

The effect of insulin-like growth factor-I, relaxin and luteinizing hormone on vascular endothelial growth factor secretion by cultured endometrial stromal cells on different days of early pregnancy in pigs

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Received: 8 April 2008; accepted: 26 June 2008

SUMMARY

The effect of insulin-like growth factor-I (IGF-I), relaxin (RLX) and luteinizing hormone (LH) on vascular endothelial growth factor (VEGF) *in vitro* secretion by endometrial stromal cells in pigs was investigated on days 10-12 and 20-22 of gestation. LH-stimulated stromal cell secretion of VEGF did not differ among tested days of early pregnancy. However, IGF-I- and RLX-mediated release of VEGF depended on the day of pregnancy. It seems that IGF-I and RLX may be considered as potent activators of VEGF-mediated angiogenesis in porcine endometrium, and their action may be more pronounced during maternal recognition of pregnancy. *Reproductive Biology* 2008, 8, 2:163-170.

Keywords: angiogenesis, VEGF secretion, endometrium, early pregnancy

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INTRODUCTION

Angiogenesis is a complex process that is essential for the development of functional endometrium as well as establishment and maintenance of pregnancy (for review see: [6]). Among other angiogenic factors, vascular endothelial growth factor (VEGF) seems to play a paramount role in angiogenesis and in vessel permeability. VEGF is expressed in a wide range of cells and tissues (for review see: [5]) including cyclic and pregnant endometrium of several domestic animals [1, 14]. Recently, we have demonstrated an up-regulation of VEGF protein level and increased VEGF immunoreactivity in endometrial compartments of a gravid porcine uterus during peri-implantation period. Moreover, increased expression of VEGF was also found during early stages of placentation [8]. Since there is little information concerning the regulation of VEGF secretion by endometrium during different stages of early pregnancy in pigs, this study was undertaken to examine the effect of insulin-like growth factor-I (IGF-I), relaxin (RLX) and luteinizing hormone (LH) on VEGF *in vitro* secretion by porcine endometrial stromal cells collected on days 10-12 (time of maternal recognition of pregnancy) and days 20-22 of gestation.

MATERIALS AND METHODS

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 (Chiron Corp., Berkeley, CA, USA). Purified porcine RLX was a generous gift from Dr O.D. Sherwood (University of Illinois at Urbana-Champaign, IL, USA). Porcine LH (pLH B-1) was a gift of USDA Hormone program (Bethesda, MD, USA).

Primary cell culture and treatment

The endometrial tissue was collected on days 10-12 (n=5) and 20-22 (n=3) of pregnancy from crossbred gilts of established breeding time. Pregnancy was

confirmed by the presence of at least four conceptuses in the uterine horns. Isolation of porcine endometrial stromal cells was described previously [3]. Briefly, stromal cell suspensions obtained after trypsin (0.25%; Biomed, Lublin, Poland) and collagenase (0.06%) digestions were pooled together, washed three times with fresh medium M199, counted and plated in 24-well culture plates at a density of 2.5×10^5 cells/well in Medium 199 containing 2% BSA (ICN, Biomedicals, Inc., Costa Mesa, CA, USA), 10% newborn calf serum and 20 $\mu\text{g/ml}$ gentamycin. Forty-eight hours after cell plating, the fresh Medium 199 was added and used as a control. Treatment media contained 10, 50 or 100 ng/ml IGF-I, 25, 50 or 100 ng/ml RLX, or 1, 10, 50 or 100 ng/ml LH. Following 24-hour incubation period (37°C, 95% air/5% CO₂), medium was collected and stored at -40°C. Cells were lysed with 100 mmol/l NaOH and total cellular protein content was measured [4]. All procedures and protocols involving the use of animals were approved by the Local Ethical Review Committee and were in compliance with the national guidelines for agricultural animal care.

Radioimmunoassay (RIA) of VEGF

Concentrations of VEGF in media were measured by RIA [2]. Briefly, the recombinant bovine VEGF₁₆₄ was used for preparation of rabbit antiserum and iodination by the iodogen method. Labeled VEGF was separated from free iodine with prepacked disposable NAP-10 column containing Sephadex G-25 (Amersham-Pharmacia, Freiburg, Germany). The standard was used in a range of 0.063-8 ng/ml. The RIA incubation buffer 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. The antibody and tracer were added within two separate days, each incubated overnight at 4°C. Bound and free VEGF were separated using the double antibody technique (secondary antibodies – self made, goat anti-rabbit antiserum; dilution 1:200) and 6% polyethylene glycol (Serva, Heidelberg, Germany). The intra-assay variation was 3.4% and the inter-assay variation 15%. The ED₅₀ and sensitivity of the assay were 0.6 and 0.1 ng/ml, respectively. Dilution of samples containing endogenous VEGF or added VEGF from blood plasma, follicular fluid and

tissue extracts produced curves parallel to the standard curve. The average recovery of exogenous VEGF was 94%.

Statistical analysis

The data are expressed as mean \pm SEM of values obtained in three to five experiments (pigs), each treatment performed in duplicates. Levels of VEGF were standardized to protein concentration per well (pg/ μ g protein). Statistical analyses were conducted using two-way ANOVA for repeated measurements followed by Bonferroni's multiple comparison post hoc test (GraphPad PRISM v. 4.0, GraphPad Software, Inc., San Diego, CA). Differences were considered to be statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Both IGF-I and RLX stimulated VEGF secretion by endometrial stromal cells and their action was affected by the treatment concentrations ($p < 0.0004$ and $p < 0.001$, respectively). Basal secretion of VEGF was not affected by the day of pregnancy ($p > 0.05$). In addition, there was no day \times treatment interaction for VEGF endometrial release. Although, on days 10-12 of pregnancy, all doses of IGF-I effectively stimulated VEGF release by endometrial stromal cells (10, 50 and 100 ng/ml; $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively), augmentation of VEGF secretion on days 20-22 of pregnancy was observed exclusively when the highest dose of IGF-I (100 ng/ml; $p < 0.01$) was used. Interestingly, RLX-stimulated VEGF secretion was detected only on days 10-12 of pregnancy and with doses 50 and 100 ng/ml ($p < 0.01$ and $p < 0.001$, respectively). This may suggest higher sensitivity of peri-implantation endometrium to IGF-I and RLX resulting in up-regulation of VEGF expression and further possible activation of angiogenesis in the uterus. Presently, no data concerning uterine concentration of RLX receptors during early pregnancy is available, but it was reported that IGF-I receptors are maintained at a constant level [13]. Thus, differentiated stimulation of VEGF secretion by endometrial cells during different days of early pregnancy, at least for IGF-I, is probably not dependent upon concentration of its binding sites.

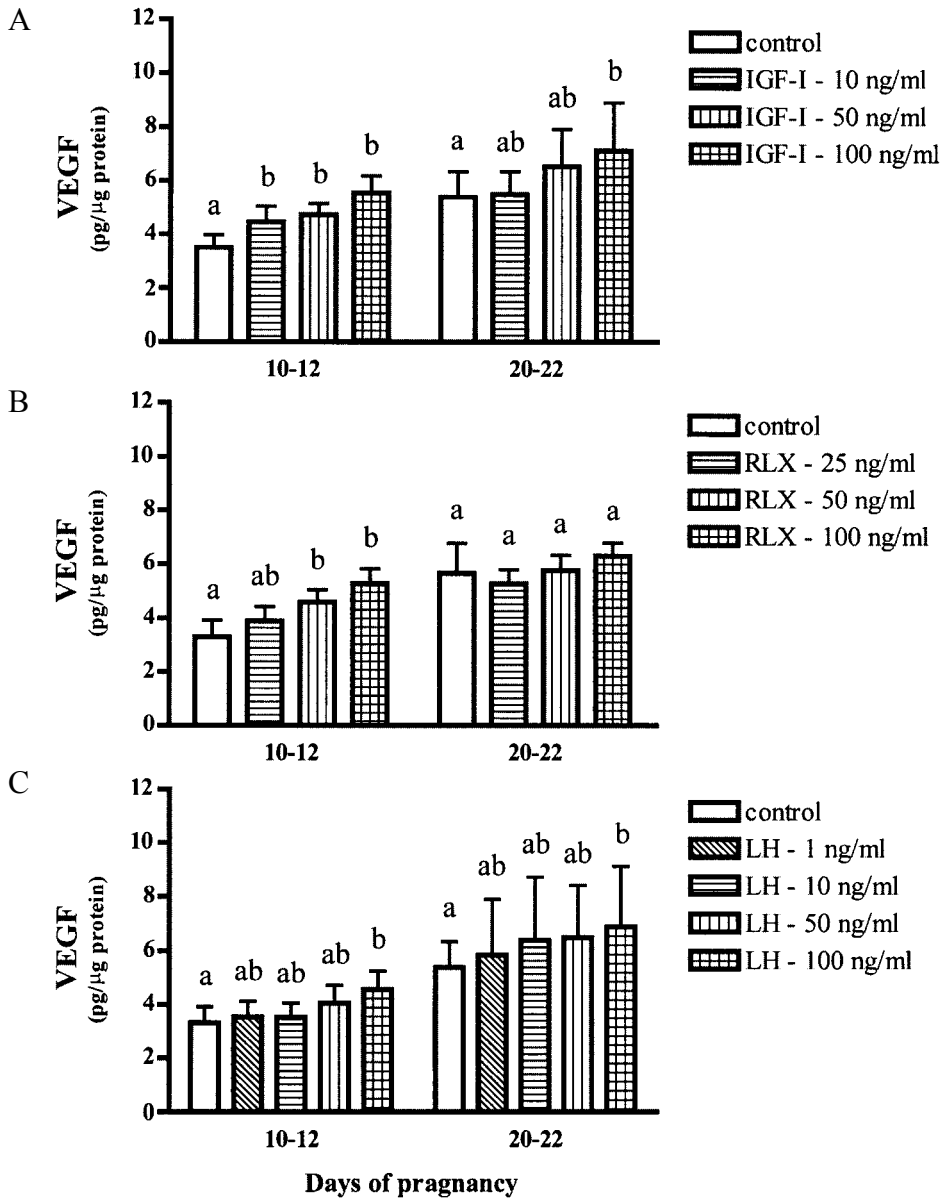


Figure 1. Insulin-like growth factor-I (IGF-I)- (A), relaxin (RLX)- (B) and luteinizing hormone (LH)-induced (C) changes in vascular endothelial growth factor (VEGF) secretion (means \pm SEM) by cultured porcine endometrial stromal cells obtained on days 10-12 (n=5) and 20-22 (n=3) of pregnancy. Cells were incubated with IGF-I, RLX or LH for 24 hours (37°C, 95% air/5% CO₂). Different superscripts designate significant differences (p<0.05) within each examined period (10-12 or 20-22 days) of pregnancy.

Ligand-independent activation of estrogen receptors (ERs) as well as activation of ER-mediated transcription of target genes *in vitro* were shown for both IGF-I and RLX [9, 10]. There are no consensus estrogen response elements (EREs) within the VEGF promoter. However, multiple consensus or near-consensus sequences for transcription factors interacting with ER in the regulation of gene transcription were identified e.g. Sp1 and AP-1 (for review see: [11]). Interestingly, the maintained ER level in the porcine uterus is high on days 10-12 of pregnancy and decreases afterwards [7, 12]. It cannot be excluded that pregnancy day-dependent stimulation of VEGF secretion by endometrial stromal cells observed in our study may result from a different availability of ERs and, consequently, different VEGF gene transcription. Nevertheless, additional studies should be undertaken to elucidate the relationship between ER expression and IGF-I and RLX-induced VEGF secretion in the porcine uterus.

LH action on VEGF release by cultured endometrial stromal cells was affected by treatment concentration ($p < 0.03$). There was no day \times treatment interaction. During each tested period of pregnancy, LH stimulated VEGF secretion only when applied at the highest dose (100 ng/ml; $p < 0.05$; fig. 1C) which suggests that uterine sensitivity to relatively high doses of LH, manifested in the induced VEGF secretion, is not affected by the day of pregnancy.

Summarizing, the presented results suggest that both IGF-I and RLX may be considered as potential pregnancy status-dependent activators of angiogenesis in the porcine uterus. Whether it is accomplished by direct action of these factors on VEGF expression or by the interaction with ER or other transcription factors must be further analyzed.

ACKNOWLEDGEMENTS

We thank Mrs. K. Gromadzka-Hliwa, Mr. J. Klos and Mr. M. Blitek for their excellent technical assistance in the laboratory and Mrs. Gabi Schwentker for expert technical assistance in RIA. The research collaboration in this study was supported by Roman Herzog Research Fellowship and Return Fellowship from Hertie and Alexander von Humboldt Foundations (to

M.M.K.). This research was supported by the State Committee for Scientific Research in Poland (grant No PBZ-KBN-084/P06/2002 from 2003 to 2005).

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