Endocrine disruption of uterine insulin-like growth factor expression in the pregnant gilt

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Abstract

Early exposure of pregnant gilts to oestrogen, prior to the normal period of porcine conceptus oestrogen secretion, disrupts the uterine environment resulting in complete embryonic mortality during the period of placental attachment to the uterine surface. The current study evaluates the uterine insulin-like growth factor (IGF) system following endocrine disruption of early pregnancy in gilts through exposure to exogenous oestrogen on Days 9 and 10 of gestation. Endometrial IGF gene and protein expression, IGF-I receptor (IGF-IR) gene expression, and uterine luminal content of IGF binding proteins (IGFBPs) were evaluated in control and oestrogen-treated gilts on Days 10, 12, 13, 15 and 17 of gestation. Oestrogen treatment altered endometrial IGF-I and IGF-IR gene expression on Days 12 and 13 of gestation. Uterine content of IGF-I and IGF-II in control gilts was greatest on Days 10, 12, and 13 followed by a four- to sixfold decrease on Day 15 of gestation. Oestrogen treatment caused a premature proteolysis of IGFBPs within the pregnant pig uterus on Day 10 of gestation, and an earlier decline in uterine luminal IGF-I content. Results demonstrate that early exposure of pregnant gilts to oestrogen causes premature loss of uterine IGFs during the period of conceptus elongation. Timing for the release of uterine IGFs during early porcine conceptus development may play an important function in the ability of the conceptus to attach and survive during the establishment of pregnancy.

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Introduction

In the pig, establishment of pregnancy involves a specific sequence of uterine developmental and secretory events that are essential for continued conceptus growth and survival. Early porcine conceptus development involves a rapid transformation of the trophoblast from a spherical to a filamentous morphology between Days 11 and 12 of gestation (Geisert *et al.* 1982). During rapid trophoblast elongation on Day 12 the conceptus secretes oestrogen, which serves as the signal for maternal recognition of pregnancy in the pig (Bazer & Thatcher 1977). There is a spatiotemporal relationship between the increase in uterine luminal content of insulin-like growth factors (IGF-I and IGF-II) and early conceptus expansion and oestrogen synthesis between Days 10 through 13 of gestation (Letcher *et al.* 1989, Geisert *et al.* 2001). Increase in uterine luminal fluid content of IGFs on Days 11 to 12 of gestation has been proposed to be involved with the stimulation of conceptus aromatase (P450_{arom}) activity to enhance conceptus oestrogen synthesis during the period of trophoblast elongation (Green *et al.* 1995) and could be important for conceptus differentiation and attachment

to the uterine luminal surface after initial expansion through the uterine horns.

Implantation of pig conceptuses involves a superficial attachment of the trophoblast to the microvilli located on the uterine apical surface epithelium between Days 13 and 18 of gestation (Dantzer 1985). Trophoblast attachment to the uterine luminal surface epithelium is mediated by a number of endometrial cytokines, growth factors, and interactions between the developing conceptus and apical expression of endometrial integrins on the surface epithelium (Burghardt *et al.* 1997, Geisert & Yelich 1997). The IGF system has been characterized in a multitude of biological systems (Simmen *et al.* 1992, Irwin *et al.* 2001, Sato *et al.* 2002) and is composed of three ligands (IGF-I, IGF-II, and insulin), five or more regulatory binding proteins (IGFBP-1 through -6), and three or more cell surface receptors (IGF type I and II receptors, insulin receptor, and hybrid receptors) (Jones & Clemmons 1995, Butler & LeRoith 2001). The porcine conceptus has been reported to express mRNA for IGF type I receptor (IGF-IR) (Corps *et al.* 1990). Although gene expression for IGF-IR was demonstrated, Chastant *et al.* (1994) were unable to

detect the presence of IGF-IR in the trophoblast, but did immunolocalize IGF type II receptor (IGF-IIR). Conceptus expression of the IGF-IR mRNA and the presence of trophoblast IGF-RII indicate that uterine IGF secretion could serve an integral part in early porcine conceptus development and survival.

The importance of IGFs in early porcine conceptus development and uterine receptivity for implantation is demonstrated by the precise alteration in the presence of IGFBPs that occur during the period of conceptus expansion (Lee *et al.* 1998, Geisert *et al.* 2001). Uterine IGFBPs are present in the porcine uterine lumen from Day 5 to Day 10 of the oestrous cycle and during early gestation (Lee *et al.* 1998, Geisert *et al.* 2001). However, the porcine uterine luminal IGFBPs are proteolytically cleaved after Day 11 in both cyclic and pregnant gilts. Activation of proteolytic enzymes such as serine protease, tissue kallikrein and the matrix metalloproteinases degrade IGFBPs in the uterine lumen allowing IGF stimulation of the conceptuses during a critical period of development in the pig (Lee *et al.* 1998, Geisert *et al.* 2001).

Our laboratory has established that conceptus secretion of oestrogen between Days 11 and 13 of gestation plays a critical role in the normal process of implantation in pigs, and we have demonstrated that oestrogen can function as an endocrine disruptor of implantation if administered on Days 9 and 10, i.e. 48 h prior to the normal period of secretion of oestrogen and 96 h prior to initiation of implantation. Exposure of pregnant gilts to exogenous oestrogens before the normal physiological secretion of conceptus oestrogens on Day 12 results in complete embryonic mortality before Day 30 of gestation (Pope *et al.* 1986). Early exposure of gilts to oestrogen on Days 9 and 10 of pregnancy causes conceptus degeneration and fragmentation between Days 15 to 18 of gestation (Gries *et al.* 1989, Blair *et al.* 1991). Recently, the concentration and timing of oestrogen stimulation was demonstrated to function within a very narrow range to open the window for uterine receptivity in the mouse (Ma *et al.* 2003). High concentrations of oestrogen shorten the window of receptivity and cause implantation failure as a result of aberrant uterine gene expression during blastocyst attachment. We propose that oestrogen treatment of pregnant gilts on Days 9 and 10 alters the timing of the presence of IGF-I within the uterine lumen, which could be critical for continued embryonic development.

Materials and Methods

Animals

Research was conducted in accordance with the Guiding Principles for Care and Use of Animals promoted by the Society for the Study of Reproduction and approved by the Oklahoma State Institutional Care and Use Committee. Crossbred cyclic gilts of similar age (8–10 months) and weight (100–130 kg) were checked twice daily for

oestrous behaviour (oestrus onset = Day 0 of oestrous cycle) with intact males. Gilts were mated with fertile crossbred boars at first detection of oestrus and again 24 h post-detection. Bred gilts ($n = 40$) were randomly assigned to one of the following two treatment groups: vehicle (Veh), i.m. injection of corn oil (2.5 ml) on Days 9 and 10 of gestation, or oestrogen treatment (E), 5 mg i.m. injection of oestradiol cypionate (AJ Legere, Scottsdale, AZ, USA) on Days 9 and 10 of gestation.

Surgical procedure

Bred gilts were hysterectomized through a midventral laparotomy on Days 10, 12, 13, 15 or 17 of gestation as previously described by Gries *et al.* (1989). Following initial induction of anaesthesia with a 1.8 ml i.m. administration of a cocktail consisting of 2.5 ml Xylazine (100 mg/ml; Miles Inc., Shawnee Mission, KS, USA) and 2.5 ml Vetamine (Ketamine HCl, 100 mg/ml; Molli Krodt Veterinary, Mundelein, IL, USA) in 500 mg Telazol (Tiletamine HCl and Zolazepam HCl; Fort Dodge, Syracuse, NE, USA), anaesthesia was maintained with a closed circuit system of halothane (5% flurothane) and oxygen (1.0 l/min). Uteri were exposed via a midventral laparotomy and a randomly selected uterine horn and its ipsilateral ovary were excised. Uterine luminal contents and conceptuses were flushed from the horn by infusing 20 ml phosphate buffered saline (PBS, pH 7.4) through the lumen and collecting the flushings into a Petri dish. Conceptuses were removed from the flushings, snap frozen in liquid nitrogen and stored at -80°C . Uterine flushings were centrifuged at 1000 *g* for 10 min at 4°C , the supernatant was collected and was stored at -20°C . Endometrial tissue was removed from the antimesometrial side of the uterine horn, immediately snap frozen in liquid nitrogen and stored at -80°C until utilized for RNA extraction.

Endometrial RNA extraction

Total RNA was extracted from endometrial tissue using RNeasy reagent (Ambion, Inc., Austin, TX, USA). Approximately 0.5 g endometrial tissue was homogenized in 5.0 ml RNeasy using a Virtishear homogenizer (Virtis Company Inc., Gardiner, NY, USA). RNA was rehydrated in nuclease-free water and stored at -80°C . Total RNA was quantified with a spectrophotometer at an absorbance of 260 nm and purity was verified based on the 260/280 ratio.

Quantitative one-step reverse transcription-polymerase chain reaction (RT-PCR)

Quantitative analyses of endometrial IGF-I and IGF-IR mRNA were conducted using quantitative real-time RT-PCR as previously described (Hettinger *et al.* 2001). The PCR amplification was performed in a reaction volume of 15 μl using an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA).

The transcripts were evaluated using dual labelled probes containing 6-Fam (5' reporter dye), and TAMRA (3' quenching dye). Primer and probe sequences for the amplification of IGF-I and IGF-1R (Table 1) were generated from porcine cDNA sequences available in the NCBI database. Total RNA (100 ng) was assayed in duplicate using thermocycling conditions for one-step cDNA synthesis of 30 min at 48°C and 95°C for 10 min, followed by 45 repetitive cycles of 95°C for 15 s and 60°C for 1 min. Ribosomal 18S RNA was assayed in each sample to normalize RNA loading as previously described by Ross *et al.* (2003).

Template amplification was quantified by determining the threshold cycle (C_T) based on the fluorescence detected within the geometric region of the semilog plot. In the geometric region, one cycle is equivalent to the doubling of PCR target template. Relative quantification and fold gene expression difference between treatment and day were determined for the endometrial samples using the comparative C_T method (Table 2). Differences in mRNA expression of IGF-I and IGF-1R were determined by subtracting the target C_T of each sample from its respective ribosomal 18S C_T value, which provides the sample ΔC_T value. Calculation of the $\Delta\Delta C_T$ involves using the highest sample ΔC_T value as an arbitrary constant to subtract from all other ΔC_T sample values. Fold differences in gene expression of the target gene are equivalent to $2^{-\Delta\Delta C_T}$.

Radioimmunoassay of IGF content of uterine flushings

Uterine content of immunoreactive IGF-I in uterine luminal flushings was concentrated and determined in a single radioimmunoassay after acid-ethanol extraction (16 h at 4°C) as previously described by Echternkamp *et al.* (1990). The intra-assay coefficient of variation was 2.8%. The content of IGF-II in uterine flushings was determined in a single assay by radioimmunoassay as described by Spicer & Echternkamp (1995). The intra-assay coefficient of variation was 3.0%.

Ligand blotting

Uterine flushings were prepared for ligand blotting by concentrating 4 ml of the flushing using a Centricon 10 concentrator (Amicon, Beverly, MA, USA) with a 10 000 molecular weight cut off. Protein content in the concentrated uterine flushing sample was determined by

using the Bio-Rad Protein Assay Kit II (Bio-Rad, Hercules, CA). IGF-BPs in the uterine flushings were analysed by one-dimensional SDS-PAGE as described by Echternkamp *et al.* (1994). Protein (50 µg) from the concentrated uterine flushing was mixed with 21 µl non-reducing denaturation buffer (BioRad, Hercules, CA, USA). Bovine follicular fluid, diluted 10-fold, was utilized as a positive control to identify band size and IGF-BPs in the porcine uterine flushings. Samples were denatured by heating to 100°C for 3 min, centrifuging at 4657 g for 3 min and were then separated using 12% (w/v) PAGE for 65 min at a constant current of 25 mA per gel. After separation, proteins in the gel were electrophoretically transferred to nitrocellulose paper (Midwest Scientific, St Louis, MO, USA) and subsequently ligand blotted (16 h at 4°C) using recombinant human ¹²⁵I-labelled IGF-I and IGF-II. The next day, the nitrocellulose blots were washed, dried, and exposed to X-ray film at -80°C for 21 days.

Statistical analysis

Data were analysed by least square ANOVA using the Proc Mixed procedure of SAS (SAS 1985). The statistical method used to analyse uterine gene expression for IGF-I and IGF-1R, and IGF-I and IGF-II protein in the uterine flushings included effects of day, treatment, and day × treatment interaction.

Results

Conceptus development

Normal conceptuses were present in the uterine flushings collected from bred vehicle-treated (Veh) gilts across all days of pregnancy evaluated. Conceptuses of normal appearance were collected from oestrogen-treated (E) gilts on Days 10, 12 and 13 of gestation. However, conceptus tissues were in various stages of fragmentation when collected on Days 15 and 17 of gestation.

Quantitative RT-PCR

A day × treatment interaction ($P < 0.003$) was detected for quantitative RT-PCR analysis of endometrial IGF-I gene expression in E and Veh gilts (Table 2). Oestrogen treatment on Days 9 and 10 of gestation decreased endometrial IGF-I gene expression approximately 3-fold compared with Veh gilts on Day 12 (Table 2). However,

Table 1 Porcine PCR primer and probe sequences used for quantitative RT-PCR.

Gene		Forward primer/reverse primer/probe	GeneBank Accession No.
IGF-I	Forward	5'-GCCTTGACTGTGATATGCGTGTT-3'	X64400
	Reverse	5'-AGAAAGACAAGTTAGCGTCCGGAGT-3'	
	Probe	5'-ACACTGACGGATGCTGAAGGCGGGCACCAT-3'	
IGF-1R	Forward	5'-GCATGGCATACTCAACGCCAATA-3'	TC131942
	Reverse	5'-TGTGAAGTCTTCGGCCACCATACA-3'	
	Probe	5'-TTTGTCCACAGACCTCGCTGCCCGGAA-3'	

Table 2 Endometrial IGF-I and IGF-IR gene expression of vehicle (Veh) and oestrogen-treated (E) gilts.

Target	Treatment	Day	Average target C _T *	18S C _T *	ΔC _T + [†] _P	ΔΔC _T §	Fold change‡
IGF-I	Veh	10	28.25 ± 0.029	18.95 ± 0.50	9.30 ± 0.42 ^{bc}	-1.87	3.66
	E	10	27.84 ± 0.19	18.67 ± 0.38	9.17 ± 0.12 ^{bc}	-1.99	3.97
	Veh	12	28.30 ± 0.27	18.32 ± 0.21	9.98 ± 0.27 ^{bc}	-1.18	2.27
	E	12	29.00 ± 0.39	17.84 ± 0.24	11.16 ± 0.55 ^a	0.00	1.00
	Veh	13	28.66 ± 0.19	18.19 ± 0.21	10.47 ± 0.18 ^b	-0.69	1.61
	E	13	28.24 ± 0.53	19.13 ± 0.82	9.11 ± 0.28 ^c	-2.05	4.14
	Veh	15	28.01 ± 0.37	18.54 ± 0.20	9.47 ± 0.31 ^{bc}	-1.69	3.23
	E	15	27.72 ± 0.30	18.86 ± 0.18	8.86 ± 0.35 ^c	-2.30	4.92
	Veh	17	27.86 ± 0.25	18.57 ± 0.24	9.29 ± 0.28 ^c	-1.87	3.66
	E	17	27.72 ± 0.43	18.84 ± 0.41	8.88 ± 0.34 ^{bc}	-2.28	4.86
IGF-IR	Veh	10	21.22 ± 0.06	18.95 ± 0.50	2.27 ± 0.26 ^{bc}	-0.83	1.78
	E	10	21.36 ± 0.34	18.67 ± 0.38	2.69 ± 0.14 ^{ab}	-0.41	1.33
	Veh	12	21.05 ± 0.34	18.32 ± 0.21	2.73 ± 0.31 ^{ab}	-0.37	1.29
	E	12	20.82 ± 0.17	17.84 ± 0.24	2.98 ± 0.23 ^a	-0.12	1.09
	Veh	13	21.30 ± 0.15	18.19 ± 0.21	3.10 ± 0.13 ^a	0.00	1.00
	E	13	20.90 ± 0.50	19.13 ± 0.82	1.69 ± 0.20 ^d	-1.42	2.68
	Veh	15	20.99 ± 0.04	18.54 ± 0.20	2.36 ± 0.08 ^b	-0.74	1.67
	E	15	20.50 ± 0.13	18.86 ± 0.18	1.74 ± 0.15 ^{cd}	-1.37	2.58
	Veh	17	20.77 ± 0.18	18.57 ± 0.24	2.23 ± 0.19 ^{bc}	-0.07	1.05
	E	17	20.66 ± 0.19	18.84 ± 0.41	1.82 ± 0.20 ^{cd}	-1.28	2.43

* C_T = Cycle threshold: cycle number where gene amplification crosses the threshold set in the geometric portion of the amplification curve.

† ΔC_T = Target transcript C_T - 18S ribosomal C_T: Normalization of C_T for target gene relative to ribosomal 18S RNA C_T.

[‡] = Statistical analysis of normalized expression levels. Values with different superscripts for each of the target genes differ significantly ($P < 0.05$).

§ ΔΔC_T = Mean ΔC_T value - highest mean ΔC_T value (IGF-I = Day 12 E and IGF-IR = IR = Day 13 Veh was used as a calibrator to set baseline for comparing mean differences in the ΔC_T values across treatments and Day.

‡ = Fold change in gene expression was determined by formula $2^{-\Delta\Delta C_T}$.

on Day 13 of gestation, endometrial IGF-I gene expression increased 2.5-fold in E compared with Veh gilts. Endometrial IGF-I expression was similar between treatments on Days 10, 15 and 17 of gestation.

A day × treatment interaction ($P < 0.0004$) was detected for endometrial IGF-IR gene expression (Table 2). IGF-IR mRNA expression was greater on Days 13 and 15 of gestation in E compared with Veh gilts (Table 2). Gene expression between Veh gilts on Days 13 and 15 of gestation was significantly different; however, in E gilts no change was observed between these two days.

Uterine luminal content of IGF-I and IGF-II

Content of IGF-I in the uterine flushings of E and Veh gilts (Fig. 1) was affected by day ($P < 0.01$) and treatment ($P < 0.03$). Uterine luminal IGF-I content in Veh gilts was greatest on Days 10, 12 and 13 (484, 611 and 690 ng respectively) of gestation, which was followed by a four- to sixfold decline on Days 15 (150 ng) and 17 (109 ng) of gestation. The content of IGF-I in uterine flushings from E gilts was similar to Veh gilts on Day 10, but uterine IGF-I content decreased 48–72 h earlier in E gilts ($P < 0.03$). The amount of IGF-I in uterine flushings of E-treated gilts sharply declined on Day 12 and was 10-fold less on Day 13 (60 ng) of gestation compared with Veh (690 ng) gilts. The content of IGF-I in uterine flushings was similar across treatments on Days 15 and 17 of gestation.

A treatment × day interaction ($P < 0.04$) was detected for the content of IGF-II in uterine flushings (Fig. 2). Pregnant gilts treated with oestrogen on Days 9

and 10 of gestation had a sevenfold decrease in uterine luminal content of IGF-II on Day 13 of gestation compared with Veh gilts (52 vs 370 ng). Uterine content of IGF-II was similar on all other days of gestation evaluated in the study.

Ligand blotting

The presence of IGFBPs in the uterine lumen of Veh- and E-treated gilts was evaluated through ligand blot analysis with ¹²⁵I-labelled IGF-I and IGF-II. The ligand blot revealed the presence of two major bands at 46 and 34 kDa (IGFBP-2 and -3) in uterine flushings of Veh gilts on Day 10 of pregnancy but bands were absent on Days 12, 13, 15 or 17 of gestation (Fig. 3). Ligand blot indicated

Figure 1 Content (means ± S.E.M.) of IGF-I in the uterine flushings from Veh-treated (grey bars) and E-treated (black bars) pregnant gilts. A significant day ($P < 0.01$) and treatment ($P < 0.03$) effect was detected. Columns without common superscripts differ.

Figure 2 Content (means $\pm$ S.E.M.) of IGF-II in the uterine flushings from Veh-treated (grey bars) and E-treated (black bars) pregnant gilts. A significant ($P < 0.04$) day by treatment interaction was detected. Columns without common superscripts differ.

that oestrogen treatment caused an earlier loss of IGFBPs as no bands were detected in uterine flushings from any of the days studied.

Discussion

During conceptus trophoblast elongation and oestrogen release to establish pregnancy in the pig, a number of endometrial cytokines and growth factors are released into the uterine lumen to support conceptus development and survival (see review by Geisert & Yelich 1997). The high concentration of IGF-I within the uterine lumen prior to and during rapid trophoblast expansion on Days 10 to 12 of pregnancy is thought to have a direct effect on augmenting conceptus steroidogenesis via enhancement of P450_{arom} gene expression and enhancement of aromatase activity (Ko *et al.* 1994).

The presence of IGF-IIR in the trophoblast (Corps *et al.* 1990) suggests that uterine and/or conceptus IGF secretion can regulate early conceptus development. In the present study, uterine luminal content of IGF-I and IGF-II peaked on the day of conceptus elongation (Days 12 to 13) in Veh gilts. The content of IGF-I and IGF-II in uterine flushings decreased dramatically following conceptus elongation and the initiation of placental attachment to the uterine surface epithelium on Day 15 of gestation. Results from Veh gilts in the present study are consistent with previous publications on luminal uterine IGF content in cyclic and pregnant pigs (Letcher *et al.* 1989, Geisert *et al.* 2001). Although there is a slight decline in endometrial IGF-I

gene expression on Days 12 and 13 of pregnancy, gene expression on Days 15 and 17 returns to expression levels detected on Day 10. Thus, the decrease in IGF-I in uterine flushings following Day 13 is not related to a depression in endometrial IGF-I gene expression.

The high content of IGFs in the uterine lumen of the pig is associated with detection of IGFBPs in the uterine lumen. Insulin-like growth factor binding proteins regulate the biological activity of the IGF ligands *in vivo* (Firth & Baxter 2002). The IGFBPs share structural homology and have a high binding affinity for the IGF ligands. On Day 10 of the oestrous cycle and pregnancy, IGFBP-2 and IGFBP-3 are detected in the uterine luminal fluids of cyclic and pregnant pigs when uterine luminal content of IGF-I and IGF-II are high (Lee *et al.* 1998, Geisert *et al.* 2001). However, there is almost a complete loss of uterine luminal IGFBPs on Day 12 of either the oestrous cycle or pregnancy (Geisert *et al.* 2001). The disappearance of IGFBPs observed in uterine luminal flushings on Day 12 is due to an increase in IGFBP proteolysis rather than to down-regulation of IGFBP mRNA (Lee *et al.* 1998). The proteolysis of IGFBPs in the porcine uterine lumen may occur through activation of serine protease, tissue kallikrein and/or the metalloproteinases (Lee *et al.* 1998, Geisert *et al.* 2001). IGFs are regulated and stabilized through tertiary binding to IGFBPs. The degradation of IGFBPs within the uterine lumen may, in part, be responsible for the decreased content of IGF-I and IGF-II collected in uterine flushings after Day 13. Ballard *et al.* (1991) demonstrated that the normal 10 min half-life of ¹²⁵I-IGF-I could be extended to greater than 15 h when bound to IGFBPs. Furthermore, IGFBP may play a role in prevention of premature binding and signalling of the ligands through the IGF-IR at the cellular level (Conover *et al.* 1990). It is possible that IGFBPs help sequester IGF-I and -II in the uterine lumen for release during the sensitive period of conceptus differentiation and trophoblast elongation. Thus the decline in luminal IGF content following Day 12 does not reflect a lack of endometrial IGF-I gene expression, but rather the loss of IGFBPs to sequester IGFs in the uterine lumen. The spatiotemporal association of uterine IGFs and IGFBPs at the critical period in early porcine conceptus development and the alteration observed following oestrogen administration in the present study suggests that the uterine IGF system serves an important biological role in the establishment and maintenance of pregnancy.

The abundant presence of IGF-I receptors and ligand mRNA appear to parallel each other in the endometrium, indicating that IGFs may have an autocrine role in uterine function during the period of conceptus expansion (Simmen *et al.* 1992). In the current study, endometrial IGF-IR gene expression increased after the decrease in luminal IGFs. Circulating concentrations of IGF-I and IGF-II are generally thought to depress expression of IGF-IR locally (Rosenfeld *et al.* 1982), which may explain the up-regulation of the IGF-IR mRNA on Days 15 and 17. Maintenance of endometrial IGF-I gene expression and loss of

Figure 3 Ligand binding of ¹²⁵I-labelled IGF-I and IGF-II to IGFBPs in uterine flushings of individual gilts collected on selected days of pregnancy (Days 10, 12 and 13 in Veh (V) and oestrogen-treated (E) gilts). Bovine follicular fluid (FF) served as a positive control.

luminal IGFBPs may allow IGFs to stimulate uterine tissue rather than sequestering the growth factors in the lumen during pregnancy.

Although oestrogen plays a major function in regulating the block to luteolysis and uterine changes in secretion and morphology for implantation in the pig, inappropriate exposure to oestrogen (i.e. delivered prior to the normal time of conceptus secretion on Days 11 to 12 of gestation) has a detrimental effect on conceptus survival. Consumption of feed containing the oestrogenic mycotoxin, zearalenone, causes total embryonic loss in swine (Long & Diekmann 1984). Pope *et al.* (1986) first demonstrated that administration of oestrogen to gilts on Days 9 and 10 of gestation resulted in complete embryo mortality before Day 30 of gestation; however, no effect was observed when oestrogen was administered on Days 12 to 13 (time of endogenous conceptus oestrogen secretion). Work in our laboratory demonstrated that premature exposure of the pregnant uterus to oestrogen (on Days 9 and 10) does not affect conceptus elongation on Day 12, but results in conceptus degeneration on Day 15 of pregnancy (Morgan *et al.* 1987, Gries *et al.* 1989). The cause of the early conceptus degeneration following endocrine disruption with oestrogen is not known. Blair *et al.* (1991) indicated that early oestrogen administration causes a loss of the uterine epithelial surface glycocalyx that could interfere with conceptus attachment to the uterine surface. Loss of porcine conceptuses at the time of placental attachment following oestrogen treatment is similar to the implantation failure caused by oestrogen in the mouse. Recently, the concentration and timing of oestrogen stimulation was demonstrated to function within a very narrow range to open the window for uterine receptivity in the mouse (Ma *et al.* 2003). High concentrations of oestrogen shorten the window of receptivity and cause implantation failure as a result of aberrant uterine gene expression during blastocyst attachment. The Ma *et al.* (2003) study provided direct evidence that oestrogen not only opens the window of uterine receptivity in the mouse, but also points to the fact that its concentration and timing are critical for ensuring proper downstream events essential for blastocyst implantation and survival.

Our results indicate that administration of oestrogen to gilts on Days 9 and 10 of pregnancy causes premature proteolysis of uterine luminal IGFBPs. The disappearance of IGFBPs may, in part, be responsible for the early decline of IGF-I and IGF-II ligand on Days 12 and 13 in oestrogen-treated gilts. Corthorn *et al.* (1997) demonstrated that oestrogen stimulated tissue kallikrein activation in the uterine epithelium of the rat. Oestrogen activation of the uterine proteolytic enzymes such as tissue kallikrein and the matrix metalloproteinases that degrade the IGFBPs (Geisert *et al.* 2001) could be the mechanism by which the early porcine conceptus releases IGFs for its development *in utero*. The advanced increase in endometrial IGF-IR gene expression in oestrogen-treated (Day 13) compared with vehicle-treated

(Day 15) gilts is consistent with the early decline of the IGFs in the uterine lumen. The precise nature of the loss of uterine luminal IGFs following conceptus elongation suggests that the release of IGFs during Days 12 and 13 of pregnancy is very critical for subsequent development and survival of pig embryos. Although we cannot demonstrate a causal effect of premature loss of IGFs with later embryonic death from our current study, early oestrogen administration clearly causes a dramatic decline in IGFs before the critical period of conceptus elongation and differentiation. Mice devoid of the IGF-IIR undergo *in utero* mortality during gestation (see review by Jones & Clemmons 1995). Studies have demonstrated that the IGF-IIR is fundamental in embryonic development in the mouse (Barlow *et al.* 1991), and plays a major role in tissue remodelling and translocation of newly synthesized cathepsins to the lysosomes (Dahms *et al.* 1989). Thus, alteration in the normal synchrony of IGF release in the uterine lumen during early pregnancy may cause aberrant endometrial and/or conceptus gene expression during implantation. Microarray analysis of the endometrium from oestrogen-treated gilts has indicated that a number of genes are up- and down-regulated on Day 13 compared with controls (JW Ross, MD Ashworth and RD Geisert, unpublished observations). Therefore, endocrine disruption is not solely limited to the IGF system although IGFs could play roles in the alteration of many other genes as well.

The present study suggests proteolysis of the IGFBPs is clearly an endocrine disrupted event caused by the administration of oestrogen during early pregnancy in the pig. Bioavailability of endometrial IGFs prior to and during conceptus elongation and differentiation may be a fundamental essential for continued development and survival in the pig.

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