Relaxin (RLX) and estrogen affect estrogen receptor α , vascular endothelial growth factor, and RLX receptor expression in the neonatal porcine uterus and cervix

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Abstract

The porcine female reproductive tract undergoes estrogen receptor (ER) α -dependent development after birth (postnatal day = PND 0), the course of which can determine adult uterine function. Uterotrophic effects of relaxin (RLX) in the porcine neonate are age specific and may involve ER activation. Here, objectives were to determine effects of RLX and estrogen administered from birth on uterine and cervical growth and expression of ER α , vascular endothelial growth factor (VEGF), and the RLX receptor (RXFP1). On PND 0, gilts were treated with the antiestrogen ICI 182 780 (ICI) or vehicle alone and, 2 h later, were given estradiol-17 β (E) or porcine RLX for 2 days. Neither RLX nor E affected uterine wet weight or protein content on PND 2. However, RLX, but not E, increased cervical wet weight and protein content when compared with controls. Pretreatment with ICI did not inhibit RLX-stimulated cervical growth. Uterine and cervical ER α increased in response to RLX, but not E. Both RLX and E increased VEGF in the uterus and cervix on PND 2. Pretreatment with ICI increased VEGF in both tissues and increased RLX-induced cervical VEGF. In the uterus E, but not RLX, increased *RXFP1* mRNA. In the cervix, E increased *RXFP1* gene expression whereas RLX decreased it. Results indicate that the neonatal uterus and cervix are sensitive to E and RLX and that growth responses to RLX in these tissues differ by PND 2. Effects of RLX on uterine and cervical ER α and VEGF expression may be important for neonatal reproductive tract development.

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Introduction

Growth of the female reproductive tract (FRT) is sensitive to both estrogen and relaxin (RLX; Vasilenko & Mead 1987, Cullinan-Bove & Koos 1993, Hall & Anthony 1993, Pillai *et al.* 1999), and organizational events responsible for both tissue patterning and programming are estrogen receptor (ER) dependent (Couse & Korach 1999, Tarleton *et al.* 1999). Thus, factors that affect ER expression and activation can determine the developmental trajectory of these tissues (Schonfelder *et al.* 2002, Suzuki *et al.* 2002, Nikaido *et al.* 2005). Evidence that ER antagonists block uterotrophic effects of RLX in the rat (Pillai *et al.* 1999) and neonatal pig (Yan *et al.* 2006a) and that RLX is required to support myometrial ER α expression in RLX-null mice (Siebel *et al.* 2003) indicates that RLX should be included in the list of factors with the potential to affect FRT programming.

It is well established that patterns of ER expression in FRT tissues are regulated both temporally and spatially during the course of development (Yamashita *et al.* 1989, Glatstein

& Yeh 1995, Mowa & Iwanaga 2000, Okada *et al.* 2005). Moreover, aberrant activation of the ER system can have lasting consequences for FRT morphology and function (Miller *et al.* 1998, Markey *et al.* 2005). In the pig, this is supported by evidence that the neonatal uterus: i) is ER α -negative at birth (Tarleton *et al.* 1998); ii) develops sensitivity to estrogen between birth (postnatal day = PND 0) and PND 15 that is associated with the appearance and proliferation of endometrial glands and the expression of ER α in the stroma and glandular epithelium (Tarleton *et al.* 1998, 2001); and iii) responds to transient estrogen exposure from birth with affects on adult phenotype, uterine responses to conceptus signals, and uterine capacity to support pregnancy (Tarleton *et al.* 2001, 2003).

Following identification of the cognate RLX receptor (LGR7; Hsu *et al.* 2002), now designated RLX family peptide receptor 1 (RXFP1; Bathgate *et al.* 2006), its expression was confirmed in the neonatal uterus at birth, prior to the onset of ER α expression (Yan *et al.* 2006b).

Taken together with evidence that uterotrophic effects of both estrogen (Spencer *et al.* 1993, Tarleton *et al.* 2001) and RLX become more pronounced with age between birth and PND 15 (Yan *et al.* 2006a), these observations indicated that functional ER α and RLX receptor systems evolve in neonatal porcine FRT tissues during the first days of neonatal life. Recent studies showed that RLX is absent from the neonatal circulation at birth, prior to nursing, but is present in colostrum or first milk at the time of parturition and is likely transmitted into the neonatal circulation during the first 48 h of postnatal life as a consequence of nursing (Yan *et al.* 2006b). Therefore, a maternally driven lactocrine mechanism was hypothesized whereby milk-borne RLX, absorbed into the neonatal circulation during the first days of postnatal life, could act directly through RXFP1 and/or indirectly through the evolving ER system to influence developmental programming events in the neonate (Yan *et al.* 2006b).

Trophic effects of RLX on reproductive tissues, including the uterus, cervix, and vagina of prepubertal and adult animals are well documented (Sherwood 2004). Given that all of the components of a classical RLX signaling system are present in neonatal gilts at birth (Yan *et al.* 2006a), and that functional crosstalk between RLX and estrogen signal transduction systems is likely to influence FRT growth (Pillai *et al.* 1999, Yan *et al.* 2006a), studies now focus on the impact of these hormones on FRT development during early postnatal life. Here, objectives were to determine short-term effects of RLX and estradiol-17 β (E), administered for 2 days from birth, on FRT growth and the expression of ER α , vascular endothelial growth factor (VEGF), a marker of estrogen (Cullinan-Bove & Koos 1993) and RLX (Unemori *et al.* 1999) action, and RXFP1 in the uterus and cervix of neonatal gilts.

Results

Uterine and cervical tissue wet weights and protein content

When administered from birth, neither E nor RLX affected uterine wet weight as determined on PND 2 (Fig. 1A). Although E also had no effect on cervical wet weight, RLX treatment increased ($P<0.01$) cervical wet weight when compared with controls (Fig. 1B). Pretreatment of gilts with ICI did not influence the RLX-induced cervical weight gain. Results for uterine and cervical protein content mirrored those obtained for tissue wet weight data, in that neither RLX nor E affected uterine protein content (Fig. 2A) whereas RLX, but not E, increased ($P<0.01$) cervical protein content on PND 2 when compared with controls (Fig. 2B). Pretreatment with ICI had no effect on the RLX-induced increase in cervical protein content on PND 2.

Figure 1 Treatment effects on (A) uterine and (B) cervical wet weight on PND 2. Data are expressed as LSM $\pm$ S.E.M. (n =three to eight animals per group). Body weight was included as a covariate in statistical analyses. Results of preplanned contrasts follow for uterus and cervix: C versus E ($P<0.5$ and 0.41), C versus R ($P<0.4$ and 0.01), E versus ICI/E ($P<0.69$ and 0.71), R versus ICI/R ($P<0.15$ and 0.65), and E versus ICI ($P<0.65$ and 0.14) respectively.

Uterine and cervical expression of ER α and VEGF protein

Representative Western blots and results of densitometric analyses for ER α and VEGF are presented in Figs 3 and 4. Treatment with RLX, but not E, from birth increased ($P<0.04$) the relative abundance of uterine ER α protein on PND 2 when compared with controls (Fig. 3A and B). Likewise, RLX, but not E, increased ($P<0.01$) cervical ER α expression on PND 2 (Fig. 3C and D). Responses to RLX and E were unaffected by pretreatment with ICI in either the uterus or cervix (Fig. 3).

The relative abundance of dimeric VEGF protein (46 kDa) in both uterine (Fig. 4A and B) and cervical tissues (Fig. 4C and D) increased in response to RLX ($P<0.01$) and E ($P<0.01$) when compared with controls on PND 2. Interestingly, treatment with ICI alone increased VEGF expression in both the uterus ($P<0.02$) and cervix ($P<0.01$). For the uterus (Fig. 4B), ICI pretreatment had a marginal negative effect on E-induced VEGF expression ($P<0.07$). By contrast, pretreatment with ICI increased ($P<0.01$) RLX-induced cervical VEGF expression (Fig. 4D).

Uterine and cervical expression of RXFP1 mRNA

Data illustrating effects of E and RLX administered for 2 days from birth on uterine and cervical RXFP1 gene expression in gilts on PND 2 are presented in Fig. 5. In the

Figure 2 Treatment effects on (A) uterine and (B) cervical protein content on PND 2. Total protein content for each tissue is expressed as LSM $\pm$ S.E.M. (n =three to eight animals per group). Results of preplanned contrasts follow for uterus and cervix: C versus E ($P<0.36$ and 0.54), C versus R ($P<0.89$ and 0.01), E versus ICI/E ($P<0.78$ and 0.31), R versus ICI/R ($P<0.42$ and 0.56), and E versus ICI ($P<0.92$ and 0.35) respectively.

Figure 3 Treatment effects on (A) uterine and (B) cervical ER α expression on PND 2. (A and C) Representative Western blots for individual animals for each tissue and treatment category. Both 67 kDa and 43 kDa bands indicative of immunoreactive ER α and β -actin (included as a loading reference), respectively, are indicated (B and D). Summaries of densitometric data for ER protein expressed as LSM $\pm$ s.e.m. (n =three to eight animals per group). Data were adjusted for potential loading variation by including data for actin as a covariate in statistical analyses. Results of preplanned contrasts follow for uterus and cervix: C versus E (P <0.28 and 0.92), C versus R (P <0.04 and 0.01), E versus ICI/E (P <0.35 and 0.78), R versus ICI/R (P <0.24 and 0.75), and E versus ICI (P <0.82 and 0.55) respectively.

uterus (Fig. 5A), E increased (P <0.01) RXFP1 mRNA levels when compared with tissues obtained from controls. By contrast, RLX did not affect uterine RXFP1 gene expression (Fig. 5A). In the cervix (Fig. 5B), E induced an increase (P <0.06) in RXFP1 gene expression, whereas RLX induced a decrease (P <0.01) in this response.

Discussion

While trophic effects of RLX on the reproductive tract are well documented (Sherwood 2004), the mechanism of RLX action in promoting uterine and cervical growth remains elusive. In this study, we report differential

Figure 4 Treatment effects on (A) uterine and (B) cervical VEGF protein on PND 2. (A and C) Representative Western blots for individual animals for each tissue and treatment category. Both 46 kDa and 43 kDa bands indicative of immunoreactive VEGF and β -actin (included as a loading reference), respectively, are indicated (B and D). Summaries of densitometric data for VEGF protein expressed as LSM $\pm$ s.e.m. (n =three to eight animals per group). Data for VEGF were adjusted for potential loading variation by including data for actin as a covariate in statistical analyses. Results of preplanned contrasts follow for uterus and cervix: C versus ICI (P <0.02 and 0.01), C versus E (P <0.01 and 0.01), C versus R (P <0.01 and 0.01), E versus ICI/E (P <0.07 and 0.33), R versus ICI/R (P <0.05 and 0.01), and E versus ICI (P <0.73 and 0.67) respectively.

Figure 5 Treatment effects on (A) uterine and (B) cervical RXFP1 mRNA on PND 2. Data are expressed as LSM $\pm$ s.e.m. (n =four animals per group). Results of preplanned contrasts follow for uterus and cervix: C versus E (P <0.01 and 0.06), C versus R (P <0.52 and 0.01), and E versus R (P <0.01 and 0.01) respectively.

effects of RLX on the uterus and cervix in the neonatal pig, in which both ER α (Tarleton *et al.* 1998) and RXFP1 expression are developmentally regulated (Yan *et al.* 2006b). Previous studies showed that the neonatal gilt is sensitive to uterine growth-promoting actions of RLX and that neonatal uterotrophic responses to RLX are both age specific and related functionally to the relative presence and state of activation of the ER α system (Yan *et al.* 2006a). For example, RLX, administered to gilts for 2 days from birth, increased uterine luminal epithelial height, but not uterine weight or protein content, on PND 2. By contrast, administration of RLX for 2 days beginning on PND 12, after the onset of uterine ER α expression, increased uterine weight, protein content, and luminal epithelial height on PND 14 (Yan *et al.* 2006a). Moreover, the latter effects were inhibited by pretreatment with ICI. These findings were consistent with data for rats showing that RLX-stimulated uterine edema is ER dependent (Pillai *et al.* 1999).

In the present study, when treatments were initiated on PND 0, RLX increased cervical but not uterine wet weight and protein content. Additionally, cervical responses to RLX were not affected by pretreatment with ICI. These data suggest that tissue-specific trophic actions of RLX observed in the cervix of newborn gilts are unlikely to involve crosstalk with the ER signaling system at this early postnatal stage of development. Whether ER activation is involved in RLX-stimulated cervical growth later in life remains to be investigated.

ER α expression was detected in both uterine and cervical tissues by PND 2 and increased in response to RLX administered from birth. To our knowledge, this is the first report that RLX increases ER α expression *in vivo*. Observations are consistent with the fact that, in the absence of RLX, myometrial ER α expression was attenuated in RLX-null mice during late pregnancy (Siebel *et al.* 2003). By contrast, studies in rats have shown that RLX decreased uterine ER β (*ESR2*) mRNA levels without affecting ER α (*ESR1*) mRNA levels (Pillai *et al.* 2002). Moreover, in the cervix and vagina of RLX-null mice, chronic infusion of RLX had no effect on ER α gene expression and decreased ER β mRNA in late gestation (Parry *et al.* 2005). Although low uterine ER β gene expression in adult porcine tissues has been

reported (Cardenas & Pope 2005), there is no evidence for porcine uterine expression of ER β protein in the first 2 weeks of neonatal life (Yan *et al.* 2006a). Collectively, these observations reinforce the importance of comparative studies. Data clearly support the idea that RLX can affect patterns of ER expression in target tissues.

Although ER α protein was detectable in the uterus and cervix by PND 2, there was no evidence that treatment with ICI alone affected uterine or cervical ER α protein expression. For the uterus, this may be explained by the fact that ICI treatment was administered on PND 0, when uterine ER α expression is low to undetectable (Tarleton *et al.* 1998, Yan *et al.* 2006a), whereas tissues were obtained on PND 2. Data describing the ontogeny of porcine cervical ER α expression from birth are sorely lacking. However, if data for the uterus provide a valid reference, the absence of a detectable effect of ICI on ER α expression in neonatal FRT tissues observed here should not be surprising. Absence of a fully functional ER α signaling system may also explain why pretreatment with ICI did not affect uterine or cervical ER α expression in response to E or RLX.

Results also indicated that VEGF expression can be used as a marker of both E and RLX action in neonatal porcine uterine and cervical tissues. Moreover, present data suggest that VEGF expression is a more sensitive marker of E action in the uterus and cervix on PND 2 than either organ wet weight or protein content. The fact that RLX stimulates both uterine and cervical VEGF expression in the neonate is consistent with similar evidence for angiogenic activity and/or VEGF expression in human endometrial stromal cells (Unemori *et al.* 1999), in the marmoset endometrium of pregnancy (Einspanier 2001) and at wound sites (Unemori *et al.* 2000).

Since ICI alone did not influence uterine or cervical wet weight, protein content, or ER α protein expression in gilts on PND 2, the observed positive effects of ICI on both uterine and cervical VEGF protein expression were somewhat unexpected. However, ICI alone was also found to increase uterine epithelial proliferation, as reflected by an increase in proliferating cell nuclear antigen (PCNA) labeling index, in gilts on PND 14 (Masters *et al.* 2007). Such effects may be explained by the fact that ICI can alter the transcription of estrogen-responsive genes via activation of SP1 promoter elements (Kim *et al.* 2003, Fleming *et al.* 2006). Like PCNA (Shipman-Appasamy *et al.* 1991), VEGF expression is positively regulated by SP1 activation (Milanini *et al.* 1998, Shi *et al.* 2001). A gene expression profiling study of mammary epithelial cells revealed 268 genes for which expression was regulated via a nonclassical ER α pathway (Glidewell-Kenney *et al.* 2005). The largest number of these genes, including some for which expression was not responsive to E, were activated by ICI (Glidewell-Kenney *et al.* 2005). Clearly, effects of ICI are complex, not necessarily or exclusively antiestrogenic, and may be biologically context

dependent. This may explain, at least in part, the agonist-like effects of ICI observed here for VEGF protein expression.

Given that objectives of the present study included evaluation of the short-term effects of E and RLX administered from birth on uterine and cervical responses, effects of these treatments on RXFP1 expression in these tissues were evaluated. Moreover, while uterine RXFP1 expression was documented in tissues obtained at birth (Yan *et al.* 2006b), similar data for neonatal porcine cervical tissues are lacking. The fact that E administered from birth increased RXFP1 expression in both the uterus and cervix on PND 2 supports and extends previous studies indicating the importance of estrogen in sensitizing reproductive tissues to RLX (Mercado-Simmen *et al.* 1982, Downing & Hollingsworth 1992, 1993). Results are consistent with the idea that estrogen priming can facilitate (Vasilenko *et al.* 1980, Vasilenko & Mead 1987) or enhance responsiveness of RLX target tissues (Adams *et al.* 1989). The observation that cervical RXFP1 expression on PND 2 was reduced following RLX administration from birth is new. These data are consistent with studies reported in rats in which infusion of unlabeled RLX reduced uterine and cervical uptake of radiolabeled RLX when compared with saline-infused controls (Downing & Hollingsworth 1993). Likewise, in RLX-null mice, both cervical and vaginal RXFP1 expression increased during late pregnancy when compared with RLX-replete wild-type controls (Parry *et al.* 2005). Furthermore, continuous infusion of RLX for 6 days in pregnant RLX-null mice decreased cervical and vaginal RXFP1 mRNA levels on gestation day 18.5 when compared with saline-treated RLX-null controls (Parry *et al.* 2005).

Collectively, these studies point to a potential negative regulatory mechanism through which RLX downregulates the expression of its own receptor. In studies of human endometrial cells, RLX had no effect on endometrial stromal RXFP1 expression while RLX increased RXFP1 mRNA in a dose- and time-dependent fashion in decidual cells collected at term (Mazella *et al.* 2004). However, using endometrial stromal cells under conditions known to promote decidualization *in vitro*, Ivell and colleagues reported that RLX did not influence RXFP1 transcript levels (Bartsch *et al.* 2004). This is consistent with data presented here showing that RLX had no effect on uterine RXFP1 expression *in vivo*. The explanation for these differences in effects of RLX on RXFP1 expression remains unclear but may be due to differences between species, endocrinological status of the tissues, and/or experimental conditions.

In conclusion, data presented here show that the neonatal porcine uterus and cervix are differentially sensitive to E and RLX from birth. In addition, effects of E and RLX on cognate RLX receptor expression during this period of neonatal life are tissue specific. This study also

provides further evidence for the interaction between E and RLX signaling pathways in which RLX stimulates ER α expression and E increases RXFP1 expression in the uterus and cervix of neonatal pigs. The fact that E and RLX presented to neonatal gilts from birth can increase uterine and cervical ER α expression by PND 2 has significant developmental implications. While the importance of ER α activation for cervical development remains to be investigated, it is clear that postnatal ER α expression is required for normal uterine growth and endometrial development (Bartol *et al.* 2006). Recent studies also showed that the neonatal uterus is RXFP1-positive at birth, that RLX is present in porcine milk from the first day of lactation, and that RLX is detectable in the peripheral circulation of nursing pigs within 48 h of birth (Yan *et al.* 2006b). Taken together with present observations, these data suggest that critical early events associated with growth, development, and programming of FRT tissues in the neonatal pig could be facilitated through the actions of milk-borne RLX delivered to the neonate from the maternal system via a lactocrine mechanism (Yan *et al.* 2006b).

The extent to which RLX or other milk-borne growth factors may be affecting the development of FRT or other RLX receptor-positive somatic tissues is unknown. Studies are underway to determine if the absence of milk-borne growth factors from birth, including RLX, affects early neonatal uterine or cervical gene expression events associated normally with FRT development and, if so, whether resulting phenotypes can be rescued by repletion of RLX or milk.

Materials and Methods

Materials

Porcine RLX (CM-A fraction; 3000 U/mg) was prepared at the Department of Biomedical Sciences (University of Guelph, Ontario, Canada) by extraction and purification from the ovaries of pregnant sows (Sherwood & O'Byrne 1974). Purity was confirmed by SDS-PAGE, which revealed a single band at ~6.2 kDa. Biological activity of the RLX 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-17 β was purchased from Sigma-Aldrich. ICI 182 780 was kindly provided by Dr Alan Wakeling, Zeneca Pharmaceuticals (Macclesfield, Cheshire, UK). Mouse anti-human ER α (Ab-15) MAB was purchased from NeoMarkers Inc. (Fremont, CA, USA). Goat anti-human VEGF (sc-152-G) and β -actin (sc-1615) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). For ER α detection, an anti-mouse secondary antibody from Santa Cruz Biotechnology Inc. (sc-2005) generated a nonspecific signal at 64 kDa, similar to the size of ER α , when used alone (Marriott *et al.* 2007). Therefore, appropriate secondary antibodies conjugated to horseradish peroxidase that did not generate a nonspecific signal (Cat # 62-6620; Zymed Inc., San Francisco, CA, USA) were used for immunoblotting. Nitrocellulose membranes were obtained from

Bio-Rad Laboratories. The Renaissance Western Blot Chemiluminescence Reagent Plus kit was obtained from Perkin-Elmer Life Science (Boston, MA, USA). X-Omatic films were purchased from American Imaging (South Plainfield, NJ, USA). TRI Reagent was obtained from Sigma-Aldrich. RNeasy Mini Kit and RNase-Free DNase Set were obtained from Qiagen Inc. SuperScript III First-Strand Synthesis System for RT-PCR was obtained from Invitrogen. SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA, USA). All other chemicals were purchased from Sigma-Aldrich and Invitrogen.

Animals

Yorkshire-Landrace gilts were obtained from the Swine Unit of the New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, NJ, USA. Gilts were randomly assigned to one of six treatment groups on PND 0 as follows: (1) Control (C; DMSO:ETOH 4:1 vehicle, given i.p., and PBS, given i.m., $n=6$); (2) ICI 182 780 (ICI; 1 mg/kg body weight (BW), given i.p. in DMSO:ETOH 4:1 vehicle as a single injection 2 h prior to start of hormone treatment, $n=4$); (3) Estradiol-17 β (E; 50 μ g/kg BW, given i.p. in DMSO:ETOH, 4:1 vehicle every 24 h for 48 h, $n=6$); (4) ICI/E ($n=3$); (5) porcine RLX (R; 20 μ g/kg BW, given i.m. in PBS every 6 h for 48 h, $n=8$); and (6) ICI/R ($n=5$). During treatment, neonatal gilts were maintained with sows and allowed to suckle naturally. Timing, dosage, and route of ICI administration were based on studies in rodents (Gibson *et al.* 1991, Pillai *et al.* 1999). Additionally, ICI was effective in blocking the E-induced uterine growth response observed in neonatal gilts that received the same E treatment regimen for 2 days prior to collection of ER-positive uterine tissues on PND 14 (Yan *et al.* 2006a). The dose and timing of RLX administration were based on studies in prepubertal gilts and rodents (Hall *et al.* 1990, Pillai *et al.* 1999). Gilts were weighed and killed 3 h after the last injection on PND 2. Each uterus and cervix was trimmed free of fat and associated ligaments and weighed. Tissues were frozen in liquid nitrogen and stored at -80°C . All procedures involving animals were reviewed and approved as appropriate by the Rutgers University Animal Care and Facilities Committee (Protocol # 88-079). Procedures were conducted in accordance with the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (1999; Federation of Animal Science Society, Savoy, IL, USA).

Protein extraction and evaluation of ER α and VEGF expression

Tissue samples were homogenized in ice-cold lysis buffer (4 ml/g tissue) containing 1% Triton X-100, 10% glycerol, 150 mM Tris-HCl, 300 mM NaCl, 1 mM MgCl₂, pH 7.5. The resulting tissue lysate was passed (five times) through a 20-gauge needle fitted to a syringe, incubated on ice for 1 h and centrifuged (12 000 g , 4°C) for 5 min. Protein concentration of the supernatant was measured using a detergent-compatible protein assay kit (DC Protein Assay, Bio-Rad Laboratories). Tissue protein content was calculated based on the protein concentration and wet weight of the organ.

To document ER α and VEGF protein expression, uterine and cervical proteins (30 μ g) were resolved on 12.0% Bis-Tris-HCl-buffered polyacrylamide electrophoresis gels under reducing

conditions in the presence of SDS and transferred onto nitrocellulose membranes. After blocking in 10% nonfat dry milk in Tris-buffered saline containing Tween-20 (TBST; 25 mM Tris (pH 7.5), 0.14 mM NaCl, 3 mM KCl, 0.05% Tween-20), membranes were probed with either mouse anti-human ER α antibody (2 μ g/ml) or goat anti-human VEGF antibody (0.2 μ g/ml) overnight at 4 °C. After washing with TBST, blots were incubated with either horseradish peroxidase-conjugated anti-mouse (0.3 μ g/ml) or anti-goat secondary antibody (0.75 μ g/ml) for 1 h at room temperature, and bound antibodies were detected by ECL. Membranes were stripped with buffer (100 mM 2- β -mercaptoethanol, 2% SDS, and 62.3 mM Tris-HCl) and reprobed with goat anti-human β -actin (0.2 μ g/ml) to determine the amount of protein loaded on the gels. Signals on films were quantified using Scion image densitometric software (Scion Corporation, Frederick, MD, USA).

RNA isolation and cDNA generation

Total RNA was isolated from 30 to 50 mg of tissue samples using TRI Reagent followed by RNeasy Mini Kit and traces of DNA were removed using the RNase-Free DNase Set. RNA concentration and purity were evaluated by spectrophotometry. RNA integrity was checked by agarose gel electrophoresis and ethidium bromide staining to visualize sharp, clear 28S and 18S rRNA bands. RT was performed with 5 μ g RNA per sample using the (PTC-200) Peltier Thermal Cycler (Bio-Rad Laboratories Inc.) and SuperScript III First-Strand Synthesis System for RT-PCR. All procedures were carried out following manuals/guidelines provided by the manufacturers.

Real time quantitative RT-PCR

Real time quantitative RT-PCR (qRT-PCR) was performed using an Applied Biosystems Gene Amp 7000 Sequence Detection System and the SYBR Green method following universal thermal cycling parameters recommended by the manufacturer. Primers for quantitative qRT-PCR were designed using Primer Express Software (Applied Biosystems) and synthesized by Invitrogen Corp. Porcine RXFP1 (GenBank accession number: CA994862) forward – 5'-GCATCACTTTGAGGCAGAGACA-3' – and reverse – 5'-CCTCGGCAAAGACATTGCAT-3' – primers were used to generate a 69 bp amplicon. Similarly, porcine cyclophilin (GenBank accession number: AU058466) forward – 5'-TTA-TAAAGGTTCTGCTTTCACAGAA-3' – and reverse – 5'-TGCCATTATGGCGTGTGAAG-3' – primers were used to generate an expected 77 bp amplicon. To ensure specific amplification, multiple controls including water only, no primers and no template were included in assays. Primer quality was evaluated by amplifying serial dilutions of the cDNA template (1, 1:2, 1:5, 1:10) and dissociation curves for each set of primers were checked to ensure that there was no amplicon-independent amplification (i.e., generation of primer dimers). The PCR amplification products were analyzed by agarose gel electrophoresis to further confirm the absence of nonspecific amplification. Values for cycle threshold (C_T), the point at which exponential amplification of the PCR products begins, were determined using Applied Biosystems software. Porcine cyclophilin was used as an internal control in qRT-PCR analyses to

normalize cDNA input and PCR efficiency. Data were analyzed by the comparative CT method ($\Delta\Delta C_T$) for relative quantitation of gene expression in which the sample having the minimum expression level was chosen as the reference calibrator (Livak & Schmittgen 2001). Therefore, data from these analyses are presented as relative expression levels.

Statistical analyses

All quantitative data were subjected to analyses of variance using general linear model procedures available with SAS (SAS 2002–2003). For uterine and cervical wet weight, protein content and both ER α (*ESR1*) and VEGF gene expression data, generated by densitometry of Western blots, statistical models considered variation due to the main effects of treatment, including pretreatment with ICI. Treatment effects were identified by performing a set of preplanned contrasts that included comparisons of specific groups as follows: control versus E; control versus RLX; E versus ICI; E versus ICI/E; and R versus ICI/R. When results of these analyses indicated a likely main effect of ICI and potential differences in the magnitude of E- and RLX-induced effects, a second set of contrasts was run with comparisons that included: control versus ICI and E versus R. Analyses of uterine and cervical RXFP1 expression data, evaluated at the transcriptional level, considered variation due to the main effects of E, RLX, and their interactions. All error terms were identified based upon the expectations of the mean squares for error and data were expressed as least square means with standard errors.

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