Proteolysis of insulin-like growth factor-binding proteins (IGFBPs) within the pig uterine lumen associated with peri-implantation conceptus development

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Pig conceptuses undergo morphological development from spherical to filamentous forms during days 10 to 12 of pregnancy, coincident with a high content of mRNAs encoding insulin-like growth factor (IGF)-I in the uterine endometrium and secretion of IGF-I into the uterine lumen. The potential regulation by developing conceptuses of the bioavailability of IGF-binding proteins (IGFBPs) within the uterine microenvironment was investigated. Uterine luminal flushings (ULFs) were obtained between days 10 and 18 of pregnancy and the presence of specific IGFBPs was determined by ligand blot analysis. ULFs collected at days 10 and 11 of pregnancy contained 46 and 43 kDa IGFBP-3, several IGFBPs of about 30 kDa including IGFBP-2, and an unidentified 26 kDa IGFBP; IGFBP-3 was the most abundant. By day 12, however, IGFBPs were substantially diminished or undetectable. Examination of the morphology of flushed conceptuses revealed that the loss of IGFBPs in ULF was associated with the transition from spherical to filamentous morphology. The abundance of IGFBP-3 mRNA in uterine endometrium, as monitored by blot-hybridization, was not altered in a similar way, suggesting that lack of IGFBP-3 in ‘filamentous’ ULF resulted from proteolysis rather than from decreased expression of the IGFBP-3 gene. Consistent with this, incubation of ‘spherical’ ULF with or without added ‘filamentous’ ULF at 37°C resulted in the disappearance of endogenous IGFBP-3 only in ‘spherical + filamentous’ ULF. The protease activity in ‘filamentous’ ULF was inhibited by EDTA, but unlike matrix metalloproteinases, was not zinc ion-dependent or inhibited by 1,10-phenanthroline. Moreover, this activity was partially inhibited by the serine protease inhibitor aprotinin, but not by 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), a known inhibitor of plasmin. The IGFBP proteolytic activity of ULF may therefore comprise a group of enzymes including an unidentified serine protease. The results suggest that elongating pig conceptuses induce IGFBP proteolytic activity which may increase the intrauterine bioavailability of IGF.

Introduction

Insulin-like growth factors (IGF)-I and -II are evolutionarily conserved regulatory peptides that are believed to play important autocrine, paracrine and endocrine roles in cell division, differentiation, metabolism and gene expression (Jones and Clemmons, 1995). We previously reported that physiological amounts of IGF-I are present in pig uterine luminal fluids (Simmen et al., 1989; Ko et al., 1994a; Green et al., 1995), which peak to maximal concentrations during days 10–12 of pregnancy. During this time, conceptus elongation occurs concomitant with maternal recognition of pregnancy as signalled by conceptus-derived oestrogens (reviewed in Bazer et al., 1991).

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The coincidence of maximal IGF-I secretion and conceptus elongation on the one hand, and the demonstrated stimulatory effect of IGF-I on conceptus aromatase gene expression on the other (Green et al., 1995), provide support for a role for this protein in pig peri-implantation blastocyst development and the establishment of pregnancy (reviewed in Simmen et al., 1995).

IGF-binding proteins (IGFBPs) 1–6 are a group of structurally and functionally related peptides that bind IGF with high affinity and modulate IGF bioavailability and action (Rechler, 1993). IGFBPs usually inhibit IGF action by competing with the IGF receptor for their cognate ligands, although, under certain conditions, IGFBP-3 and its proteolytic fragment can modulate cell growth independently of IGF ligand (Sell et al., 1993; Rechler, 1997; Zadeh and Binoux, 1997). A low-affinity IGFBP, namely IGFBP-7, bearing structural homology to the
classic IGFBindPs was recently described by Oh et al. (1996); however, a biological function similar to those for other IGFBindPs has not been elucidated.

IGFBindPs in many biological fluids and cell conditioned culture media are found to be partially proteolysed (Jones and Clemmons, 1995). Of particular biological significance is the observation that partially proteolysed IGFBindPs lose their high-affinity binding for IGF resulting in enhanced release of bound IGF (Blat et al., 1994; Lassarre and Binoux, 1994; Lee and Rechner, 1996). Previously, we reported that mRNAs encoding IGFBindPs 2–6 are synthesized by pig uterine endometrium (Simmen et al., 1992; Song et al., 1996). In the present study, it is shown that IGFBindP proteins are present in the pig uterine lumen at early pregnancy and that these BPds are proteolysed in close temporal association with conceptus morphological changes occurring at about day 12 of pregnancy.

Materials and Methods

Materials

IGF-I and IGF-II, glycosylated hIGFBindP-3 and rabbit polyclonal antisera to bIGFBindP-2 and hIGFBindP-3 were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit antiserum no. 3695 to rIGFBindP-2 (Romanus et al., 1986) was from M. M. Rechner (NIDDK, Bethesda, MD). Ultraconcentrator Centricon-3 cartridges were obtained from Amicon Co. (Beverly, MA). Immunoprecipitin, a 10% suspension of protein A-coated Staphylococcus aureus, was obtained from Life Technologies (Gaithersburg, MD). [125I]-labelled IGF-II and 14C-labelled Rainbow® molecular mass standards were from Amersham Co. (Arlington Heights, IL). Pre-stained molecular mass standards were from Bio-Rad Laboratories (Hercules, CA). Unstained molecular mass standards, BSA, zinc chloride and aprotonin were from Sigma Chemical Co. (St Louis, MO). 1,10-Phenanthroline was purchased from Genzyme (Boston, MA); 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) and EDTA were from Boehringer Mannheim (Indianapolis, IN).

Animals

Prepuberal gilts (Hampshire × Duroc × Yorkshire) were monitored daily for onset of oestrous activity. Animals exhibiting two consecutive oestrous cycles of normal duration (18–22 days) were mated with boars at oestrus and again 12 and 24 h later. The day of onset of oestrus was defined as day 0. Animals were slaughtered at the university abattoir on the indicated days of pregnancy or of the oestrous cycle. Reproductive tracts were removed, immersed in ice and transferred to a laminar flow hood. Conceptuses were flushed from the uterus and classified according to size, morphology or to both factors and the corresponding endometrium obtained by dissection. Four to six gilts were killed on each day of pregnancy or the oestrous cycle, except on days 11–12 when approximately twenty gilts were slaughtered to obtain luminal flushings from uteri bearing the various morphological stages of conceptuses. Animal use protocols were approved by the University of Florida Institutional Animal Care and Use Committee.

Uterine luminal flushings

For blasts shown in Fig. 1, conceptuses were flushed from each uterine horn with 20 ml of sterile 0.9% (w/v) saline. For blasts shown in Fig. 2, conceptuses were flushed from each uterine horn with 20 ml of sterile phosphate-buffered saline (PBS: 8.0 g NaCl, 200 mg KCl, 1.44 g Na2HPO4, 240 mg KH2PO4, l l−1, pH 7.4). Flushings were transferred to 50 ml conical tubes and cleared of conceptus debris by low speed centrifugation at 4°C for 10 min. Flushings were stored at −80°C until used. Uterine luminal flushings (ULF) were classified according to conceptus size (largest diameter) and morphology as follows: small spherical, all conceptuses ≤5 mm; large spherical, all conceptuses > 5 mm but ≤10 mm; transitional, all conceptuses > 10 mm but ≤35 mm; filamentous, all conceptuses > 35 mm. ULF from uteri bearing conceptuses of heterogeneous morphology were not used in this study. Pools, 50 ml of ULF from ‘spherical’ (small and large) and ‘filamentous’ bearing uteri were generated by mixing equal volumes of ULF from seven and three individual gilts, respectively. Subsequently, the ‘spherical’ and ‘filamentous’ pools were concentrated 10-fold with Centricon-3 cartridges.

Ligand blotting

Ligand blotting was performed as previously described (Lee et al., 1991; Ko et al., 1994b). One millilitre of an individual ULF corresponding to day 10, 11, 12, 15 or 18 of pregnancy (Ko et al., 1994a) was dialysed against 30 mmol sodium phosphate buffer 1 l−1 (pH 7.4), lyophilized and reconstituted in 50 µl H2O. One half was subjected to the ligand blot procedure using [125I]-labelled IGF-II that had been prepared in these laboratories (Lee et al., 1991; Ko et al., 1994b).

In a subsequent experiment, individual ULF samples were classified according to size, morphology or both factors of flushed conceptuses and concentrated tenfold using Centricon-3 cartridges. An aliquot (40 µl) of concentrated ULF was subjected to ligand blotting using [125I]-labelled IGF-II prepared by Amersham Co. or [125I]-labelled IGF-II prepared in our laboratories. No differences were observed in the binding of [125I]-labelled IGF-II from Amersham Co. and that prepared in our laboratory.

Immunoprecipitation and immunoblotting

Individual IGFBindP species were identified by incubating 0.1–0.25 ml of concentrated pooled ‘spherical’ ULF overnight at 4°C with 5 µl rabbit antisera no. 3695 to rIGFBindP-2, rabbit antisera to hIGFBindP-3, or nonimmune rabbit serum. Immune complexes were collected with immunoprecipitin followed by ligand blotting as described by Yang and Rechner (1993) and Lee et al. (1997). In addition, individual ULFs classified
according to conceptus morphology were subjected to western blot analysis using rabbit antiserum to bovine IGFBP-2 and \[^{125}I\]-labelled protein A.

RNA dot blot hybridization

Extraction of endometrial RNA, preparation and hybridization of RNA dot blots, and generation of IGFBP-3 hybridization probe were as described by Song et al. (1996). The probe was a full-length cDNA encoding human IGFBP-3 (Wood et al., 1988), previously validated for pig tissue RNA by northern hybridization (Lee et al., 1993; Song et al., 1996). RNA dot-blotss were subjected to autoradiography and phosphorimage analysis (Green et al., 1995, 1996).

Assay of IGFBP protease activity in ULF

IGFBP protease activity in ULF was examined by ligand blotting after incubation at 37°C. The ‘filamentous’ ULF (20 µl) was preincubated for 3 h at 37°C in the absence or presence of 5 mmol AEBSP \(1^{-1}\) (serine protease inhibitor), EDTA or 1 mmol 1,10-phenanthroline \(1^{-1}\) (metal ion chelator) (30 µl total volume, in 50 mmol PBS \(1^{-1}\)) after which ‘spherical’ ULF (20 µl) was added and the mixture further incubated at 37°C for 18 h, followed by ligand blotting. In a second approach, glycosylated hIGFBP-3 was added to ‘filamentous’ ULF that had been preincubated for 3 h at 37°C in the absence or presence of protease inhibitors or activators, and the mixture was further incubated for 18 h at 37°C, followed by ligand blotting with \[^{125}I\]-labelled IGF-II.

Statistical analysis

Phosphorimage data were subjected to least-squares ANOVA using the General Linear Models procedures of the Statistical Analysis System (SAS, 1985) as described by Green et al. (1995, 1996). Effects of pregnancy and conceptus morphology were evaluated by orthogonal contrasts.

Results

IGF-binding proteins in pregnant pig uterine luminal flushes

ULFs collected at defined days of the peri-implantation period were examined for IGF-binding proteins by use of the ligand blot technique. ULFs corresponding to days 10 and 11 of pregnancy contained an easily detectable IGFBP doublet co-migrating with the 46 kDa pre-stained molecular mass standard, a triplet with a molecular mass between 30 and 34 kDa and a 26 kDa IGFBP (Fig. 1). Of the IGFBPs detected, the 46 kDa IGFBP doublet was the most abundant in all samples. ULFs representing days 12, 15 and 18 of pregnancy had much less or no detectable IGFBPs (Fig. 1).

For determining whether the observed decline in ULF IGFBPs at day 12 of pregnancy was coincident with one or more morphological stages of conceptus development, ULFs from a second group of pregnant pigs were classified according to morphology of flushed conceptuses rather than day of pregnancy and examined for IGFBPs. This group of ULFs spanned days 10.5 to 12 of pregnancy and contained conceptuses representing small spherical, large spherical, transitional (ovoid–tubular) and early filamentous morphological stages. Upon ligand blotting, small and large ‘spherical’ ULFs predominately exhibited the 46 and 43 kDa IGFBP-3 and small amounts of 34 kDa IGFBP-2 and IGFBPs of about 30 kDa (Fig. 2). Of the known IGFBPs, only IGFBP-3 has a molecular mass greater than 40 kDa. Although the 46 and 43 kDa IGFBP-3 glycosylation variants were not recognized by the hIGFBP-3 antiserum owing to lack of antibody crossreactivity (data not shown), this IGFBP doublet in pig serum was previously identified as IGFBP-3 using homologous antiserum (Coleman and Etherton, 1991; Lee et al., 1991). The 34 kDa IGFBP and an additional IGFBP of about 30 kDa in ULF were recognized by rIGFBP-2 antiserum no. 3695 in immunoprecipitation and by hIGFBP-2 antiserum in Western blotting (data not shown). One of the IGFBPs of about 30 kDa, therefore, represents a truncated IGFBP-2 protein. These IGFBPs were diminished or absent in ULFs containing transitional conceptuses and were completely absent in those containing conceptuses at the filamentous stage. Thus, disappearance of IGFBPs in ULF is temporally associated with transition to the filamentous morphological stage.

Abundance of endometrial mRNA encoding IGFBP-3

For examining whether the observed alterations in IGFBP-3 content of ULF associated with conceptus development were a consequence of changes in endometrial IGFBP-3 gene expression, a dot-blot containing endometrial RNAs from day 12 cyclic pigs, day 12 pregnant pigs containing spherical conceptuses and day 12 pregnant pigs containing filamentous conceptuses was hybridized with a radiolabelled IGFBP-3 cDNA probe. There were no significant differences in the abundance of steady-state IGFBP-3 mRNA for uterine endometrium among the physiological or developmental stages examined (Fig. 3). Therefore, the rapid changes in uterine luminal IGFBP-3 content, occurring in concert with conceptus morphological development, are due to mechanisms other than altered endometrial IGFBP-3 mRNA synthesis or degradation.

A cation-dependent IGFBP protease activity in ‘filamentous’, but not ‘spherical’, ULF

The absence of any detectable IGFBP-3 in ‘filamentous’ ULF despite constitutive endometrial IGFBP-3 mRNA synthesis, suggested that there is an IGFBP protease activity in this fluid. This was examined by incubation of ‘spherical’ ULF at 37°C with or without added ‘filamentous’ ULF, followed by ligand blotting. The amount of endogenous IGFBP-3 in ‘spherical’ ULF was not altered by incubation at 37°C (Fig. 4, lanes 2 versus 3), suggesting that ‘spherical’ fluid lacks endogenous IGFBP protease activity. However, IGFBP-3 in ‘spherical’ ULF disappeared after co-incubation with ‘filamentous’ ULF (Fig. 4, lanes 4 versus 5), indicating that the latter contains a protease activity for IGFBPs. This activity was partially inhibited by the metal ion chelator EDTA (Fig. 4, lane 7), but not by another...
Fig. 1. Insulin-like growth factor (IGF)-binding proteins in peri-implantation uterine luminal flushes (ULFs) from pigs. Equivalent volumes of reconstituted ULFs were subjected to the ligand blot procedure using [\textsuperscript{125}I]-labelled IGF-II. Each lane represents ULF from an individual uterus collected at the indicated day of pregnancy ($n = 3$ for each day in both blots, except for day 18 in both blots ($n = 2$) and day 12 in lower blot ($n = 2$)). Each blot represents a different group of animals. Pre-stained molecular mass standards were BSA (66 kDa), ovalbumin (46 kDa) and carbonic anhydrase (30 kDa).
metal ion chelator 1,10-phenanthroline (Fig. 4, lane 8) or by the serine protease inhibitor AEBSF (Fig. 4, lane 6).

Further characterization of the IGFBP protease activity in 'filamentous' ULF using exogenous hIGFBP-3 as substrate indicated that it was minimally inhibited by the serine protease inhibitor, aprotinin (Fig. 5, lane 4) and strongly by EDTA (Fig. 5, lane 5). However, unlike members of the matrix metalloproteinase (MMP) family (Salvesen and Nagase, 1989), the IGFBP protease activity inhibited by EDTA was not reactivated by addition of zinc ion (Fig. 5, lanes 5 versus 6) and was not inhibited by addition of tissue inhibitor of metalloproteinase-1 (TIMP-1) (data not shown).

Discussion

This study extends previous investigations, by our laboratories, of the pig uterine IGF system during pregnancy (Simmen et al., 1995). A striking result was the observed disappearance of high-affinity IGFBPs from ULF between days 11 and 12 of pregnancy coincident with the well-defined transition in pig conceptus morphology. These proteins can now be added to the expanding group of pig uterine endometrial or conceptus-derived proteins that exhibit significant changes either in tissue content or uterine luminal concentrations during the narrow window encompassing conceptus elongation (Fazleabas et al., 1983; Zavy et al., 1984; Simmen et al., 1989, 1990, 1992; Roberts et al., 1993; Ko et al., 1994a; Green et al., 1995, 1996; Choi et al., 1996; Yelich et al., 1997).

The disappearance of high-affinity IGFBPs from ULF, coincident with morphological changes of the conceptus, most likely resulted from IGFBP-specific proteolysis within the lumen. In support of this, we observed constitutive synthesis of mRNA encoding IGFBP-3 in uterine endometrium during the 'spherical' to 'filamentous' transition and an IGFBP protease
activity in ‘filamentous’ but not ‘spherical’ ULF using hIGFBP-3 or ‘spherical’ ULF IGFBPs as exogenous substrates, respectively. These observations suggest the involvement of conceptus-secreted oestrogens in the luminal disappearance of IGFBP-3, but not in endometrial IGFBP-3 RNA synthesis or degradation, as might be speculated from the down-regulation by oestrogen of IGFBP-3 mRNA and protein synthesis in rat uterus (Yallampalli et al., 1993; Huynh and Pollak, 1994).

An expected consequence of IGFBP proteolysis between days 11 and 12 of pregnancy is increased intrauterine bioavailability of IGFs and accordingly, IGF action. Proteolysis of IGFBPs is usually accompanied by enhanced release of bound IGFs (Cohick et al., 1993; Lassarre and Binoux, 1994; Lee and Rechler, 1996) and, therefore, is regarded as an IGF release mechanism (Giudice, 1995). Furthermore, Blat et al. (1994) have demonstrated that as a consequence of the enhanced release of IGF from partially proteolysed IGFBP-3, human pregnancy serum has greater IGF mitogenic activity than non-pregnancy serum. In a similar and more relevant context, we interpret our previously observed inhibitory effect of IGFBPs on IGF-I-stimulated mitogenesis in HRE-H9 rabbit endometrial cells (Ko et al., 1994b) to suggest that removal of IGFBPs via proteolysis is likely to increase the intrauterine bioavailability and actions of the IGF. It is also apparent that partially proteolysed IGFBPs
ULF with filamentous conceptuses is characterized by maximal IGF-I content (Green et al., 1995) coincident with loss of IGFBP activity as monitored by ligand blotting. This probably allows IGF-I to be present in the readily available free form during this period. The biological consequences of this increased luminal IGF-I bioavailability, however, are unknown. IGF-I may enhance oestrogen synthesis and secretion by conceptuses; this is consistent with the observation that this growth factor increases the abundance of aromatase mRNA by filamentous but not by spherical conceptuses (Green et al., 1995). It is also possible that IGF-I, with its significant mitogenic activity, promotes conceptus trophoblast or uterine epithelial hyperplasia (Ko et al., 1994b).

The IGFBP protease activity in pig ULF described here appears distinct from those previously described for other biological fluids such as serum (Fowlkes et al., 1994a; Lee and Rechler, 1996) and follicular fluid (Bensard et al., 1996, 1997). The partial inhibition of this protease activity by aprotinin suggests that a serine protease may be responsible, at least in part, for this activity. In this regard, plasmin may be involved, based on the active secretion of plasminogen activator (PA) by elongating pig conceptuses (Fazleabas et al., 1983), inhibition of PA by aprotinin (Lottenberg et al., 1988), the presence of plasminogen in the uterine lumen at this developmental time (Fazleabas et al., 1983), and the known IGFBP-3 proteolytic activity of plasmin (Campbell et al., 1993; Lalou et al., 1994). However, the inability of AEBSF, a known inhibitor of plasmin (Angelloz-Nicoud and Binoux, 1995), to inhibit the ULF protease activity is not consistent with the plasminogen–plasmin activator system. Furthermore, unlike matrix metalloproteinases (Salvesen and Nagase, 1989) that can degrade IGFBP-3 in other biological fluids (Fowlkes et al., 1994a,b; Bensard et al., 1996, 1997), the ULF IGFBP-3 protease activity was not inhibited by 1,10-phenanthroline or tissue inhibitor of metalloproteinase-1, and furthermore was not zinc ion-dependent. These results, taken together with the observed inhibition by EDTA, suggest that the ULF IGFBP protease activity may represent a group of cation-dependent enzymes including a serine protease(s).

The pig uterine microenvironment at peri-implantation contains an abundance of proteases and their corresponding inhibitors that may modulate the extent of protein degradation within the uterine lumen and the invasiveness of trophoblast (Fazleabas et al., 1982; Zavy et al., 1984; Simmen et al., 1991; Roberts et al., 1993). The present results implicate uterine luminal proteases (and by association their inhibitors) in the regulation of IGF availability and, therefore, IGF actions at the embryo–maternal interface. A similar system of IGFBPs, IGFBP proteases and protease inhibitors may be operative within the uterine lumen of early pregnant sheep (Ledgard et al., 1995), suggesting a general role for the IGF system in peri-implantation events of domestic ungulates. Further studies of IGFBP proteases and the biological consequences of their action(s) during peri-implantation development are necessary to clarify the regulation and function of the intrauterine IGF system.

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