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Characterization and biological activity of relaxin in porcine milk

Amy-Lynn Frankshun, Teh-Yuan Ho, David C Reimer[1], Joseph Chen, Salamia Lasano[2], Bernard G Steinetz[2], Frank F Bartol[3] and Carol A Bagnell

Department of Animal Sciences, Rutgers, The State University of New Jersey, 84 Lipman Drive, New Brunswick, New Jersey 08901, USA, [1]Laboratory Animal Services, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, USA, [2]Nelson Institute of Environmental Medicine, New York University School of Medicine, Tuxedo, New York 10987, USA and [3]Department of Anatomy, Physiology and Pharmacology, Cellular and Molecular Biosciences Program, Auburn University, Auburn, Alabama 36849, USA

Correspondence should be addressed to C A Bagnell; Email: bagnell@aesop.rutgers.edu

C A Bagnell and F F Bartol contributed equally to this work

Abstract

A lactocrine mechanism for delivery of maternally derived relaxin (RLX) into the neonatal circulation as a consequence of nursing was proposed for the pig. Immunoreactive RLX was detected in colostrum and in the serum of newborn pigs only if they were allowed to nurse. Milk-borne RLX concentrations are highest during early lactation (9–19 ng/ml), declining to <2 ng/ml by postnatal day 14. Whether milk-borne RLX is bioactive is unknown. Evidence that RLX concentrations in milk are higher than in maternal circulation in several species suggests the mammary gland as a site of local RLX production. It is unknown whether the porcine mammary gland is a source of RLX. Therefore, objectives were to evaluate RLX bioactivity in porcine milk during the first 2 weeks of lactation, identify the form of RLX in porcine milk, and determine whether mammary tissue from early lactation is a source of milk-borne RLX. Milk RLX bioactivity was determined using an *in vitro* bioassay in which cAMP production by human embryonic kidney (HEK293T) cells transfected with the human RLX receptor (RXFP1) was measured. RLX bioactivity was highest at lactation day (LD) 0, decreasing to undetectable levels by LD 4. Immunoblot analysis of milk proteins revealed an 18 kDa band, indicating proRLX as the primary form of RLX in porcine milk. ProRLX protein and transcripts were detected in porcine mammary tissue on LD 0 and 7. Results support the lactocrine hypothesis by defining the nature and a potential source for bioactive proRLX in porcine colostrum/milk.

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Introduction

Relaxin (RLX), a prototypical milk-borne peptide hormone (Bagnell *et al*. 2009), is found in the colostrum of several species including humans (Eddie *et al*. 1989), dogs (Goldsmith *et al*. 1994), rats (Steinetz *et al*. 2009), and pigs (Yan *et al*. 2006). Immunoreactive RLX is detectable in the systemic circulation of newborn pigs only if they are allowed to nurse (Yan *et al*. 2006). Detection of RLX in both colostrum and the neonatal circulation during the first few days of life correlates with the time of gut closure in pigs, estimated to occur at 24–48 h after birth (Lecce 1973). Similar studies in dogs showed that milk-borne RLX is transmitted from lactating bitches to their offspring via nursing and is absorbed into the systemic circulation (Goldsmith *et al*. 1994). The lactocrine hypothesis for maternal programing of neonatal tissues was proposed to explain how milk-borne RLX, transmitted from mother to offspring as a specific consequence of nursing, is absorbed into the neonatal circulation and acts on RLX receptor (RXFP1)-positive neonatal tissues to affect their development (Bartol *et al*. 2008, Bagnell *et al*. 2009).

RLX bioactivity has been measured using various techniques based on the actions of RLX in target tissues. Fevold *et al*. (1930) devised a guinea pig pubic symphysis palpation bioassay to assess mobility of the pubic symphysis subjectively after RLX-induced separation of the pubic bones. Later, a uterine contractility bioassay was developed based on RLX inhibition of myometrial contractions (Krantz *et al*. 1950). The mouse interpubic ligament bioassay was conceived as a simpler, direct measure of RLX bioactivity since estrogen-primed mice respond to RLX with a dose-dependent lengthening of the interpubic ligament (Kroc *et al*. 1959). In 1990, an *in vitro* bioassay was developed based on RLX stimulation of cAMP production using primary cultures of human endometrial

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cells (Fei *et al*. 1990) and later refined using the human monocytic THP-1 cell line (Parsell *et al*. 1996). Most recently, human embryonic kidney (HEK293T) cells stably transfected with the human RXFP1 (HEK293T-RXFP1) were used to measure cAMP response and RLX bioactivity (Hsu *et al*. 2002).

RLX, a member of the RLX family of peptides (Bathgate *et al*. 2006), is synthesized as single-chain, 23 kDa preproRLX (Gast 1982, 1983, Haley *et al*. 1982) containing a signal peptide, connecting peptide and A and B chains. The signal peptide is cleaved and translocated across the endoplasmic reticulum (Gast 1982, 1983) to form 18 kDa proRLX (Haley *et al*. 1982, Layden & Tregear 1996). Further processing of the connecting peptide results in the native 6 kDa RLX molecule found in circulation (O'Byrne *et al*. 1989). The amino acid motif Arg-X-X-X-Arg-X-X-Ile, located in the middle of the B chain, is required for RLX bioactivity (Bullesbach & Schwabe 2000).

Sources of RLX are species-specific. Whereas RLX is synthesized primarily by corpora lutea in the pig (Sherwood & O'Byrne 1974) and rat (Sherwood 1979), in other species, it is a product of the uterus (Pardo *et al*. 1980) and placenta (Eldridge & Fields 1985, Stewart & Papkoff 1986). There is also evidence for local RLX production in other tissues (Bryant-Greenwood *et al*. 1980, Sakbun *et al*. 1990). Evidence that RLX concentrations in milk are higher than in the maternal circulation (Sherwood *et al*. 1981, Eddie *et al*. 1989, Goldsmith *et al*. 1994) suggests that RLX is either produced locally by the mammary gland or synthesized elsewhere and concentrated in milk.

Support for the mammary gland as a source of RLX comes from immunolocalization studies in the guinea pig mammary gland (Peaker *et al*. 1989) and in human breast tissue (Mazoujian & Bryant-Greenwood 1990). RLX transcripts were also detected in the rat mammary gland of late pregnancy and early lactation (Gunnersen *et al*. 1995), in normal and neoplastic human breast tissue (Tashima *et al*. 1994), and in neonatal pig adipose tissue (Hausman *et al*. 2006). Whether porcine mammary tissue is a site for RLX production is unknown. To confirm and extend previous studies (Frankshun *et al*. 2009), objectives here were to evaluate RLX bioactivity in porcine milk during the first 2 weeks of lactation using an *in vitro* bioassay, identify the form of RLX found in porcine colostrum/milk, and determine whether porcine mammary tissue could serve as a source of milk-borne RLX.

Results

Validation of the cell-based RLX bioassay

Validation of the cell-based RLX bioassay involved evaluation of cell system response to increasing amounts of porcine RLX standard added to milk extract that had low RLX concentrations (LD 14 milk). A dose-dependent increase in cAMP production by transformed HEK293T-RXFP1 cells was observed consistently in response to increasing concentrations of porcine RLX (Fig. 1). A test for heterogeneity of regression between lines representing porcine RLX in assay buffer and LD 14 milk was not significant. This indicated similar patterns of increase in cAMP production by transformed HEK293T-RXFP1 cells with the addition of RLX, providing no evidence against parallelism (Fig. 1). The area under each curve was calculated and revealed a quantitative recovery of 79.6%. Defined as the minimal RLX concentration required to elicit a detectable cAMP response, sensitivity of the assay was determined to be 100 pg RLX. Production of cAMP by HEK293T-RXFP1 cells was not affected when these transfected cells were incubated with either insulin or insulin-like growth factor 1 (IGF1; data not shown). Consistently, no effect on cAMP production was detected when non-transfected HEK293T cells were incubated with porcine RLX (Frankshun *et al*. 2009). Collectively, these data show assay specificity.

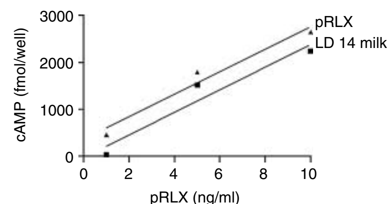

Figure 1 Determination of assay recovery for the transformed HEK293T cell-based RLX bioassay. Increasing amounts of porcine RLX were added to lactation day (LD) 14 porcine milk extract and compared to effects of a porcine RLX standard when added in increasing concentrations to the HEK293T cell bioassay. A test of heterogeneity of regression between the lines was not significant ($P>0.05$), indicating similar effects of increasing milk extract on transformed HEK293T cell cAMP generation to those observed for the porcine RLX standard.

Biological activity of RLX in porcine milk during the first 2 weeks of lactation

The cAMP response of the HEK293T-RXFP1 cell line was used to test the biological activity of milk-borne RLX as illustrated in Fig. 2. Milk from LD 0 produced an average of 3040 ± 229 fmol/well of cAMP, indicating the presence of bioactive RLX in these milk extracts. RLX bioactivity decreased ($P<0.05$) after LD 0 and was undetectable by LD 4. This bioactivity pattern was similar to that observed for immunoreactive RLX concentrations in the same milk extracts (Fig. 2, inset). Immunoreactive RLX concentrations in milk extracts were highest on LD 0, with an average concentration of 15.4 ng/ml (± 2.2 ng/ml). Milk RLX concentrations declined ($P<0.01$) from LD 0, averaging <1 ng/ml on LD 14 (Fig. 2, inset).

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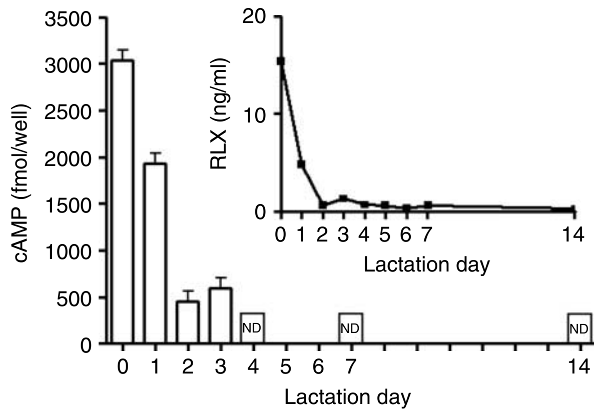

Figure 2 Relaxin bioactivity patterns in porcine milk extracts during the first 2 weeks of lactation relative to RLX concentrations determined by RIA (inset). Relaxin bioactivity decreased ($P<0.05$) within the first few days of lactation and fell ($P<0.05$) to non-detectable (ND) levels by day 4 of lactation. The bioactivity pattern was reflected by data for immunoreactive RLX concentrations in the same milk extract samples (inset). Data are expressed as LSM±S.E.M. $N=3$ sows/group.

Prorelaxin in porcine milk

An 18 kDa band, corresponding in size to proRLX (Layden & Tregear 1996), was identified in porcine milk obtained on LD 0–4, 7, and 14, with no evidence of the 6 kDa native RLX molecule (Fig. 3A). Bands indicative of both 18 kDa proRLX and 6 kDa native RLX molecules were observed for luteal tissue protein extract used as a positive control. Results for detection of immunoreactive RLX in milk extracts and luteal tissue were similar when using either the Gg or R6 anti-porcine RLX antisera; thus, only data generated using the Gg antiserum are shown (Fig. 3A). Neither form of RLX was detected in the milk replacer used as a negative control. Densitometric analysis indicated that relative levels of immunoreactive proRLX in porcine milk were highest on LD 0, decreased curvilinearly thereafter through LD 4, following a cubic pattern ($Y=-957.49x+335.96x^2-39.60x^3+1302.664$; $P<0.01$; $R^2=84\%$), and remained low through LD 14 (Fig. 3B).

Prorelaxin protein and mRNA expression in porcine mammary tissue

Immunoblot analyses of porcine mammary tissues from LD 0 and 7 revealed an 18 kDa band, corresponding in size to porcine proRLX, with no evidence of the 6 kDa native RLX molecule (Fig. 4A). Relative levels of proRLX protein in porcine mammary tissue decreased from LD 0 to 7 ($P<0.05$). Again, porcine luteal tissue protein extract contained both 18 and 6 kDa molecules, corresponding to pro- and native RLX. Cervical tissue protein from postnatal day 0, used as a negative control, showed no evidence of either 18 or 6 kDa forms of RLX. QPCR data indicating *RLX* mRNA expression by porcine mammary tissue from LD 0 and 7 are shown in Fig. 4B. There was no difference in relative levels of *RLX* mRNA expression between LD 0 and 7.

Discussion

Immunoreactive RLX was first identified in human colostrum within 3 days of parturition and in breast milk up to 6 weeks *post partum* (Eddie *et al.* 1989). Since then, RLX has been detected in the colostrum/milk of several species (Goldsmith *et al.* 1994, Steinetz *et al.* 2009) including the pig (Yan *et al.* 2006). Evidence that RLX concentrations in milk are higher than in the maternal circulation during lactation (Sherwood *et al.* 1981, Eddie *et al.* 1989, Goldsmith *et al.* 1994) led to the suggestion that RLX is either produced locally by the mammary gland or synthesized elsewhere and concentrated in milk (Eddie *et al.* 1989). Recently, the lactocrine hypothesis for maternal programing of neonatal tissues was proposed to explain how RLX, a prototypical milk-borne growth factor communicated from mother to offspring as a consequence of nursing, is absorbed into the neonatal circulation and acts on target tissues to affect their development (Yan *et al.* 2006, Bartol *et al.* 2008). This study was designed to determine whether RLX in porcine colostrum/milk is biologically active, identify the primary form of RLX present in porcine colostrum/milk, and establish whether the porcine

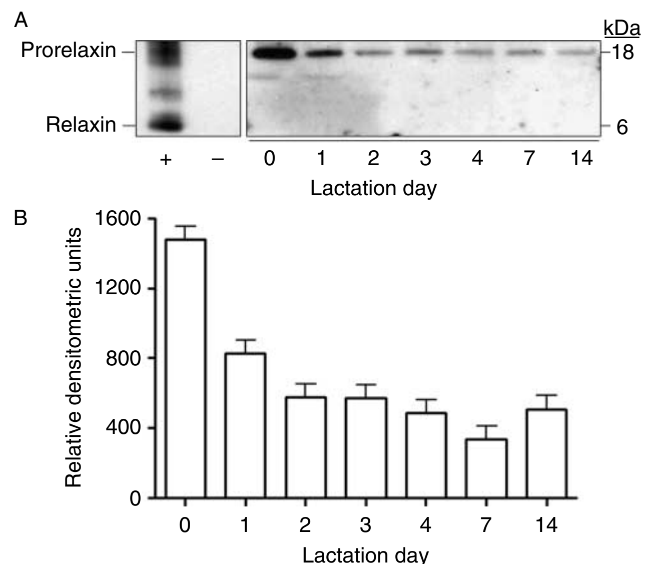

Figure 3 Immunoreactive proRLX in sow milk extracts from lactation days 0–4, 7, and 14. (A) Representative immunoblot indicating the presence of 18 kDa proRLX. (+) Positive control, pregnant sow luteal tissue extract. (−) Negative control, milk replacer. (B) Relative expression of proRLX was quantified by densitometry and expressed as relative densitometric units. LSM±S.E.M. $N=4$ animals per group. The decrease in relative abundance of proRLX between days 0 and 4 was best described by a cubic function ($Y=-957.49x+335.96x^2-39.60x^3+1302.664$; $P<0.01$; $R^2=84\%$).

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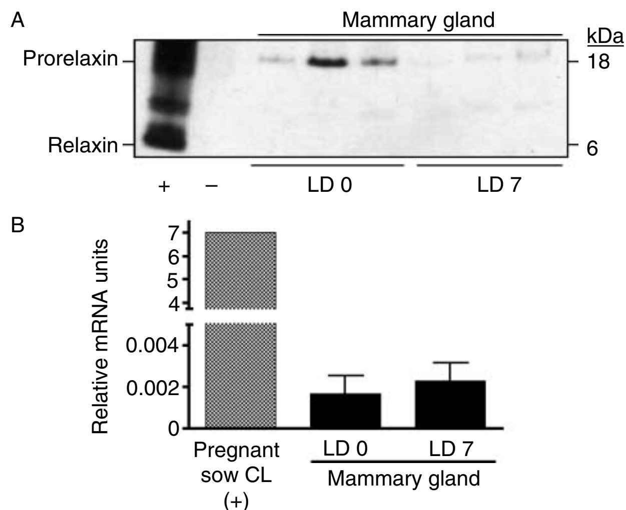

Figure 4 Prorelaxin protein and mRNA expression in sow mammary tissue from lactation days 0 and 7. (A) Immunoblot analysis of immunoreactive proRLX (18 kDa) in sow mammary tissue. (+) Positive control, pregnant sow corpus luteum. (−) Negative control, PND 0 gilt cervix. (B) Sow mammary *RLX* mRNA expression. Data are expressed as LSM±S.E.M. N=3 sows/group.

mammary gland might serve as an endogenous source of lactocrine-active RLX.

When immunoreactive porcine RLX was detected in porcine milk, it was hypothesized that such RLX was biologically active (Yan *et al.* 2006, Frankshun *et al.* 2009). This was based on the fact that the antibody used in the porcine RLX RIA detected only intact RLX, with A and B chains linked by disulfide bonds, and did not detect isolated peptide chains or reduced-alkylated RLX (O'Byrne & Steinetz 1976). Results presented here support that hypothesis by showing that RLX bioactivity was highest in milk from LD 0 and decreased within the first few days of lactation, dropping below bioassay sensitivity by LD 4. This bioactivity pattern was similar to the pattern of immunoreactive RLX concentrations determined for the same milk samples (Fig. 2). The decline in both RLX bioactivity and RLX concentrations in milk during early lactation correlates with the time of gut closure, estimated to occur between 24 and 48 h after birth in pigs (Lecce 1973). RLX concentrations in whole porcine milk were ∼17 ng/ml on LD 0 and dropped to about 2 ng/ml by LD 14 (Yan *et al.* 2006). Results are similar to those reported for dogs, in which biologically active milk-borne RLX was detected at relatively high concentrations (∼13–15 μg/ml) on LD 0, dropping to <100 ng/ml by week 4 of lactation (Goldsmith *et al.* 1994, Steinetz *et al.* 2008). Similarly, in pregnant women at term, mean RLX concentrations in milk decreased between 3 days and 6 weeks of lactation (Eddie *et al.* 1989). Collectively, results are consistent with present findings indicating that biologically active proRLX is present in porcine milk and available for lactocrine transmission to neonatal pigs.

Results of this study show clearly that the 18 kDa proRLX molecule is the primary form of RLX in porcine milk. No evidence was obtained indicating conversion of proRLX to the 6 kDa native form of RLX. Given that proRLX in porcine milk induced cAMP production in the cell-based *in vitro* bioassay used here, results suggest that cleavage of the RLX-connecting peptide is not necessary for biological activity of porcine RLX. Results agree with data published for other species. Recombinant equine proRLX secreted by the MAC-T mammary epithelial cell line (Neumann *et al.* 2006) and recombinant marmoset proRLX (Zarreh-Hoshyari-Khah *et al.* 2001) produced by insect cells were both biologically active, with no evidence for conversion to the 6 kDa form. Additionally, recombinant rat proRLX produced by COS cells (Soloff *et al.* 1992), recombinant porcine proRLX expressed by CHO cells (Vu *et al.* 1993), and recombinant human proRLX expressed by canine and human mammary cancer cells (Silvertown *et al.* 2003) were all biologically active and not processed to the native 6 kDa RLX molecule. In this regard, RLX is similar to IGF1 in that the pro-form of IGF1 displays bioactivity, but differs from insulin since proinsulin has no biological activity (Peavy *et al.* 1984). Whether secretion of proRLX into milk is a means of encrypting the RLX protein to extend its half-life, as reported for other milk peptides (Meisel 2005), is unknown.

With respect to the molecular size of porcine RLX found in circulation, multiple forms of immunoreactive RLX were identified in serum obtained from pregnant sows, including an 18 kDa putative pro-form (Bryant-Greenwood *et al.* 1980). However, subsequent studies (O'Byrne *et al.* 1989) suggested that plasma RLX found in pregnant pigs immediately prepartum is secreted by the corpus luteum in a biologically active 6 kDa form, with no evidence of higher molecular weight precursors. O'Byrne *et al.* (1989) used the rabbit anti-porcine R6 antiserum to detect native RLX in serum obtained from pregnant sows on gestational days 112–114. The R6 antiserum detects both 6 kDa native RLX, as indicated above, and high molecular weight RLX precursors of porcine luteal origin (M J Gast, personal communication). Results presented here confirm this observation, showing that both high (18 kDa) and low (6 kDa) molecular weight forms of RLX were detectable in luteal tissue extracts by immunoblotting using the R6 antiserum. These observations support the idea that 18 kDa proRLX is the primary form of RLX likely to be produced by porcine mammary tissue and present in porcine colostrum.

Whether mammary tissue is a source of RLX or is produced elsewhere and concentrated from the maternal circulation in the porcine mammary gland is unknown. Studies in the guinea pig, human, dog, and rat indicated the mammary gland as a source of RLX. RLX was immunolocalized in epithelial cells that form the mammary duct system in guinea pigs (Peaker *et al.*

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1989), and expression of both H1 and H2 human RLX genes was detected in normal and neoplastic breast tissue (Tashima *et al.* 1994) providing support for local synthesis of RLX by breast tissue. Also, milk obtained from dogs that were ovariohysterectomized at term contained as much RLX as did milk of intact dogs through 5 weeks of lactation. Thus, in dogs, the source of RLX in milk cannot be the ovaries or uterus and is likely to be the mammary gland (Goldsmith *et al.* 1994). Treatment of rats with a monoclonal anti-rat RLX prevented ovarian RLX from reaching the mammary glands via the circulation but did not affect milk RLX concentrations, suggesting that the RLX detected in rat milk is primarily a product of the mammary glands and is not derived from circulating ovarian RLX (Steinetz *et al.* 2009). Results presented here indicate that RLX gene and protein expression are low in the porcine mammary gland during early lactation. These results support previous reports of low levels of maternal plasma RLX (0.4 ng/ml) by 37 h *post partum* in pigs (Sherwood *et al.* 1981). It is possible that the proRLX protein detected at LD 0 and 7 by immunoblot represents residual protein retained in tissues from mammary secretions at the time of biopsy. Data for milk RLX concentrations in ovariohysterectomized sows have not been reported and would help in determining the source of milk-borne RLX in pigs.

The possibility that milk-borne RLX found in colostrum during early lactation is coming from the corpus luteum and not from the mammary gland cannot be ruled out. RLX gene expression was detected in the corpus luteum at LD 2 by both northern blot analysis and *in situ* hybridization (Bagnell *et al.* 1990). In addition, RLX protein was localized in luteal cells throughout the corpus luteum during the first 2 weeks of lactation (Bagnell *et al.* 1987). However, if the corpus luteum is the source of milk-borne RLX, delivered via maternal circulation to be concentrated in the mammary gland, the 18 kDa proRLX molecule should be detectable in maternal circulation. This is not the case. Only the 6 kDa form of RLX has been detected in maternal serum immediately prepartum (O'Byrne *et al.* 1989), and only the 18 kDa proRLX is detectable in porcine milk. Together, these results suggest that proRLX detected in milk does not come from the maternal circulation, but is produced locally as proRLX by mammary tissue.

Results presented here support and extend previous reports of bioactive proRLX in porcine milk that is available for delivery from mother to offspring via nursing (Yan *et al.* 2006, Frankshun *et al.* 2009). Given that neonatal porcine milk intake is estimated at up to 30% of body weight per day (Coalson & Lecce 1973) and that concentrations of RLX in porcine milk within 48 h of birth range from 5 to 15 ng/ml, individual neonates consume microgram quantities of potentially lactocrine-active RLX within 2 days of birth (Yan *et al.* 2006, Bagnell *et al.* 2009). Since RLX has trophic effects on both uterine and cervical tissues at PND 2 (Yan *et al.* 2005, 2008) and RXFP1 is expressed by porcine uterine (Yan *et al.* 2006) and cervical tissues from birth (Yan *et al.* 2008), it is reasonable to hypothesize a role for lactocrine-active RLX in development of the neonatal female porcine reproductive tract. The stage is now set for additional studies designed to test the lactocrine hypothesis and to determine specific effects of lactocrine-active RLX on reproductive development in the neonatal gilt.

Materials and Methods

Materials

HEK293T cells were obtained from the American Type Culture Collection (ATCC no. SD-3515, Manassas, VA, USA). The HEK293T-RXFP1 cell line and anti-porcine RLX serum (Gg) were generous gifts from Dr Gillian Bryant-Greenwood (University of Hawaii, Honolulu, HI, USA). In addition, a second anti-porcine RLX serum, R6, was used for both RIA and immunoblotting. Purified porcine RLX was kindly donated by Dr David Sherwood (University of Illinois, Urbana, IL, USA). Advanced DMEM-F-12 was purchased from Gibco. Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). Antibiotics were purchased from Invitrogen. TRI Reagent and 3-isobutyl-1-methylxanthine were obtained from Sigma–Aldrich. The Amersham cAMP Biotrak Enzyme Immunoassay System was purchased from GE Healthcare (Piscataway, NJ, USA). Xylazine, telazol, yohimbine, and lidocaine were purchased from Henry Schein (Melville, NY, USA). RNeasy Mini kits and RNase-Free DNase Sets 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). Primers were synthesized by Sigma–Aldrich. Detergent-compatible protein assay kits (DC Protein Assay) were obtained from Bio-Rad Laboratories. HRP-conjugated anti-rabbit secondary antibody was purchased from Zymed (Carlsbad, CA, USA). Tricine gels (10–20%) were obtained from Invitrogen. Nitrocellulose membranes were obtained from Bio-Rad Laboratories. The Renaissance Western Blot Chemiluminescence Reagent Plus kits were acquired from Perkin Elmer Life Sciences (Waltham, MA, USA). XOmatic films were purchased from American Imaging (South Plainfield, NJ, USA).

Porcine milk collection, extraction, and mammary gland biopsy

Milk was collected as previously described (Frankshun *et al.* 2009). Briefly, milk from purebred Yorkshire sows ($N=4$ sows/time point) was collected manually at the time of parturition (lactation day (LD) 0), daily through LD 7, and on LD 14. In addition, a commercial milk replacer for pigs (Advance Liqui-Wean; Milk Specialties, Carpentersville, IL, USA) was tested. Individual milk and milk replacer samples were diluted 1:1 with 200 mM EDTA (pH 7.0) to solubilize caseins and centrifuged (15 min at 4000 ***g*** at 4 °C) to remove fat (Van Cott *et al.* 1996).

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The supernatant, herein referred to as milk extract, was stored at −20 °C until subjected to further testing.

Mammary gland biopsies were collected from the same three lactating sows on LD 0 and LD 7. Sows were anesthetized with a combination of xylazine (2.2 mg/kg body weight (BW)) and telazol (5.0 mg/kg BW) delivered i.m. (Ko *et al*. 1993). The site of biopsy collection was prepared with surgical scrub (betadine and ethanol), and a local anesthetic (2% lidocaine) was applied in a ring around the site of biopsy. A one inch keyhole incision was made to expose mammary gland tissue, and a punch biopsy was used to collect the mammary tissue followed by suturing the transected area in order to control milk loss and facilitate healing. The biopsy wound site was closed intradermally using 3-0 absorbable suture. A portion of the biopsied mammary tissue was fixed in 4% paraformaldehyde for histology, and the remainder was frozen in RNA Later (Qiagen) for molecular analysis. Yohimbine (0.1 mg/kg BW i.m.) was administered to stimulate recovery from surgery. All procedures involving animals were reviewed and approved by the Rutgers University Animal Care and Facilities Committee (Protocol #88-079), and were performed in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (2010; Federation of Animal Science Societies, Champaign, IL, USA).

RLX in vitro *bioassay and validation*

HEK293T-RXFP1 cells were grown in DMEM-F-12 containing 10% FBS, 1% penicillin–streptomycin–glutamine, and 1 mg/ml geneticin. Cells (2×10^5 cells/well) were cultured overnight in a 96-well culture plate and then pretreated with 0.25 mM 3-isobutyl-1-methylxanthine for 20 min at 37 °C (Kern *et al*. 2007). After incubation (30 min at 37 °C) with milk extracts from LD 0–7 and 14 (20 µl), cells were lysed and total cellular cAMP was determined using the Amersham cAMP Biotrak enzyme immunoassay system. cAMP standards were run in duplicate, and unknown samples were run in triplicate. Inter- and intra-assay coefficients of variation (CV) were 6.3 ± 1.0 and 5.3 ± 1.5% respectively. Dose dependence, sensitivity, and specificity of the RLX bioassay were reported previously (Frankshun *et al*. 2009). Assay recovery was determined by the addition of known amounts of porcine RLX standard (1, 5, and 10 ng/ml) to LD 14 milk extracts and compared to a porcine RLX standard curve. All assay results were corrected for assay dilution effects.

RLX RIA

Immunoreactive RLX in milk extracts from LD 0–7 and LD 14 was measured using a homologous porcine RLX RIA validated for use with milk (Yan *et al*. 2006). Purified porcine RLX was used as the standard, and ^{125}I-labeled tyrosyl porcine RLX was used as the radioligand. Antiserum R6 was used at a dilution of 1:20 000, and all standards and unknown samples were run in triplicate at a final volume of 500 µl of PBS (pH 7.0) containing 1% BSA before the addition of antiserum and the radioligand. Inter- and intra-assay CV were 4.7 ± 0.8 and 1.0 ± 0.3% respectively.

Western blot analysis

Proteins (20–30 µg) in milk extracts, milk replacer extract, sow mammary tissue, and pregnant sow corpus luteum (500 ng), used as a positive control for RLX protein, were resolved on non-reducing 10–20% Tricine gels under denaturing conditions by SDS-PAGE followed by transfer onto nitrocellulose membranes (0.2 µm pore size) via electroblotting. Membranes were blocked with 10% nonfat milk powder (NFMP) 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) for 1 h at room temperature and then incubated with rabbit anti-porcine RLX serum (Gg or R6; 1:5000) in TBST–1% NFMP overnight at 4 °C. Membranes were washed with TBST and incubated with goat anti-rabbit IgG–HRP conjugate (1:10 000 in TBST–5% NFMP) for 30 min at room temperature. After washing in TBST, membrane-bound antibodies were detected by ECL according to the manufacturer recommendations. Protein loading was monitored using actin as a reference. Chemiluminescence signals were quantified densitometrically from film using Scion Image for Windows (Scion Corporation, Frederick, MD, USA).

RNA isolation, cDNA generation, and real-time RT-PCR

Total RNA was isolated as previously described (Chen *et al*. 2010). Briefly, total RNA was isolated from mammary tissue (50 mg) using TRI Reagent and the RNeasy Mini kit. RT was performed with 500 ng total 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 the manufacturer's instructions.

Real-time RT-PCR (quantitative PCR, qPCR) was performed using an Applied Biosystems Gene Amp 7000 Sequence Detection System (Applied Biosystems) with the SYBR Green method following the universal thermal cycling parameters indicated by the manufacturer as previously described (Chen *et al*. 2010). Primers for qPCR were designed using Primer Express Software (Applied Biosystems). For porcine RLX, the forward primer sequence was 5′-GCATGCGGCCGAGAATT-3′, and the reverse primer sequence was 5′-CAGGAGACGGAGCCACAGAT-3′ with an expected amplicon size of 59. For porcine 18S, the forward primer sequence was 5′-CCGCGGTTCTATTTTGTTGGTTTT-3′, and the reverse primer sequence was 5′-CGGGCCGGGTGAGGTTTC-3′ with an expected amplicon size of 399. To ensure specific amplification, controls including water only, no primers, and no template were included in the assay. Primer quality was evaluated, and dissociation curves for each primer set were checked. Data were analyzed using the relative standard curve method for quantitation of gene expression as described by Applied Biosystems (ABI User Bulletin 2, 2001). Standard curves were generated for each gene using twofold dilutions of cDNA from porcine corpus luteum of pregnancy. Target gene expression was normalized to the expression of porcine 18S, and data from qPCR analysis were presented as relative mRNA units.

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Statistical analyses

All data were subjected to ANOVA using general linear model procedures available with SAS (SAS 2009–2010, Cary, NC, USA). For assay validation data, statistical models were tested for heterogeneity of regression and considered effects of dose of RLX. For relative proRLX protein expression in milk data, statistical models accounted for variation due to sow, and best-fit least-squares regression was identified. Preplanned contrasts were performed to identify discrete effects of day: (a) LD 4 vs LD 7+LD 14; and (b) LD 7 versus LD 14. For mammary tissue protein and gene expression data, statistical models accounted for variation due to day. Error terms were identified based upon the expectations of the mean squares for error, and data were expressed as least-squares means with S.E.M.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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