown to upregulate VEGF in a number of tissues; however there is only one report demonstrating transcriptional regulation of VEGF gene expression in the uterus (Lopes et al., 2006). Interestingly, we found PGE\(_2\) to significantly increase VEGF164 mRNA levels in porcine endometrial stromal cells, whereas VEGF120 and VEGFR mRNA was not affected by the treatment. Halder et al. (2000) showed that VEGF164 is one of the primary mediators of VEGF signaling in the uterine vascular changes during implantation in mice. Thus, it appears that PGE\(_2\) may be involved in induction of vascular permeability during implantation time in the pig, by improving VEGF164 secretion from endometrial stroma.

The presence of a low concentration of OTR in the endometrium during early pregnancy (Okano et al., 1996) at first suggested that OT, known inducer of uterine PGF\(_{2\alpha}\) secretion, is involved in the process of maternal recognition of pregnancy in pigs (Carnahan et al., 1996; Edgerton et al., 1996). Further demonstration that endometrial sensitivity to OT is not regulated primarily through changes in OTR population density (Ludwig et al., 1998) and blocking of OTR did not prevent luteolysis in pigs (Kotwica et al., 1999) deflated mandatory role OT in establishment of pregnancy in pigs. This study provides the first demonstration of OT-mediated inhibition of VEGF secretion as well as mRNA expression in endometrial stromal cells from early pregnancy. In our experiments, only the highest dose of OT was effective in inhibition of VEGF secretion and mRNA expression. This concentration of OT (100 nM) is substantially higher than was reported in the peripheral circulation during luteolysis in pigs (Kotwica et al., 1990). However, as it was reported for RLX, porcine endometrium could be a source of OT during pregnancy (Boulton et al., 1996). Although it was suggested that OT is a rather redundant component of the uterine environment during maternal recognition of pregnancy, our present results suggest that high doses of OT, apart
from stimulation of luteolytic PGF$_{2\alpha}$ secretion from uterus, are able to affect the process of
uterine vascularization and vascular permeability during early pregnancy in pigs.

Although all tested factors affected secretion and mRNA expression of VEGF, VEGFR-1 and VEGFR-2 mRNA levels were maintained at a very low and constant level, without significant stimulatory or inhibitory effects. The result is not surprising since the dominant localization VEGFRs was found not in stromal, but in uterine capillary endothelial cells in pigs (Charnock-Jones et al., 2001; Kaczmarek et al., 2008). The examined factors may have also some effects on receptors in intact tissue if they are produced in stromal cells. VEGF may act therefore directly on endothelial cells and also as a chemokine in attracting endothelial cell migration during the outgrowth of new vessels in the endometrium.

In the present study, proliferation of HUVEC \textit{in vitro} was induced directly by media conditioned by stromal cells collected after IGF-I and RLX treatment. These effects were blocked by sVEGFR-1, suggesting that IGF-I- and RLX-stimulated VEGF is able to induce endothelial cells proliferation. More significant inhibition of HUVEC proliferation by media conditioned by stromal cells collected after RLX treatment, may have an explanation in our gene expression studies, where levels of highly mitogenic VEGF164 mRNA were induced by RLX in a grater manner comparing to IGF-I. In contrast, sVEGFR-2 (no naturally occurring splice variant) was not able to significantly inhibit HUVEC proliferation; however such a tendency was observed. Like other growth factor transmembrane tyrosine kinase receptors, VEGF receptors presumably undergo ligand-induced dimerization to activate transduction pathways. Thus, dimers between sVEGFR and full-length VEGF receptor block signal pathways dependent on intracellular tyrosine kinase dimerization. Both soluble receptors used in the experiment has the same ED$_{50}$ for VEGF-dependent proliferation of HUVEC provided by the supplier (10-30 ng/ml), therefore the reason of such observation in a present study may rather not lay in a dose of the sVEGFR used in a culture. However, previous results showed
that VEGFR-1 and its soluble form bind VEGF with higher affinity (approximately $K_d \approx 10-20\, pM$; de Vries et al., 1992; Kendall and Thomas, 1993) than VEGFR-1 (approximately $K_d \approx 75-125\, pM$; Terman et al., 1992). On the other hand, sVEGFR-2 seemed to increase the cell proliferation \textit{in vitro} itself, thus it is possible that its action on VEGF-stimulated proliferation of HUVEC was masked by this effect in our experiment.

In summary, the present study is the first to demonstrate that VEGF-receptor system expression in porcine endometrial stromal cells is controlled by factors creating a unique microenvironment for successful embryo implantation in pigs. IGF-I and RLX are the most potent inducers of VEGF secretion and mRNA expression, acting together with estrogens of embryonic origin. Luteoprotective PGE$_2$ was effective in stimulation of VEGF secretion as well as VEGF164 mRNA expression suggesting its participation in vascular events during implantation. In contrast, high doses of OT seem to inhibit VEGF-mediated actions in endometrial stroma during early pregnancy in pigs. These results suggest that during the periimplantation period essential crosstalk exists between examined factors that ensures uterine and embryo development, implantation and pregnancy success by affecting uterine vasculature function.
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Transcriptional regulation of uterine vascular endothelial growth factor during early


Figure legends

Fig. 1. Porcine endometrial stromal cells in culture. (A) Light microscopy of stromal cells after 48 h after plating. (B) Immunofluorescent staining of porcine stromal cells for VEGF protein expression. (C) Negative control. Magnification, x 400.

Fig. 2. IGF-I- and RLX-induced changes in VEGF secretion (pg/µg protein) by cultured porcine endometrial stromal cells obtained on days 10-12 of pregnancy. Cells were incubated with IGF-I or RLX (for both 0-100 ng/ml) for 12 h (n=3), 24 h (n=7) and 48 h (n=4). Data are presented as means ± S.E.M. * P < 0.05, ** P <0.01 compared to controls.

Fig. 3. OT- and PGE$_2$-induced changes in VEGF secretion (pg/µg protein) by cultured porcine endometrial stromal cells obtained on days 10-12 of pregnancy. Cells were incubated with OT (0-100 nM) or PGE$_2$ (0-1 µM) for 6 h (n=4), 12 h (n=3), 24 h (n=7) and 48 h (n=7). Data are presented as means ± S.E.M. * P < 0.05, ** P <0.01 compared to controls.

Fig. 4. Dose-dependent effect of IGF-I and RLX on VEGF, VEGF164 and VEGF120 expression in cultured porcine endometrial stromal cells obtained on days 10-12 of pregnancy. Cells were incubated with IGF-I or RLX (for both 0-100 ng/ml) for 24 h (n=4). Data are presented as means ± S.E.M. * P < 0.05, ** P <0.01 compared to controls.

Fig. 5. Dose-dependent effect of OT and PGE$_2$ on VEGF, VEGF164 and VEGF120 expression in cultured porcine endometrial stromal cells obtained on days 10-12 of pregnancy. Cells were incubated with OT (0-100 nM) or PGE$_2$ (0-1 µM) for 24 h (n=3). Data are presented as means ± S.E.M. * P < 0.05, ** P <0.01 compared to controls.
Fig. 6. Induction of endothelial cell proliferation by 35% of conditioned medium collected after IGF-I (A; n=5) or RLX (B; n=4) treatment of porcine endometrial stromal cells. HUVEC were cultured in control or conditioned medium in the absence or presence of sVEGFR-1 and sVEGFR-2 (100 ng/ml). VEGF (10 ng/ml) was used as a positive control. Data are presented as means ± S.E.M. * P < 0.05, ** P <0.01, *** P <0.001.
Kaczmarek et al.  
(Fig. 1)
Figure 2

Kaczmarek et al. (Fig. 2)
Figure 5

Kaczmarek et al.
(Fig. 5)
Figure 6

Kaczmarek et al. (Fig. 6)

A

Cell proliferation (% of alamarBlue™ reduction)

sVEGFR-1 - + - - - - - + -
sVEGFR-2 - - + - - - - + -
VEGF - - - + - - - - -
35% IGF-I - - - - + - + +

B

Cell proliferation (% of alamarBlue™ reduction)

sVEGFR-1 - + - - - - - + -
sVEGFR-2 - - + - - - - + -
VEGF - - - + - - - - -
35% RLX - - - - + - + +
Table 1
Gene specific primers used for Real Time PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers (5’-3’)</th>
<th>Product size</th>
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<tr>
<td>VEGF</td>
<td>Forward: CCTGATGCGGTGCGGGGGCT Reverse: TGGTGGTGGC GGCGGGCTATG</td>
<td>VEGF&lt;sub&gt;188&lt;/sub&gt;:510 VEGF&lt;sub&gt;164&lt;/sub&gt;:438 VEGF&lt;sub&gt;120&lt;/sub&gt;:306</td>
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<td>VEGF&lt;sub&gt;164&lt;/sub&gt;</td>
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<td>Forward: CACCCCGGAAATCTATCAGATC Reverse: GAGTACGTGAAAGCCGCTGGT</td>
<td>180</td>
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<tr>
<td>VEGFR-2</td>
<td>Forward: GATGCTCGCCTCCCTTGA Reverse: AGTTCCCTCTTTCCAGTCGCCTACA</td>
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<tr>
<td>β-actin</td>
<td>Forward: ACATCAAGGAGAAGCTCTGTACGT</td>
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<sup>a</sup>See text for definitions.
Table 2
Relative expression of VEGFR-1 and VEGFR-2 in porcine endometrial stromal cells after treatment

<table>
<thead>
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<th>Treatment</th>
<th>VEGFR-1 vs. β-actin</th>
<th>VEGFR-2 vs. β-actin</th>
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<td>0</td>
<td>0.017 ± 0.004</td>
<td>0.002 ± 0.0007</td>
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<tr>
<td>10</td>
<td>0.017 ± 0.005</td>
<td>0.003 ± 0.0010</td>
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<tr>
<td>50</td>
<td>0.014 ± 0.003</td>
<td>0.003 ± 0.0010</td>
</tr>
<tr>
<td>100</td>
<td>0.015 ± 0.003</td>
<td>0.003 ± 0.0007</td>
</tr>
<tr>
<td>0</td>
<td>0.018 ± 0.005</td>
<td>0.002 ± 0.0010</td>
</tr>
<tr>
<td>100</td>
<td>0.019 ± 0.004</td>
<td>0.003 ± 0.0009</td>
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<tr>
<td>0</td>
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<td>0.007 ± 0.0029</td>
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<td>0.021 ± 0.007</td>
<td>0.006 ± 0.0015</td>
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<tr>
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<tr>
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<td>0.020 ± 0.006</td>
<td>0.007 ± 0.0015</td>
</tr>
<tr>
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<td>0.006 ± 0.0021</td>
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<tr>
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<td>0.005 ± 0.0013</td>
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aData are presented as means ± S.E.M.