Tissue-specific regulation of porcine prolactin receptor expression by estrogen, progesterone, and prolactin

Josephine F Trott1,2, Katherine C Horigan1, Julia M Gloviczki1,2, Kristen M Costa1, Bradley A Freking3, Chantal Farmer4, Kanako Hayashi5, Thomas Spencer6, Joseph E Morabito1 and Russell C Hovey1,2

1Lactation and Mammary Gland Biology Group, Department of Animal Science, The University of Vermont, 570 Main Street, Burlington, Vermont 05405, USA
2Department of Animal Science, University of California, 2145 Meyer Hall, 1 Shields Avenue, Davis, California 95616, USA
3US Meat Animal Research Center, PO Box 166, Clay Center, Nebraska 68933, USA
4Agriculture and Agri-Food Canada, PO Box 90, Lennoxtown STN, Sherbrooke, Quebec, Canada J1M 1Z3
5Department of Physiology, Southern Illinois University School of Medicine, 1135 Lincoln Drive, Carbondale, Illinois 62901, USA
6Department of Animal Science, Center for Animal Biotechnology and Genomics, Texas A&M University, College Station, Texas 77843, USA

(Re correspondence should be addressed to R C Hovey at Department of Animal Science, University of California; Email: rchovey@ucdavis.edu)

Abstract

Prolactin (PRL) acts through its receptor (PRLR) via both endocrine and local paracrine/autocrine pathways to regulate biological processes including reproduction and lactation. We analyzed the tissue- and stage of gestation-specific regulation of PRL and PRLR expression in various tissues of pigs. Abundance of pPRLR-long form (LF) mRNA increased in the mammary gland and endometrium during gestation while in other tissues it remained constant. There was a parallel increase in the abundance of the pPRLR-LF protein in the mammary gland and endometrium during gestation. We determined the hormonal regulation of pPRLR-LF mRNA expression in various tissues from ovariectomized, hypoprolactinemic gilts given combinations of the replacement hormones estrogen (E2), progesterin (P), and/or haloperidol-induced PRL. Abundance of pPRLR-LF mRNA in kidney and liver was unaffected by hormone treatments. Expression of uterine pPRLR-LF mRNA was induced by E2 whereas the effect of E2 was abolished by co-administering P. The expression of pPRLR-LF mRNA in the mammary gland stroma was induced by PRL, whereas E2 induced its expression in the epithelium. In contrast to these changes in pPRLR expression, pPRL expression was relatively constant and low during gestation in all tissues except the pituitary. Taken together, these data reveal that specific combinations of E2, P, and PRL differentially regulate pPRLR-LF expression in the endometrium and mammary glands, and that the action of PRL on its target tissues is dependent upon pPRLR-LF abundance more so than the local PRL expression.


Introduction

The polypeptide hormone prolactin (PRL) regulates numerous physiological functions across multiple species. In pigs, PRL exerts its most significant effects on mammary gland growth (Farmer et al. 2000), lactation (Farmer et al. 1998) and reproduction (Young et al. 1990, Ciereszko et al. 2002), while it also modulates biological responses including stress (Kaminska et al. 2000) and maternal behavior (Farmer et al. 1998, 1999). The role for PRL during mammary gland growth and lactation in swine is crucial, where inadequate suppression of serum PRL also impairs lactation and the responsiveness of the mammary gland to PRL during lactation in pigs (Plaut et al. 1989).

PRL primarily acts via the long form (LF) of its receptor, a member of the cytokine receptor superfamily (Lesueur et al. 1991) that we recently cloned from pigs (Trott et al. 2007). The LF is the only PRLR isoform that has been identified in pigs to date. As in other species, the pPRLR is expressed in various tissues including the mammary gland from nulliparous (Trott et al. 1990, Ciereszko et al. 2002), pregnant (Plaut et al. 1989, 1990), and lactating females (Slomczynska et al. 2001), uterus (Young & Bazer 1989), kidney (Trott et al. 2007), ovary (Bramley & Menzies 1987, Slomczynska et al. 2001), and maternal behavior (Farmer et al. 1990, Ciereszko et al. 2002), while the pPRLR is expressed in various tissues including the mammary gland from nulliparous (Farmer et al. 1998, 1999). The role for PRL during mammary gland growth and lactation in swine is crucial, where inadequate suppression of serum PRL also impairs lactation and the growth of nursing pigs (Farmer et al. 1998). Conversely, administering porcine PRL to lactating sows does not increase nursing pig growth or mammary gland development, probably because PRL receptors (PRLR) in the mammary gland are ligand-saturated (Farmer et al. 1999). These results suggest that PRLR abundance (as a determinant of receptor site availability) may be a major limiting factor for responsiveness of the mammary gland to PRL during lactation in pigs (Plaut et al. 1989).
adrenal cortex (Klemcke et al. 1989), and brain (Muccioli et al. 1988). However, the factors that regulate pPRLR expression have not been assessed.

While the anterior pituitary is the principal site for the synthesis and secretion of endocrine PRL, the potential exists for local autocrine/paracrine synthesis of PRL in various target tissues. Extrapituitary sources of PRL in humans, rodents, and ruminants include uterine and brain tissues, lacrimal, sweat, and adrenal glands, immune and mammary epithelial cells, skin fibroblasts, kidney, and the ovary (Fields et al. 1993, Doppler 1994, Ben-Jonathan et al. 1996, Prigent-Tessier et al. 1999, Sakai et al. 1999, Tao et al. 2004, Kobayashi et al. 2007, Roselli et al. 2008). Interestingly, PRL appears to not be expressed in the rat liver (Kurtz et al. 1993), a tissue rich in PRLR (Bole-Feysof et al. 1998). In rats, PRL mRNA is first expressed within the mammary gland at mid-late pregnancy and is expressed throughout lactation (Iwasaka et al. 2000). Along these lines, precocious lactogenesis in neonatally-estrogenized nulliparous female mice coincides with elevated expression of PRL mRNA in the mammary gland (Hovey et al. 2005). Similarly, many breast tumors in humans produce PRL that may affect disease progression (Ginsburg & Vonderhaar 1995, Bhatavdekar et al. 2000). While the decidua from rats and humans is a rich source of PRL and PRL-related proteins, and ruminant trophoblast cells produce placental lactogen (reviewed by Soares 2004), the endometrium and placenta of pigs is considered to secrete neither PRL nor placental lactogen (Forsyth 1974, DeHoff 1986, Young & Bazer 1989). While these data support the notion that autocrine/paracrine PRL can act on various target tissues, the extent that local PRL contributes to PRL-induced responses is unclear. Furthermore, the regulation of local PRL synthesis and the mechanisms involved are not well defined beyond a recent report that in humans, differential promoters are utilized during transcription of the PRL gene in the pituitary versus extrapituitary tissues (Gerlo et al. 2006).

As an extension of our recent cloning of the pPRLR-LF gene and considering its candidate role in mediating the endocrine and autocrine/paracrine actions of PRL, we have analyzed pPRL and pPRLR-LF expression in various PRL target tissues of gilts during gestation. We also report the hormonal regulation of pPRLR-LF mRNA expression in hormone-depleted nulliparous females. Not only do these data reveal temporal and spatial changes in hormone-regulated pPRLR expression, but they also provide the first profile of PRL gene expression in a range of PRL-responsive tissues during gestation for any species.

Materials and Methods

Animals and tissues

Non-pregnant and pregnant pigs Meishan-derived (50% Meishan×50% hyperprolific Large White), crossbred Yorkshire×Landrace (F1) and Large White multiparous sows and nulliparous gilts housed at Agriculture and Agri-Food Canada were euthanized at the following stages of development; multiparous sows at 15 d after estrus (Meishan n=4), multiparous sows at 15 of gestation (Meishan n=7), gilts at d 75–76 of gestation (Large White n=2, Yorkshire×Landrace n=1, Meishan n=2) or gilts at d 89–91 of gestation (Meishan n=4). The animals were fed a commercial feed (13% CP, 3038 kcal/kg DE, 0.64% w/w lysine, 2 kg/d) throughout gestation and were housed in individual stalls according to a recommended code of practice (Agriculture and Agri-Food Canada 1993). The animals were heat checked daily to detect standing estrus in the presence of a mature boar and were then bred via artificial insemination. The animals were euthanized using a penetrating bolt, in accordance with the guidelines of the Canadian Council on Animal Care (Agriculture and Agri-Food Canada 1993), and the mammary gland tissue was snap frozen in liquid nitrogen and stored at −80°C.

Pregnant unilateral hysterectomy–ovariectomy gilts These gilts were part of a study (at the US Animal Meat Research Center, Clay Center, NE, USA) to evaluate selection lines for uterine capacity and embryo survival throughout gestation. Gils were housed, treated, euthanized and their tissues harvested as described (Freking et al. 2007). Briefly, gilts from selected and unselected lines of a 4-breed composite, with equal contributions from Chester White, Landrace, Large White, and Yorkshire breeds, were subjected to unilateral hysterectomy–ovariectomy (UHO) at ~160 d of age. Heat checking in the presence of a boar started at 201 d of age and continued until mating by natural service. Pregnant UHO gilts (n=5 per group) were euthanized on d 25, 45, 65, 85, and 105 of gestation. At necropsy, tissue samples were collected and snap frozen in liquid nitrogen. Samples were collected from adrenal glands, adipose tissue (back fat), brain, diaphragm, endometrium, heart, hypothalamus, kidney, muscle (Longissimus dorsi), liver, lung, lymph nodes, mammary gland, ovary, pituitary, spleen, thymus, and fetal placenta. The experimental procedures were performed in accordance with the US Meat Animal Research Center Animal Care Guidelines and the Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS 1999).

Bromocriptine-treated pregnant gilts Crossbred (F2) primigravid gilts, from a cross between Large White×Landrace sows and Large White boars, housed at Agriculture and Agri-Food Canada, were heat checked daily for visual appraisal of standing estrus in the presence of a mature boar and were bred via artificial insemination. Pregnant gilts received 2–bromo–α-ergocryptine methanesulfonate salt (Bromo; 10 mg; Novartis) orally at 0730, 1530, and 2330 daily between d 50 and 69 of gestation (n=9), d 70–89 of gestation (n=7) or d 90–109 of gestation (n=10) as described by Farmer & Pettilcerc (2003). Control gilts did not receive any Bromo (n=7). This dose and frequency of Bromo was previously demonstrated to be effective in lowering serum PRL during these treatment periods.
Hormone-treated Yucatan miniature pigs

Peripubertal Yucatan miniature gilts housed in groups at the University of Vermont (5-months old, Sinclair Research, ME, USA) had free access to water and were fed Pig and Sow Pellets (16% CP, 2926 kcal/kg DE, 1% w/v lysine, Blue Seal Feeds, VT; 4% BW/d provided twice daily). Yuca
tan miniature gilts were used to determine recovery for each sample (mean recovery was 77.4%).

The intra-assay coefficient of variation for the E2 and P assays were 10.9 and 8.3% respectively. The inter-assay coefficients of variation (CV) for the E2 and P assays were 10-7 and 8-3% respectively. pPRL in serum was measured using a homologous pPRL RIA, with purified pPRL (National Hormone and Peptide Program, Torrance, CA, USA) as the standard and [125I]-pPRL as the ligand. Sensitivity of the assay was 0.9 ng/ml. Intra- and inter-assay CV were 2-1 and 2-0% (Horigan et al. 2009).

RNA extraction and reverse transcription

Tissue samples were homogenized in Trizol (Invitrogen) and total RNA precipitated according to the manufacturer's instructions. Total RNA (7 μg) was treated with DNaseI (Roche Molecular Systems) and purified using a DNA-Free RNA Kit (Zymo Research Corporation, Orange, CA, USA) prior to confirmation of its integrity by formaldehyde–agarose gel electrophoresis. Total RNA (0.5-1 μg) from each sample was then reversed transcribed into cDNA using 5X RT Buffer (Promega), oligo dT (20 μg/μl, Amersham Pharmacia Biotech, Inc.), dNTP mixture (0.4 mM, Promega), random hexamers (2 μg/μl, Amersham Pharmacia Biotech, Inc.), Moloney's murine leukemia virus (MMLV) reverse transcriptase (4 U/μl Promega), and RNase inhibitor (1 U/μl, Stop Rnase Inhibitor, 5 Prime Inc., Gaithersburg, MD, USA) by incubation at 25°C for 5 min, 37°C for 60 min, and 95°C for 5 min.

Real-time TaqMan quantitative PCR

Primer and probe sets for Taqman quantitative PCR (qPCR) were designed from the predicted Yucatan miniature pig pPRLR-LF specific sequence (AY308824) and GenBank sequence for Sus scrofa 18S rRNA (AY265350) using primer express. Probes were labeled with 6-carboxyfluorescein (6-FAM) and black hole quencher-1 (BHQ-1; Biosearch Technologies, Novato, CA, USA). The primer and probe sequences are presented in Table 1. Reverse transcribed cDNA template (2 μl) was amplified by qPCR using Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.9 μM 1 primers, and 0.2 μM 1 probe. Reactions were performed on an ABI7700 Sequence
The level of PRL mRNA expression was normalized to 18S rRNA using a relative standard curve as described above.

**In situ hybridization**

In situ hybridization for pPRLR mRNA was performed on paraffin-embedded tissue sectioned at 4 μm. The methods used were described previously (Spencer et al. 1999). Briefly, deparaffinized, rehydrated sections of the mammary gland were treated with protease and then hybridized with an [α-35S]UTP labeled sense or antisense cRNA probe generated from a linearized plasmid template containing partial cDNA (717 bp) for the pPRLR extracellular domain that detects all isoforms of the pPRLR. After hybridization, washing, and ribonuclease A digestion, slides were dipped in NTB-2 liquid photographic emulsion (Kodak), stored at 4 °C for 30 d, and developed in a Kodak D-19 developer. Slides were then counterstained with Gill’s modified hematoxylin (Stat Lab, Lewisville, TX, USA), dehydrated through a graded series of alcohols to citrisolve, and coverslipped. Images of the representative fields were recorded using a Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX, USA) fitted with a Nikon DXM1200 digital camera.

**Mammary gland and endometrial membrane preparation**

Mammary gland parenchyma and endometrium (1–2.5 g) from pregnant UHO gilts (d 45, 65, 85 and 105 of gestation; n = 2) was homogenized in 25 ml Tris–EDTA buffer (containing 1 mmol/l MgCl2, 1 mmol/l EDTA, 0.25 mol/l sucrose, 1 mmol/l glutathione, pH 7.4). The homogenate was filtered through a cheesecloth and centrifuged at 11 000 g for 20 min at 4 °C. The supernatant was then filtered through a cell strainer (100 μm; BD Falcon, Franklin Lakes, NJ, USA) and ultracentrifuged at 100 000 g for 1 h at 4 °C. The pellet was resuspended in 25 ml buffer (25 mmol/l Tris, 0.02% w/v Na2S2O3, pH 7.2) and analyzed for pPRLR by western blotting.

**Immunoprecipitation of PRL**

Mammary gland samples from the UHO gilts on d 25 and 105 of gestation and sows at d 21 of lactation (n = 2) were homogenized in buffer (25 mmol/l Tris pH 7.5, 2 mmol/l MgCl2, 1 mmol/l EDTA, 1 mmol/l DTT, 0.5 μg/ml leupeptin (Roche), 0.7 μg/ml pepstatin A (Roche), 1 μg/ml aprotinin (Roche), 10 μg/ml soybean trypsin inhibitor (Roche), 20 μg/ml phenylmethylsulfonyl fluoride (Roche), 1% v/v Triton X-100). Total protein (0.75 mg) was incubated with 0.02% w/v rabbit polyclonal IgG α-pPRL antiserum (National Hormone and Peptide Program) for 5 h at 4 °C before the addition of 30 μl protein A/G PLUS Agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and agitation overnight at 4 °C. The immunoprecipitated complex was washed four times with PBS and the pellet boiled in loading dye prior to electrophoresis and western blotting.

**Table 1** Sequences of primers (F and R) and probes (P; 6-FAM/BHQ-1 labeled) used for real-time q-PCR

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Primer and probe sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPRLF F</td>
<td>CGCCGCTTGGCTGGAA</td>
</tr>
<tr>
<td>pPRLR R</td>
<td>GCCAGTCTGGTGTTTTTG</td>
</tr>
<tr>
<td>pPRL P</td>
<td>ACGGCAGGGCATCTGGTTCCACCT</td>
</tr>
<tr>
<td>18S rRNA F</td>
<td>ACCGGTACACATCCAAGGA</td>
</tr>
<tr>
<td>18S rRNA R</td>
<td>CAAATTACAGGGCCTCGAAA</td>
</tr>
<tr>
<td>18S rRNA P</td>
<td>CGCGAAATACCACCTCCCGA</td>
</tr>
<tr>
<td>pigPRL F</td>
<td>GAGTCTACCTGCTCCACTACATA</td>
</tr>
<tr>
<td>pigPRL R</td>
<td>CCTGGGCTACCTTTATCCA</td>
</tr>
</tbody>
</table>

Detection System (Applied Biosystems) using cycling conditions of 50 °C for 2 min, 95 °C for 10 min then 40 cycles of 95 °C for 15 s and 58 °C for 1 min. Each PCR run included a no-template control containing all reagents except cDNA and a reverse transcription negative control reaction that was performed in the absence of MMLV reverse transcriptase. Melting curve analysis showed a single amplification product that was sequenced to confirm its identity as pPRL mRNA.
Western blotting

Protein concentrations were determined against BSA standards using Coomassie Protein Assay Reagent (Pierce, Rockford, IL, USA). Proteins were resolved using either 10% (pPRLR; 80 μg) or 16% (pPRL) SDS-PAGE and transferred to Hybond-P (Amersham Pharmacia Biotech, Inc.) before blocking with 5% w/v milk in TBST. Porcine PRLR were detected using a mouse monoclonal IgG α-PRLR antibody (raised against the extracellular domain of hPRLR; Zymed Laboratories, Invitrogen) and a HRP-conjugated donkey IgG (H+L) α-mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA). Porcine PRL was detected using a rabbit polyclonal IgG α-pPRL antiserum (National Hormone and Peptide Program) and a HRP-conjugated donkey IgG (H+L) α-rabbit antibody (Jackson ImmunoResearch). Actin was detected using a HRP-conjugated goat IgG α-actin antibody (I-19; Santa Cruz Biotechnology). Immunoreactivity was detected using enhanced chemiluminescence (Super Signal West Pico Chemiluminescent Substrate, Pierce). Molecular size determinations were made using MagicMark immuno-reactive markers (Invitrogen). Autoradiographs were digitized and protein band intensities of pPRLR-LF and reactive markers (Invitrogen) were quantified by densitometry using Image J (Research Services Branch, NIH).

Statistical analyses

qPCR data were log10 transformed to normalize data where necessary, and analyzed with procGLM (SAS version 9.2, SAS Institute Inc., Cary, NC, USA). Data for pPRLR-LF mRNA levels in the mammary gland, (Figs 1 and 5) were analyzed by ANOVA using breed as a blocking factor (Fig. 1 only) followed by a Tukey–Kramer multiple means comparison. pPRL and pPRLR expression for UHO gilts (Figs 2 and 8) was analyzed within each tissue by multiple regression analysis. Relative levels of PRLR-LF protein (Fig. 3) were analyzed within each tissue by multiple regression analysis. Two–way ANOVA was also used to test for the main effects of tissue type and day of lactation followed by Tukey–Kramer multiple means comparison. Data from intact hormone-treated Yucatan miniature pigs (Study 1; Fig. 4) were analyzed using student’s t-test. Data from ovariectomized hormone-treated Yucatan miniature pigs (Study 2; Fig. 6) were analyzed using ANOVA (blocked for date) followed by a Tukey–Kramer multiple means comparison. In addition the main effects of E2, P, and Hal and their interactions were tested by factorial analysis. Levels of statistical significance are indicated in the results and figure legends.

Results

Tissue-specific regulation of pPRLR-LF transcription during gestation

Given that the mammary gland is a primary target for PRL, we first examined pPRLR-LF mRNA levels within the mammary glands of nulliparous and pregnant pigs. On d 15 of gestation the level of pPRLR-LF mRNA was not different to that in nulliparous females sampled on d 15 after estrus (Fig. 1). During gestation the level of pPRLR-LF mRNA expression in the mammary glands was increased at d 75-76, and was maximal on d 89–91 (P<0.05). We next examined pPRLR-LF mRNA in various other tissues from gilts at different stages of gestation. The highest levels of pPRLR-LF mRNA were present in adrenal glands, endometrium and mammary glands (P<0.05), while the lowest levels were in brain, hypothalamus, liver and ovary (P<0.05; Fig. 2). A low level of pPRLR-LF mRNA was also detectable in adipose and spleen but it was barely detectable in other tissues including lung, muscle, diaphragm, lymph nodes, thymus, and heart (data not shown). The level of pPRLR-LF mRNA in the mammary glands increased linearly over time (P<0.05; Fig. 2D), consistent with the data presented in Fig. 1. There was a similar linear increase in pPRLR-LF mRNA expression in the endometrium during gestation (P<0.05; Fig. 2B). There was an overall increase in the pPRLR-LF mRNA expression during gestation in the hypothalamus that followed a cubic regression curve (P<0.05) whereby it rose early, and then again later, during gestation (Fig. 2C). Levels in the placenta rose in mid gestation and dropped again, following a quadratic regression curve (P<0.05; Fig. 2E). By contrast, the level of pPRLR-LF mRNA in adrenal, brain, kidney, liver, ovary, and pituitary remained unchanged during gestation. These data indicate that transcription of the pPRLR-LF in various PRL–responsive tissues from pigs is differentially regulated during gestation.

Figure 1 Levels of pPRLR-LF mRNA within the pig mammary gland during gestation. Mammary gland tissue was collected from multiparous cycling sows at d 15 after estrus and from pregnant gilts and sows. Total RNA was DNase-treated and analyzed for the level of pPRLR-LF mRNA by quantitative RT-PCR with normalization for the corresponding level of 18S rRNA. Data are means ± S.E.M. (n=4–7 per stage). **Means with different superscripts are significantly different (P<0.05). gest, gestation.
Figure 2 Levels of pPRLR-LF mRNA in various tissues of UHO gilts during gestation. (A) Total RNA was DNase-treated and analyzed for the level of pPRLR-LF mRNA by quantitative RT-PCR with normalization for the corresponding level of 18S rRNA. Data are means ± S.E.M. (n=4–5). (B) Linear regression of pPRLR-LF mRNA levels in endometrium with 95% confidence intervals (Endomet; first coefficient = 0.1346, P<0.05). (C) Cubic regression curve of pPRLR-LF mRNA levels in hypothalamus with 95% confidence intervals (Hypothal; first coefficient = 0.1229, P<0.05; second coefficient = −0.002, P<0.05; third coefficient = 0.00001, P<0.05). (D) Linear regression of pPRLR-LF mRNA levels in the mammary gland with 95% confidence intervals (M Gland; first coefficient = 0.0551, P<0.05). (E) Quadratic regression curve of pPRLR-LF mRNA levels in placenta with 95% confidence intervals (first coefficient = 0.0431, P<0.05; second coefficient = −0.000325, P<0.05).
We sought to establish whether the levels of pPRLR-LF protein in the mammary gland and endometrium during gestation coincided with the pPRLR-LF mRNA levels. As shown in Fig. 3, pPRLR-LF in the mammary gland and endometrium of pregnant gilts was detected as a protein of \( \sim 85 \) kDa, consistent with our earlier findings (Trott et al. 2007). The abundance of pPRLR-LF protein in the mammary gland and endometrium during gestation paralleled the increasing levels of mRNA reported above. Therefore, translation of the pPRLR-LF in the mammary gland and endometrium increases during gestation and mirrors changes in mRNA abundance.

**Hormonal regulation of pPRLR-LF mRNA levels in various tissues**

The finding that pPRLR-LF mRNA expression during gestation increased within the mammary glands and endometrium, but not in liver or kidney, led us to examine the hormonal regulation of pPRLR-LF mRNA expression in these tissues. Two studies were conducted using hormone-treated Yucatan miniature pigs. In Studies 1 and 2, the administration of exogenous E\(_2\) increased its level in serum (\( P<0.05 \); Study 1, 471 ± 179 vs 29 ± 7 pg/ml; Study 2, 3432 ± 440 vs 22 ± 2 pg/ml). In Study 1, the administration of exogenous P tended to increase its level in serum up to 24 h after injection (\( P<0.07 \); 26 ± 9 vs 7 ± 2 ng/ml). In Study 2, we used medroxyprogesterone 17-acetate that precluded its detection in serum due to unavailability of a suitable assay. In Study 2, serum PRL levels were increased by treatment with Hal compared with saline controls (\( P<0.0001 \); 30.5 ± 5.4 vs 2.4 ± 0.8 ng/ml) and decreased by Bromo-treatment compared with saline controls (\( P<0.05 \); 0.8 ± 0.04 vs 2.4 ± 0.8 ng/ml).

We first measured pPRLR-LF mRNA expression in ovary-intact nulliparous Yucatan miniature gilts that were treated for 3 d with exogenous E\(_2\) or P. While the level of pPRLR-LF mRNA differed across the tissues examined, it was unchanged in mammary gland, kidney, ovary, uterus, and liver following treatment with E\(_2\) or P (Fig. 4), although in the uterus it tended to be reduced by P (\( P<0.08 \)).
Bromo administered to gilts between d 90 and 109 of gestation inhibits mammary gland growth and subsequent galactopoiesis (Farmer et al. 1998, Farmer & Petitclerc 2003). We therefore anticipated a reduction in pPRL-LF mRNA within the mammary glands of pregnant gilts treated with Bromo from d 90 to 109 of gestation. As shown in Fig. 5, administering Bromo during pregnancy for these 20 d had no effect on pPRL-LF mRNA expression at d 109 compared with the control gilts, despite its previously-documented negative effect on mammosogenesis (Farmer & Petitclerc 2003). Treating pregnant gilts for 20 d with Bromo also did not change the number of PRL binding sites on the mammary gland membrane preparations at d 109 of gestation (Farmer & Petitclerc 2003).

Next we sought to define the individual hormones that regulate pPRL-LF mRNA expression *in vivo*. Given the potential confounding effects of a hormonal background in ovari-intact females, we examined pPRL-LF mRNA expression in tissues from ovariectomized Yucatan miniature gilts treated with either Bromo or Hal in the presence or absence of E2 and/or P. There was a positive main effect of Hal on pPRL-LF mRNA levels in the mammary gland (P=0.001; Fig. 6A). Levels of pPRL-LF mRNA in the uterus were increased by treatment with E2 (P=0.001) or E2+Hal (P<0.01; Fig. 6B) when compared to those in ovariectomized+Bromo gilts. There was also a positive main effect of E2 on pPRL-LF mRNA levels in the uterus (P=0.001). Intriguingly, there was an interaction between E2 and P (P<0.05) whereby P reduced the positive effects of E2, regardless of whether PRL was present or not (Fig. 6B). The level of pPRL-LF mRNA in kidney and liver was unaltered by any combination of exogenous E2, P, or Hal relative to that in hormone-depleted gilts (Fig. 6C and D), consistent with the data in Figs 2 and 4. In a separate qPCR analysis using primers within the extracellular domain of the pPRLR, there was no difference between the changes in pPRLR-LF mRNA and total pPRLR mRNA levels in the mammary glands following the various hormone treatments (data not shown).

Given that the mammary glands are a primary target for PRL, we also examined total pPRLR mRNA distribution within the mammary glands of the hormone-treated ovariectomized gilts by *in situ* hybridization. As shown in Fig. 7, ovariectomy and treatment with Bromo down-regulated the expression of pPRLR mRNA within the stroma (Fig. 7A versus B–E). Treatment with E2 induced a marked increase in pPRLR mRNA expression specifically in the mammary epithelium (Fig. 7C, E, G, I versus B). There was no obvious effect of P alone (Fig. 7D), or in combination with E2 (Fig. 7E). By contrast, treatment with Hal (Fig. 7F–I) restored stromal pPRLR mRNA expression to levels similar to or greater than the saline controls (compare to Fig. 7A). These data indicate that dynamic changes in pPRLR mRNA expression and distribution within the mammary glands is regulated by specific endocrine cues, whereby E2 induces its expression in the mammary epithelium whereas hyperprolactinemia induces its expression in the stroma.

### Expression of PRL mRNA in various tissues

We also sought to establish whether hormonal and developmental regulation of pPRLR-LF expression in various porcine tissues is accompanied by changes in the local expression of paracrine/autocrine PRL. We first analyzed PRL mRNA expression in tissues from gilts at various stages of gestation. As anticipated, the highest levels of PRL mRNA expression were detected in the pituitary (P<0.0001; Fig. 8). There was no change in PRL mRNA abundance during gestation in any of the tissues examined apart from a trend for pituitary PRL mRNA expression to increase linearly (P=0.07). With respect to the overall abundance of PRL mRNA in the extra-pituitary tissues, levels in hypothalamus were higher than in kidney, liver, and ovary (P<0.05) while all other tissues had similar PRL mRNA levels.

### Immunoreactive PRL in the mammary gland

We evaluated immunoreactive PRL in extracts of mammary gland from gilts at different stages of gestation and lactation. As shown in Fig. 9, immunoreactive PRL was detected in the mammary gland at 25 and 105 of gestation and d 21 of lactation. The pPRL was 23 kDa while some slightly smaller forms were also detected (19–23 kDa).

### Discussion

PRL elicits a wide range of important biological functions, particularly during lactation and reproduction. Among other considerations, pigs are unique among the Artiodactyla order in their requirement for pituitary PRL to
maintain their corpora lutea and thus their pregnancy (Anderson et al. 1967, Forsyth 1986). In these studies we hypothesized that developmental changes in pPRLR-LF expression in pigs during gestation were hormonally-regulated, and that local PRL expression contributed to PRL-regulated mammogenesis.

Our results reveal that the endocrine environment differentially regulates pPRLR-LF expression in the mammary glands and uterus during development, and likely directs the pregnancy-associated changes in pPRLR-LF mRNA levels in the hypothalamus and placenta. The levels of pPRLR mRNA and protein increased in the mammary glands and endometrium during gestation, consistent with the findings from a previous study using a heterologous radioreceptor assay that measured pPRL binding (DeHoff 1986). The increasing expression of pPRLR-LF mRNA in the mammary glands of pigs during gestation contrasts with its profile in this tissue from mice wherein PRLR-LF mRNA levels decrease during gestation (Hovey et al. 2001). However, our data are remarkably similar to the profile for PRLR during gestation in sheep (Cassy et al. 1998) and rats (Varas & Jahn 2005).

The increase in pPRLR levels occurs prior to the peak of PRL in serum at parturition (DeHoff et al. 1986, Plaut et al. 1989, Farmer et al. 2000), and likely prepares the mammary gland for lactogenesis. Within the porcine endometrium, an increase in pPRLR-LF mRNA levels during gestation mirrors the increase in pPRLR-LF mRNA expression in the ovine endometrium (Stewart et al. 2000). This profile of increasing pPRLR-LF mRNA fits with the hypothesis that uterine PRLR are essential for maintaining pregnancy during late gestation (Reese et al. 2000). Given that E2 stimulates, and P suppresses, expression of pPRLR-LF mRNA in the uterus, the increasing expression of pPRLR-LF during gestation is likely driven by increasing serum E2 and decreasing serum P concentrations, starting around d 40 of gestation (Eldridge-White et al. 1989).

The pregnancy-associated increase in pPRLR-LF mRNA abundance in the porcine hypothalamus is consistent with data from rats where PRLR-LF mRNA expression during gestation increases in the arcuate nucleus and choroid plexus (Augustine et al. 2003) as well as in oxytocin–expressing neurons in the paraventricular nuclei of the hypothalamus.

Figure 6 Levels of pPRLR-LF mRNA in the (A) mammary gland, (B) uterus, (C) kidney, and (D) liver of Yucatan miniature gilts following hormone treatment. Immediately after ovariectomy, gilts were treated with bromocriptine (Br) for 9 d followed by 5 d of treatment with various combinations of 17ß-estradiol (E2), medroxyprogesterone 17-acetate (P) and/or Br (hypoprolactinemic) or (Hal, hyperprolactinemic). Sham-operated females received injections of saline (Sal) for the 14-d treatment period. Total RNA was DNase-treated and analyzed for the level of pPRLR-LF mRNA by quantitative RT-PCR with normalization for the corresponding level of 18S rRNA. Data are means ± S.E.M. (n = 3–4). * Means with different superscripts are significantly different (P<0.05). Levels of pPRLR-LF mRNA in tissues from Sal-treated animals were set to 100%.
PRL regulates its own secretion at the level of the hypothalamus, where the wide influence of PRL on brain functions is thought to be required for neurobiological adaptations during preparation for pregnancy and lactation (Grattan et al. 2008). The detection of pPRL mRNA expression in the brain and hypothalamus was expected given data from rats (Grattan & Kokay 2008), sheep (Roselli et al. 2008), and mice (Chen et al. 2004). Combined, these data suggest that PRL may mediate some actions in the brain by acting as a neuropeptide as well as a neuroendocrine hormone (Grattan & Kokay 2008).

Our results indicate that E2 and PRL both induce pPRL mRNA expression in the mammary glands, albeit in different cell types. In the mammary glands of mice ovariectomized during early pregnancy, both E2 and PRL upregulate PRLR-LF mRNA expression, while it is suppressed by P (Mizoguchi et al. 1997). PRL upregulates PRLR levels in the mammary glands of pseudopregnant rabbits, while P prevents this PRL-induced upregulation (Djiane & Durand 1977). Given these data and our present findings, it appears that the suppressive effect of P on PRLR expression is restricted to the pregnant state. The stimulation of PRLR expression by E2 has previously been attributed to its indirect effect on PRL secretion from the pituitary (Sheth et al. 1978, Hayden et al. 1979). However, here we show that E2 has a direct positive effect on pPRLR expression, specifically in mammary epithelial cells, during hypoprolactinemia induced by co-administered Bromo. While it is possible that this effect of E2 on pPRLR mRNA levels in the mammary epithelium is specific to pigs, it is more likely that this cell type-specific induction of PRLR mRNA is not revealed using methods that analyze the entire tissue, as we found in this study. The lack of any effect of Bromo on pPRLR-LF mRNA levels in the

Figure 7 In situ hybridization analysis of pPRLR mRNA expression within the mammary glands from hormone-treated ovariectomized Yucatan miniature gilts. Protected transcripts in mammary glands from gilts treated with combinations of replacement hormones were visualized by liquid emulsion autoradiography, and imaged under bright-field (hematoxylin staining) and dark-field (α-35S-PRLR signal) illumination. A representative section from an E2 + Hal treated gilt hybridized with radiolabeled sense strand cRNA (sense) was the negative control. Gilts were either (A) ovary-intact, saline treated, or ovariectomized and treated with (B) Br, (C) Br + E2, (D) Br + P, (E) Br + E2 + P, (F) Hal, (G) Hal + E2, (H) Hal + P, (I) Hal + E2 + P. Scale bar = 100 μm. E2, 17β-estradiol; Br, bromocriptine; P, medroxyprogesterone 17-acetate; dl, ductal lumen; st, stroma.

(Kokay et al. 2006). PRL regulates its own secretion at the level of the hypothalamus, where the wide influence of PRL on brain functions is thought to be required for neurobiological adaptations during preparation for pregnancy and lactation (Grattan et al. 2008). The detection of pPRL mRNA expression in the brain and hypothalamus was expected given data from rats (Grattan & Kokay 2008), sheep (Roselli et al. 2008), and mice (Chen et al. 2004). Combined, these data suggest that PRL may mediate some actions in the brain by acting as a neuropeptide as well as a neuroendocrine hormone (Grattan & Kokay 2008).
mammary glands of pregnant gilts may be due to the dramatic rise in E2 concentrations (0–4500 pg/ml) occurring in the second half of pregnancy (Eldridge-White et al. 1989) that is probably stimulating the increase in pPRLR-LF mRNA expression observed during gestation. We hypothesize that Bromo may suppress pPRLR-LF mRNA levels specifically in the mammary stroma of late pregnant gilts, a change that is not obvious using whole-tissue q-PCR analyses but that would be detectable using in situ hybridization.

In contrast to the mammary glands, the major determinant of pPRLR-LF mRNA expression in the uterus is E2, as has been reported by others (Young et al. 1990). However, whereas Young et al. (1990) found that long-term (11 d) treatment with E2 reduced the number of PRLR binding sites in the endometrium, we observed a significant increase in pPRLR-LF mRNA expression after 5 d treatment with E2. This discrepancy may reflect differences in treatment length, time between the final hormone injection and subsequent tissue collection (3 vs 24 h), or the dose used, where our study produced levels of E2 at least as high as those found on d 110 of pregnancy (Winn et al. 1993).

Our data from ovariectomized, PRL-deficient gilts indicate that P alone does not regulate pPRLR expression in the mammary gland or uterus. This finding is consistent with the outcome of PRL binding to the endometrium from ovariectomized, P-treated gilts (Young et al. 1990). We now demonstrate that P suppresses the ability of E2 to induce pPRLR-LF mRNA expression specifically within the uterus. This negative effect of P also likely accounts for its tendency to downregulate pPRLR-LF mRNA expression within the uterus of ovary-intact females. On the other hand, expression of pPRLR-LF mRNA levels in the liver and kidneys was insensitive to the various combinations of E2, P, and PRL, despite the fact that all these tissues are targets for PRL (Bole-Feysot et al. 1998). In fact, early studies using hypophysectomized rats showed that PRL upregulates PRLR in the liver (Posner et al. 1975). While it is possible that species differences exist for the hormonal responsiveness of liver PRLR, it is also possible that regulation of liver PRLR expression is not as sensitive to changes in PRL concentrations as mammary or endometrial PRLR. Such differential responsiveness emphasizes the coordinated control of pPRLR expression during gestation. These differences likely involve cell type- and tissue-specific, hormone-sensitive regulatory factors as well as differential promoter utilization within the PRLR gene (Hu et al. 1996, 2002, Ormandy et al. 1998).

We identified that lung, muscle, diaphragm, lymph nodes, thymus, and heart contained barely-detectable levels of pPRLR-LF mRNA during gestation while endometrium, adrenal, and mammary glands had the highest levels. These profiles of PRLR mRNA abundance across different tissues at different stages of development are essentially the same as reported for human tissues (Peirce & Chen 2001, Trott et al. 2003). Similar to our findings in pigs, rabbits express low levels of PRLR in the liver whereas the levels in the ovary are high (Dusanter-Fourt et al. 1991). By comparison, some of the highest levels of the PRLR-LF are found in the liver.
and ovaries of rats (Nagano & Kelly 1994). Consistent with our previous work (Hovey et al. 2001), we now show that pPRLR levels are differentially-regulated during development in a tissue-specific manner, thereby precluding a direct comparison of PRLR levels in various tissues across species. However, the question does remain as to whether there are tissue-specific roles for the PRLR within and between species.

Several groups have proposed that the extrapituitary synthesis of PRL may serve as a local mechanism for PRLR activation (Clevenger & Plank 1997, Ormandy et al. 2003). Within this study we detected a tendency (P=0·07) for PRL gene expression in the pituitary to increase linearly during gestation, whereas PRL gene expression was constant in all other tissues. The dramatic rise in serum PRL during late gestation to a peak of ~100–200 ng/ml around parturition generally begins around d 105, when levels are still only ~15 ng/ml. However, there is a tendency for levels to rise from ~d 70–75 to ~d 105–110 (DeHoff et al. 1986, Farmer et al. 2000). Thus, the linear increase in pituitary PRL mRNA expression that we observed from d 25 to d 105 of gestation may be reflected by this small rise in serum PRL later in gestation prior to the peak at parturition. During gestation there was a tissue-specific increase in pPRLR-LF expression within the endometrium, hypothalamus and mammary gland without any change in their expression of pPRL mRNA. These data suggest that endocrine PRL is likely the major effector of PRL action on peripheral tissues in pigs, and that responsiveness to PRL is conferred primarily via tissue-specific changes in pPRLR-LF transcription and translation.

Also noteworthy is our finding that PRL gene expression in the placenta of gestating pigs was no higher than in other extrapituitary tissues. This finding affirms limited evidence that the pig placenta is not a major source of lactogenic hormones (Forsyth 1974, DeHoff 1986), where these determinations were made using either radioreceptor assays or by co-culturing placenta with mouse or rabbit lobuloalveolar tissue and measuring a lactogenic response. The low levels of PRL mRNA that we measured in placenta does not preclude the synthesis of other PRL-related proteins by this tissue, although other evidence also indicates that pigs depend on pituitary PRL to maintain their corpora lutea and pregnancy (Anderson et al. 1967). Certainly the rise and fall in placental PRLR mRNA levels during gestation, as also occurs for PRLR in rat decidual tissue (Jayatilak & Gibori 1986, Gu et al. 1996), suggests that this tissue is differentially responsive to PRL during pregnancy. What remains unclear is how, or why, PRL transcription is suppressed in the placenta of pigs, given that many other species express a relative abundance of PRL in this tissue (Prigent-Tessier et al. 1999, Soares 2004). Likewise, the potential role for the placenta to produce various PRL-related proteins requires consideration given their more recent identification in other livestock species ( Larson et al. 2006, Ushizawa et al. 2007a,b).

Our in situ hybridization analysis revealed the novel finding that PRL upregulated the expression of PRLR mRNA primarily in the stromal compartment of the mammary gland whereas E2 upregulated its expression specifically in the epithelium. This approach revealed hormone-induced changes that were not apparent in whole tissue analysis by qPCR. While the role for stromal PRLR in the mammary gland is unclear, it apparently does not contribute to branching morphogenesis in mice (Ormandy et al. 2003). On a related note, we did find that pPRLR-LF mRNA was expressed in adipose tissue (data not shown), as occurs in humans (Ling et al. 2000, Zinger et al. 2003). Furthermore, our current data align with our previous demonstration of stage-specific PRL isoform expression in the stroma of mammary glands from neonatal mice (Hovey et al. 2001). Combined, these data support the potential for endocrine PRL to modulate crosstalk between epithelial and stromal cells in the mammary gland, although the precise contribution(s) of this relationship to mammary gland function remains unresolved.

Declaration of interest

The authors have nothing to disclose.

Funding

This project was supported in part by the National Research Initiative Competitive Grant no. 2004-35206-14140 and no. 2008-35206-18895 from the USDA Cooperative State Research, Education, and Extension Service.

Acknowledgements

We wish to thank Dr A F Parlow of the National Hormone & Peptide Program for providing purified pituitary PRL and the rabbit polyclonal α-PRL antiserum and Dr Tom Famula for statistical advice.

References

Agriculture and Agri-Food Canada 1993 Recommended Code of Practice for the Care and Handling of Farm Animals-Pigs. Publ. No. 1898E. Ottawa, ON: Agriculture and Agri-Food Canada.


Received in final form 22 April 2009
Accepted 28 April 2009
Made available online as an Accepted Preprint 28 April 2009