Uterotrophic effects of relaxin related to age and estrogen receptor activation in neonatal pigs

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Abstract

While uterotrophic effects of relaxin are well documented, the mechanism through which relaxin promotes uterine growth is incompletely understood. Studies in rats suggest that relaxin-stimulated uterine edema depends on estrogen receptor (ER) activation. Here, neonatal pigs were used to investigate the interaction between relaxin and ER signaling pathways. Gilts were treated either at birth (postnatal day (PND) 0) (study 1) before the onset of endometrial ERα expression, or on PND 12 (study 2) after the onset of ERα expression. In study 1, gilts were treated with estradiol-17β or porcine relaxin for two days and uteri were collected on PND 2. In study 2, PND 12 gilts were treated with a single injection of the ER antagonist ICI 182,780 (ICI) or vehicle. Two hours later, gilts were given either estradiol-17β or porcine relaxin for two days. When administered for two days from birth (study 1), neither estradiol-17β nor relaxin affected uterine weight or protein content. However, uterine luminal epithelial height was greater in relaxin- than in vehicle-treated gilts. In contrast, in study 2, both estradiol and relaxin increased uterine weight, protein content and uterine luminal epithelial height on PND 14. These effects were inhibited by pre-treatment with ICI in both estradiol- and relaxin-treated gilts. The results indicate that uterotrophic effects of relaxin in the neonatal pig are related to age and to both the relative presence and state of activation of the ER system in developing uterine tissues between birth and PND 14.

Introduction

Relaxin, a 6000 Da polypeptide hormone, promotes growth of reproductive tissues. Uterotrophic actions of relaxin, first described in the rat (Steinetz et al. 1957), include both uterine water imbibition (Steinetz et al. 1957, Zarrow & Brennan 1957) and increased uterine dry matter content associated with protein, collagen and glycogen accretion (Steinetz et al. 1957, Vasilenko et al. 1980, 1981, Vasilenko & Mead 1987). Early studies indicating that relaxin-rich extracts promoted endometrial growth in juvenile and ovariectomized rhesus monkeys (Hisaw & Hisaw 1964, Dallenbach-Hellweg & Hisaw 1966) were later confirmed by work involving the purified hormone (Goldsmith et al. 2004), which showed that relaxin also stimulated endometrial angiogenesis. Consistently, relaxin administered in the peri-implantation period increased endometrial thickness in the macaque (Hayes et al. 2004). Relaxin also stimulated uterine remodeling and growth in prepubertal (Hall et al. 1990), pregnant (Galvin et al. 1991, Min et al. 1997) and ovariectomized, steroid-treated prepubertal pigs (Hall et al. 1992).

Mechanisms regulating uterotrophic effects of relaxin are unclear. However, it is clear that some of these effects mimic those induced by estrogen. For example, like estradiol, relaxin increased expression of uterine insulin-like growth factor-I (Ohleth et al. 1997, Kowalski et al. 2004), gap junctions (Risek et al. 1995, Lenhart et al. 1999), vascular endothelial growth factor (Cullinan-Bove & Koos 1993, Unemori et al. 1999), and uterine vascularization (Vasilenko & Mead 1987, Cullinan-Bove & Koos 1993). Estrogen receptors, which function classically as hormone-regulated transcription factors, can be activated both directly by estrogen and indirectly via crosstalk with peptide growth factor signaling pathways (Ignar-Trowbridge et al. 1992, Ma et al. 1994, Smith 1998). Crosstalk involving estrogen-independent, growth factor-dependent estrogen receptor (ER) activation is well documented (Smith 1998). Data indicating that pretreatment of ovariectomized rats with the ER antagonist ICI 182,780...
inhibited relaxin-induced uterine edema were interpreted to indicate that relaxin could be acting, in part, through crosstalk with the ER system (Pillai et al. 1999). Similar effects have not been documented in other species. Moreover, the extent to which uterotrophic effects of relaxin may be affected by the relative presence and/or state of ER activation in developing uterine tissues remains undefined.

In the pig, uterine development between birth (postnatal day (PND) 0) and PND 15 involves differentiation of endometrial glandular epithelium (GE) from luminal epithelium (LE) and proliferation of GE through endometrial stroma towards the developing myometrium (Tarleton et al. 1999, Bartol et al. 2006). Porcine endometrial ER architecture, defined in terms of the spatial relationships between ER-positive and ER-negative stromal and epithelial cells in situ (Tarleton et al. 2001), changes dramatically during this period. Uterine tissues are ERα-negative at birth. However, differentiation of GE from LE shortly after birth is marked by the onset of ERα expression in endometrial stroma and nascent GE (Tarleton et al. 1998). Additionally, inhibition of uterine wall development and endometrial gland genesis by administration of ICI 182,780 to gilts from birth through PND 13 (Tarleton et al. 1999) showed that ERα is not only a marker, but is also a mediator of endometrial development during this period. Transient ERα activation in neonatal gilts, induced by administration of estrogen for 14 days from birth, altered endometrial function and compromised uterine capacity for conceptus support in adults (Tarleton et al. 2003). Uterine responses to estrogen in neonatal gilts were exposure period-specific and related directly to endometrial ER architecture characteristic of the period during which exposure occurred (Spencer et al. 1993, Tarleton et al. 2001). Collectively, data indicate that ER-dependent developmental events characteristic of the period from birth through PND 14 are critical determinants of porcine uterine capacity. Available data can be interpreted to suggest that ERα expression patterns characteristic of the period from PND 0 through PND 14 may affect the nature of uterine tissue responses to growth factors capable of activating the ER. Recent preliminary data indicate that the neonatal porcine uterus is relaxin receptor (LGR7)-positive throughout this period (Wiley et al. 2003, Bagnell et al. 2005, Bartol et al. 2006). Thus, to the extent that the effects of relaxin may be mediated, in part, via crosstalk between LGR7 and ER signaling systems, the neonatal porcine uterus provides a valuable model with which to study these relationships, particularly as they pertain to uterine growth and endometrial programming (Bartol et al. 2006). Therefore, the objectives of this study were to determine if the effects of relaxin could be related functionally to the relative presence and/or state of activation of the ER system when administered either before or after the onset of ERα expression in the neonatal porcine uterus.

Materials and Methods

Materials

Purified porcine relaxin (CM-A fraction; 3000 U/mg) was prepared at the Department of Biomedical Sciences (University of Guelph, ON, Canada) by extraction and purification from ovaries of pregnant sows (Sherwood & O’Byrne 1974). Purity was confirmed by SDS-PAGE, which revealed a single band at approximately 6.2 kDa. The biological activity of the relaxin preparation was ascertained by inhibition of spontaneous uterine motility in vitro (Wiqvist & Paul 1958), and immunoreactivity was verified by RIA (Porter et al. 1992). Estradiol-17β was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). ICI 182,780 was kindly provided by Dr Alan Wakeling, Astra Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK). Mouse anti-human ERα (Ab-15) monoclonal antibody was purchased from NeoMarkers, Inc. (Fremont, CA, USA). Horseradish peroxidase-conjugated anti-mouse secondary antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Nitrocellulose membranes were obtained from Bio-Rad Laboratories (Hercules, CA, USA). The Renaissance Western Blot Chemiluminescence Reagent Plus kit was obtained from Perkin Elmer Life Sciences (Boston, MA, USA). X-Omatic films were purchased from Eastman Kodak Company (Rochester, NY, USA). All other chemicals were purchased from Sigma-Aldrich Co. and Invitrogen (Carlsbad, CA, USA), unless otherwise specified.

Animals

Yorkshire-Landrace gilts from the Swine Unit of the New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, NJ, USA and the Mississippi Agricultural and Forestry Experiment Station, Mississippi State University, Starkville, MS, USA were used in these studies. In study 1, PND 0 gilts were assigned to one of three treatment groups as follows: (1) control (C; vehicle alone; DMSO:ethanol (ETOH), 4:1 and PBS; n = 6); (2) estradiol-17β (50 µg/kg body weight (BW), in DMSO:ETOH, 4:1 vehicle; given i.p. every 24 h for 48 h; n = 7); (3) porcine relaxin (20 µg/kg BW, in PBS; given i.m. every 6 h for 48 h; n = 8). In study 2, gilts were randomly assigned to begin one of six treatments on PND 12, as follows: (1) control (C; vehicle alone; n = 7); (2) ICI 182,780 (ICI; 1 mg/kg BW, given i.p. in DMSO:ETOH, 4:1 vehicle; given as a single injection 2 h prior to start of hormone treatment; n = 5); (3) estradiol-17β (50 µg/kg BW, i.p., every 24 h for 48 h; n = 5); (4) ICI/estradiol (n = 5); (5) porcine relaxin (20 µg/kg BW, given i.m. every 6 h for 48 h; n = 6); and (6) ICI/relaxin (n = 10). Timing, dosage and route of ICI administration were based on studies in rodents (Gibson et al. 1991, Pillai et al. 1999), while those for relaxin administration were based on studies in prepubertal gilts and rodents (Hall et al. 1990, Pillai et al. 1999). In both studies, gilts were weighed...
Protein extraction and evaluation of uterine ER expression status

Protein content of uterine tissue was determined using procedures described previously (Ryan et al. 1996). Uterine tissues were homogenized in 5 volumes (1 g/5 ml) boiling lysis buffer (1% SDS, 10 mM Tris, 1 mM CaCl₂, pH 7.4) followed by heating in a microwave (2 cycles of 5–7 s at high power). Samples were then sonicated and centrifuged (12 000 g, 4 °C) for 30 min. Protein concentration was measured using a detergent-compatible protein assay kit (DC Protein Assay, Bio-Rad Laboratories). Uterine protein content was calculated based on the protein concentration and wet weight of the uterus.

To document ERα expression status, uterine proteins (30 μg) obtained from control gilts on PND 0, 2 and 14 were resolved on 12.0% Bis-Tris–HCl-buffered polyacrylamide electrophoresis gels under reducing conditions and transferred onto nitrocellulose membranes. After blocking in 10.0% nonfat dry milk in Tris–HCl-buffered saline containing Tween-20 (TBST; 25 mM Tris (pH 7.5), 0.14 mM sodium chloride (NaCl), 3 mM potassium chloride (KCl), 0.05% Tween-20), membranes were incubated with mouse anti-human ERα antibody (1:100) overnight at 4 °C. After washing with TBST, blots were incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (1:2000) for 1 h at room temperature, and bound antibodies were detected by enhanced chemiluminescence on film. Efforts to identify ERβ in neonatal porcine uterine tissue, using the same immunoblotting procedures, failed to reveal evidence for ERβ expression at the protein level.

Histology

Paraffin-embedded uteri were processed for morphometric analysis as described previously (Lenhart et al. 1998). Uterine tissue sections (~5 μm thick) were stained with hematoxylin and eosin and mounted using Permount solution. Assessment of uterine luminal epithelial growth was determined by measuring the height of the epithelial layer from the apical surface to the basal surface adjacent to the stroma using an ocular micrometer with bright field microscopy (Ryan et al. 2001). Uterine tissues from 3–5 animals per treatment group were randomly selected and processed for morphometric analysis. For each animal, three sections from the mid-portion of one uterine horn were analyzed. Ten random sites were measured on each section.

Statistical analysis

All statistical analyses were performed using General Linear Models procedures (SAS 1989). For study 1, uterine wet weight and protein content data obtained from gilts on PND 2 were subjected to one-way analysis of variance in order to determine effects of treatment, using body weight as a covariate. In study 2, uterine wet weight and protein content data obtained from gilts on PND 14 were subjected to analyses of variance for a 2 × 3 factorial design. The statistical model considered variation associated with the main effects of ICI pre-treatment, hormone treatment and their interactions, with body weight as a covariate in all analyses. For analysis of uterine luminal epithelial height, data were evaluated by analysis of variance for a completely nested design. When appropriate, mean comparisons were performed using the Student-Newman-Keuls test for multiple comparisons and P values are provided in the text. All quantitative data are expressed as least square means ± standard errors (LSM ± s.e.m.).

Results

Confirmation of uterine ER expression status

Results of an immunoblot analysis of ERα expression in uterine tissues obtained from control gilts on PND 0, 2 and 14 are presented in Fig. 1. Analyses confirmed, as anticipated, that ERα protein was undetectable on PND 0. In contrast, a single 67 kDa band corresponding to ERα protein was detectable for tissues obtained on both PND 2 and PND 14. The results establish that treatments were administered beginning prior to onset of ERα expression in study 1 and after the onset of ERα expression in study 2.

Study 1 – effects of relaxin and estradiol-17β on uterine growth responses at PND 2

When administered from birth, neither estradiol nor relaxin affected uterine wet weight (Fig. 2) or protein content (data not shown) as determined on PND 2. Representative photomicrographs of the uterine wall illustrating

![Figure 1](https://www.reproduction-online.org)
the effects of relaxin and estrogen administered from birth on uterine LE height at PND 2 are shown in Fig. 3A. Morphometric analyses showed that both relaxin and estradiol treatment increased ($P < 0.001$) uterine LE height as compared with controls (Fig. 3B). Moreover, LE height in relaxin-treated gilts was greater than that observed in estradiol-treated gilts (relaxin: $39.2 \pm 0.4$ vs estradiol: $28.6 \pm 0.2$ mm; $P < 0.001$).

Study 2 – effects of relaxin, estradiol-17β, and ICI 182,780 on uterine growth responses at PND 14

Data illustrating the effects of relaxin and estradiol administered for two days from PND 12 on uterine weight in gilts on PND 14, both with and without pre-treatment with ICI, are presented in Fig. 4A. In contrast with results for study 1, administration of estradiol daily for two days increased uterine weight on PND 14 relative to controls (estradiol: $1747.0 \pm 107.8$ vs control: $924.6 \pm 104.4$ mg; $P < 0.001$). Likewise, relaxin treatment increased uterine weight ($1440.3 \pm 97.2$ mg; $P < 0.001$) to a level comparable to that in estradiol-treated gilts. Pre-treatment with ICI, the ER antagonist, inhibited uterine weight gain in
response to both estradiol ($P < 0.001$) and relaxin ($P = 0.01$) in PND 14 gilts. When administered alone, ICI did not affect uterine weight.

Data for uterine protein content on PND 14 are summarized in Fig. 4B. Again, both estradiol ($P < 0.001$) and relaxin ($P < 0.001$) treatment increased uterine protein content on PND 14 when compared with controls (control: $36.3 \pm 6.1$ mg; relaxin: $63.4 \pm 5.4$ mg; estradiol: $76.5 \pm 6.7$ mg). Administered alone, ICI pretreatment did not affect uterine protein content. However, pretreatment with ICI inhibited the effects of both estradiol ($P = 0.001$) and relaxin ($P = 0.008$).

Representative photomicrographs of the uterine wall illustrating the effects of relaxin, estradiol, and ICI, administered alone and in combination from PND 12, on uterine LE height at PND 14 are shown in Fig. 5A. Morphometric analyses showed that relaxin ($P < 0.001$), but not estradiol, increased uterine LE height when compared with vehicle-treated controls. Additionally, an interaction between hormone treatment and ICI pretreatment was detected (Fig. 5B). When administered alone, ICI decreased ($P < 0.001$) LE height. Pretreatment with ICI inhibited ($P < 0.001$) relaxin-induced effects on LE height and, as might be expected, also reduced ($P < 0.001$) LE height when administered in advance of estrogen.

**Discussion**

Although uterotrophic effects of relaxin are well documented, the mechanism through which relaxin promotes uterine growth remains elusive. This study was designed to investigate uterotrophic effects of relaxin when administered beginning either before or after the onset of ER$\alpha$ expression in the neonatal porcine uterus. Recently, evidence was presented for low uterine ER$\beta$ gene expression in adult porcine uterine tissues detected by quantitative PCR (Cardenas & Pope 2004). Here, immunoblot analysis failed to reveal evidence for ER$\beta$ expression at the protein level in neonatal uterine tissues. However, results showed clearly that uterine ER$\alpha$ protein, undetectable by immunoblot analysis on PND 0, was present in tissues obtained on PND 2 and PND 14.

The data are consistent with earlier studies (Tarleton et al. 1998), in which temporospatial patterns of ER$\alpha$ expression were determined for porcine uterine tissues from birth through PND 15 using in situ hybridization and immunohistochemical techniques. When treatment began before the onset of ER$\alpha$ expression (PND 0), relaxin increased uterine LE height but had no effect on either uterine wet weight or protein content on PND 2. However, when treatment began after the onset of ER$\alpha$ expression (PND 12), relaxin increased all three measures of uterine growth on PND 14. Thus, the uterotrophic effects of relaxin were age-specific and more pronounced, in terms of the range of responses measured, when treatments were initiated after the onset of uterine ER$\alpha$ expression. The age-specific effects of relaxin observed here were similar to those observed for estrogen (Tarleton et al. 2001). Moreover, pretreatment of gilts with the ER antagonist ICI 182,780 inhibited tropic effects of relaxin on PND 14. Collectively, these studies suggest that the age-specific, uterotrophic effects of relaxin are developmentally regulated and that the effects of relaxin in this system are dependent, in part, on ER activation. These findings agree with research in rats, involving a similar experimental approach with ICI 182,780, in which relaxin-stimulated uterine edema was interpreted to be ER-dependent (Pillai et al. 1999).

The present data not only support the idea that relaxin can act, in part, through the ER signaling system, but reinforce the idea that ER$\alpha$ activation is necessary to facilitate the uterotrophic effects of relaxin. In the rat, uterotrophic effects of relaxin were observed with or without prior estrogen priming (Vasilenko et al. 1980, Adams et al. 1989), although priming enhanced relaxin effects (Adams et al. 1989). Similar relationships were described for the pig (Hall et al. 1990, 1992, Galvin et al. 1991, Ohleth et al. 1997). Additionally, data generated in ovariectomized, pubertal gilts (Zaleski et al. 1995) indicating that ten days of relaxin treatment failed to promote uterine growth in the absence of ovarian steroids (estrogen or progesterone), support the view that uterotrophic actions of relaxin are at least facilitated by steroid hormone receptor
activation. Studies involving the MCF-7 breast cancer cell line showed that relaxin could stimulate expression of an estrogen response element-luciferase reporter gene (Koos & Pillai 2001), indicating that signaling pathways affected by relaxin can also activate the ER in the absence of estrogen.

The fact that relaxin treatments initiated both prior to and after the onset of endometrial ERα expression increases uterine LE height on PND 2 (study 1) and PND 14 (study 2) indicates that the porcine endometrium is relaxin-sensitive at or very shortly after birth and that endometrial relaxin receptor (LGR7) expression should be expected during this period. Tropic effects of relaxin on LE height reported here agree with earlier studies from our laboratory involving older prepubertal gilts (Ryan et al. 2001). Data indicating that cervical and vaginal luminal epithelia remained atrophic in pregnant, relaxin-null as compared with wild-type mice (Zhao et al. 2000) support a role for LGR7-mediated signaling in LE development. Specific, saturable relaxin binding sites were demonstrated in uterine LE cells of pregnant gilts (Min & Sherwood 1996). However, direct evidence of porcine uterine LGR7 expression was documented only recently in neonatal gilts, with pronounced uterine stromal LGR7 mRNA observed from birth through PND 14 (Wiley et al. 2003, Bartol et al. 2006). Studies in human and primate endometria showed specific immunolocalization of LGR7 in uterine stroma underlying LE cells (Ivell et al. 2003), a pattern also observed in the neonatal pig by PND 14 (Bartol et al. 2006). Thus, relaxin may act through its cognate receptor to promote uterine and LE growth. Still, the fact that relaxin-induced increases in LE height in PND 14 gilts were blocked by pretreatment with ICI 182,780 indicates that such effects involved ER activation. These data, taken together with evidence of uterine LGR7 expression during an established critical period for ERα-dependent, porcine uterine development (Tarleton et al. 2003), makes the neonatal gilt a valuable model with which to investigate crosstalk between LGR7 and ER signaling systems. Evidence that LGR7 is expressed by uterine tissues from birth and that the neonatal porcine uterus responds to exogenously administered relaxin raises the question of whether there is a natural source of relaxin in the neonatal pig. Studies in other species indicate that relaxin of maternal origin could be an important source of the hormone in the neonate by way of ingestion of colostrum and milk. Relaxin is detectable in human (Eddie et al. 1989) and canine (Goldschmidt et al. 1994) colostrum and milk, and mammary tissue has been suggested as a local source of relaxin (Tashima et al. 1994). Like other peptide growth factors in milk, relaxin may contribute to early neonatal development (Burrin et al. 1997, Anderson et al. 1999). Preliminary data from this laboratory indicates that bioactive relaxin is present in porcine colostrum (Yan et al. 2005). Relationships between suckling, relaxin delivery to the neonatal circulation and reproductive tract development are under investigation.

In conclusion, data presented here indicate that neonatal uterotropic responses to relaxin in pigs are developmentally regulated and can be related functionally to the relative presence and/or state of activation of the ER system. These findings support the hypothesis that activation of ERα is important for the uterine growth-promoting actions of relaxin. These studies provide a foundation for further research using the neonatal gilt as a model to study relaxin/ER interactions. The extent to which recently characterized G-protein coupled receptor 30 mediated responses to estrogen (Revankar et al. 2005, Thomas et al. 2005) may be involved in neonatal uterine responses to either estrogen or relaxin remains to be investigated.

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References


Reproduction antagonist.

signaling pathways - epidermal growth-factor action involves the


junction protein expression during follicular development in the

SK-ER3.

growth and differentiation of the human neuroblastoma cell line

Insulin-like growth factors activate estrogen receptor to control the

Biology of Reproduction 42 769–774.


Pillai SB, Rockwell LC, Sherwood OD & Koos RD 1999 Relaxin stimulates uterine edema via activation of estrogen receptors: blockade of its effects using ICI 182,780, a specific estrogen receptor antagonist. Endocrinology 140 2426–2429.


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