Changes in content of mRNA encoding oxytocin in the pig uterus during the oestrous cycle, pregnancy, at parturition and in lactational anoestrus

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The aim of this study was to show that the pig uterus synthesizes oxytocin. Uteri were obtained from 2–7 pigs at regular intervals during the oestrous cycle, throughout pregnancy, at parturition and in lactational anoestrus. Localization of mRNA encoding oxytocin was by in situ hybridization and oxytocin concentrations were measured by radioimmunoassay. As reproductive status changed, mRNA encoding oxytocin varied significantly (P < 0.05). Uterine tissue type was a significant factor in determining synthesis of mRNA encoding oxytocin (P < 0.001). In luminal epithelia, concentrations of mRNA encoding oxytocin were greater at oestrus than during day 14 of the luteal phase (P < 0.01) or at any stage of pregnancy (P < 0.05), with concentrations minimal at parturition. This trend was also exhibited in uterine circular muscle. In longitudinal muscle, concentrations of mRNA encoding oxytocin were lower during late pregnancy than at oestrus (P < 0.05) or during the luteal phase (P < 0.05). Concentrations were minimal at parturition. The oxytocin content in endometrial and myometrial tissue was positively correlated across reproductive status (P < 0.02, r = 0.402, n = 35). These data are the first indication that the uterine endometrium and musculature of the pig express mRNA encoding oxytocin. The luminal epithelium of animals at oestrus was particularly rich in mRNA encoding oxytocin, whilst late pregnant and parturient animals did not show a rise in mRNA encoding oxytocin. Local uterine synthesis of oxytocin may therefore be more important in control of the oestrous cycle than in pregnancy or at parturition in pigs.

Introduction

Oxytocin is thought to act in an endocrine manner during maternal recognition of pregnancy and at parturition (see Flint 1995 for review). In recent years, paracrine roles for oxytocin have been proposed since its synthesis has been demonstrated in peripheral sites such as the thymus (Geenen et al., 1987), adrenals (Hawthorn et al., 1987), placenta (Lefebvre et al., 1992a) and ovary (Ott and Scott, 1910; Flint and Sheldrick, 1982; Wathes et al., 1983). Uterine mRNA encoding oxytocin has been reported in rats (Lefebvre et al., 1992b) and humans (Chibbar et al., 1993) but was not present in pregnant sheep (Wathes et al., 1996). The role of uterine oxytocin synthesis remains unclear but it has been suggested that this oxytocin may obviate the need for oxytocin of neurohypophyseal origin to act as an initiating stimulus for parturition (Wathes et al., 1996) since endometrial and placental oxytocin concentrations increase at term in rats and humans (Lefebvre et al., 1992b; Chibbar et al., 1993).

The aims of this study were to determine whether the pig uterus synthesizes mRNA encoding oxytocin and, if so, to investigate how the concentration varies according to reproductive status.

Materials and Methods

Animals

Animals from the Babraham Large White pedigree herd were used in this study. These animals (gilts n = 28 and sows n = 14) were reared under minimal disease conditions and were either kept in groups in straw yards, or individually penned with wood shavings. In all cases, water was available ad libitum and the pigs were fed on a sow’s ration, as dictated by normal husbandry practice.

Cyclic animals were allowed daily contact with a boar and monitored for signs of behavioural oestrus (day 0). If mating
was not allowed, animals were slaughtered on day 0 \((n = 6)\),
day 7 \((n = 3)\) or day 14 \((n = 6)\) of the oestrous cycle. Other
animals were mated twice and killed at a known stage of
pregnancy \(\text{early pregnant, 20–40 days, } n = 7; \text{ mid-pregnant, 41–80 days, } n = 3; \text{ late pregnant, 81–112 days, } n = 5; \text{ parturient, the birth of at least one piglet, } n = 2\), or approximately 7
weeks after birth when still in lactational anoestrous \((n = 4)\) to
obtain the uterus and \(n = 6\) to obtain brain tissue as a positive
control). The stage of pregnancy was confirmed by the size of
the fetuses \((\text{Binns, 1969})\). Whenever possible, tissues were
obtained from animals that were killed for clinical or managerial
reasons and, in all cases, animals were killed for reasons
unrelated to reproductive performance. The animals were killed
by an intravenous injection of Lethobarb \((\text{Solvay Duphar }
\text{Veterinary, Southampton, UK})\).

**In situ hybridization**

Immediately after the death of the animal, the uterus was
removed under sterile conditions, cut longitudinally into
sections 2 cm thick and frozen in iso-pentane \((\text{BDH, Poole})\) on
liquid nitrogen. The frozen sections were transported on dry
ice and stored at \(-80^\circ\text{C}\) until required. The uterus was
sectioned \((12 \mu\text{m} \text{ thick})\) on a cryostat \((\text{Bright Instruments Ltd., }
\text{Huntingdon})\), freeze-thaw mounted onto poly-l-lysine coated
slides and air dried. Slides were fixed in 4% \((w/v)\) phosphate-
buffered paraformaldehyde \(pH 7.4\) \((4^\circ\text{C for 5 min})\), washed
three times in PBS \((1–4 \text{ min})\), dehydrated in an ascending
ethanol series and stored at \(-80^\circ\text{C}\) in air tight containers until
use.

**In situ hybridization** was carried out as described by
\text{Sirinathsinghji et al. (1990)} using a specific \(^{35}\text{S}\)-labelled 45mer
antisense oligonucleotide probe complementary to bases
771–816 of the ovine oxytocin–neurophysin 1 cDNA gene
\((\text{Ivell et al., 1990})\). This antisense oligonucleotide probe was
shown to be specific by Northern analysis \((\text{Broad et al., 1993})\).
The gene encoding oxytocin is highly conserved between
species such that the oligonucleotide probe derived from ovine
oxytocin–neurophysin 1 cDNA gene was 91% homologous to
the pig oxytocin gene sequence \((\text{bases 92–137; \text{Rehbein and}
\text{Richter, 1990})\). The complementary sense sequence was also
hybridized under identical conditions and used to determine
the nonspecific hybridization. The oligonucleotide probe was
labelled using deoxynucleotidyl transferase \((\text{Gibco, Paisley})\) and
\(^{[35}\text{S}]\text{dATP (NEN)}, \text{purified using Sephadex G-50 exclusion}
gel columns and diluted to a concentration of \(3 \times 10^4 \text{ d.p.m.}
\mu l^{-1} \text{ (v/v)}\) in a hybridization buffer containing 4 x standard
saline citrate \((\text{SSC}), 50\% \text{ deionised formamide (Sigma, Poole)}, \text{50 mmol sodium phosphate}
\((\text{pH 7.0}), 10 \text{ mmol sodium pyrophosphate}
\((\text{pH 7.0}), 5 \text{ x Denhardt's solution (Sigma), 200 }\mu \text{g salmon sperm}
\text{DNA ml}^{-1} \text{ (Sigma), 100 }\mu \text{g polyadenylic acid ml}^{-1}, 40 \text{ mmol diithiothreitol}
\text{ml}^{-1} \text{ (Sigma) and 10} \% \text{ (w/v) dextran sulphate (Pharmacia, Upsala). Sections}
\text{were left overnight in an incubator at } 42^\circ\text{C and then washed}
twice in } 1 \times \text{ SSC, once at room temperature and once at 55^\circ\text{C for}
30 \text{ min each. Sections were briefly washed in } 1 \times \text{ SSC then}
0.1 \times \text{ SSC, dehydrated in an ascending alcohol series, dried and}
exposed to X-ray film (Kodak XAR-5, Hemel Hempstead) for
21 days. Sections required for quantification were dipped in K-5
liquid emulsion \((\text{Ilford, London})\) and stored at 4°C in lightproof
boxes containing silica gel for 2 weeks. Sections were then
developed in phenisol \((\text{Ilford}), \text{fixed and counterstained with}
\text{haematoxylin and eosin).}

**Quantification of in situ hybridization**

Each emulsion dipped slide \((\text{three replicates per animal})\) was
examined under the microscope \((\times 40 \text{ magnification})\) to con-
firm the tissue type being quantified. Hybridization signal was
visualized from the autoradiographs by using the Macroauto-
radiography package for densitometric analysis \((\text{Seescan,}
\text{Cambridge})\). The autoradiographs were projected onto a computer
screen and representative areas from each tissue type
were outlined on each section. The computer program sub-
tracted the background optical density of blank film and then
calculated the optical density for the encircled areas \((\text{six}
readings per tissue type, per slide). The optical density readings
were converted into concentrations \((\text{nCi g}^{-1})\) by the computer
when they were calculated against \(^{14}\text{C} \text{standards (ARC Ltd., St.}
\text{Louis MO) on a linear scale ranging from 8.0 to 293.0 nCi g}^{-1}\.}
The uterine tissue types examined were longitudinal muscle, circular muscle, luminal epithelium and endometrial stroma (including uterine glands). Sections of brain tissue (hypothalamus) were hybridized with the mRNA encoding oxytocin sense or antisense probe and used as negative and positive controls, respectively. The specificity and distribution of the hybridized antisense oligonucleotide probe in the hypothalamus was observed but the signal strength was not quantified.

Statistical analysis of in situ hybridization results

Mean concentrations of mRNA encoding oxytocin were calculated from 18 readings per tissue per pig (derived from six
readings per tissue type for three slides). The effect of group, tissue type and group–tissue type interaction for the concentrations of mRNA encoding oxytocin were examined using analysis of variance (ANOVA) by Genstat 5 (Rothamstead Experimental Station, IACR, Harpenden). Comparisons to determine levels of significance were achieved by t test.

**Extraction of oxytocin from endometrial and myometrial tissue**

Each sample was removed from the $-80^\circ$C freezer and allowed to partially thaw so that the endometrium and myometrium could be separated using a pair of forceps. Sections were then ground in a percussion mortar. The weight

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**Fig. 3. (a)–(c)**
Concentrations of mRNA encoding oxytocin in the pig uterus

Fig. 3. Expression of mRNA encoding oxytocin in the pig uterus during the oestrous cycle, throughout pregnancy and in lactational anoestrus measured against $^{14}$C standards and expressed as nCi g$^{-1}$ in (a) luminal epithelium. Significant differences: a versus b $P < 0.05$. Values obtained from parturient animals were minimal (that is, below the minimum standard of 8.0 nCi g$^{-1}$). (b) Circular muscle. Significant differences: $P < 0.01$ a versus c; $P < 0.05$ a versus b, c versus d, c versus e. Values obtained from parturient animals were minimal (that is, below the minimum standard of 8.0 nCi g$^{-1}$). (c) Longitudinal muscle. Significant differences as determined by t tests as follows: $P < 0.01$ c versus b; $P < 0.05$ a versus b, b versus e, c versus d. Values obtained from parturient animals were minimal (i.e. below the minimum standard of 8.0 nCi g$^{-1}$). Values are means ± SEM of 2–7 animals at each time point.

of the thawed sample was recorded before it was placed in extraction medium (3 ml 15% trifluoroacetic acid in 1 mol formic acid 1$^{-1}$) and treated according to the method of Wathes et al. (1986). Briefly 60 000 d.p.m. [3H]oxytocin was added and the samples boiled for 5 min in a water bath and centrifuged at 25 000 g for 30 min at 4°C. The supernatant was removed and freeze-dried. The extract was redissolved in 700 µl oxytocin assay buffer and extracted using C₁₈ Sep Pak cartridges (Waters Associates, Millipore Corporation, Waters Chromatograph Division) and the radioimmunoassay carried out as described by Thornton et al. (1986) using an antiserum described by Sheldrick and Flint (1981, 1986). The detection limit for the radioimmunoassay was 15.6 pg ml$^{-1}$ and the intra-assay coefficient of variation 3.58%.

Statistical analysis of oxytocin content in tissues

Assay results obtained from either endometrial or myo¬metrial tissues were examined using ANOVA to determine the effect of group. Results were not significant for either endometrium ($P = 0.68$) or myometrium ($P = 0.84$). Regression analysis for linear and quadratic trends with respect to time was then performed on this data using Minitab 9.2 (Minitab Inc., State College, PA). In addition, log$_{10}$ transformations were performed and the largest and smallest means for each tissue type compared by t tests. Correlation coefficients ($r$) were calculated using Minitab.

Results

In situ hybridization

The characteristic distribution of oxytocin in the pig hypothalamus (Fig. 1) was similar to that reported by van Eerdenburg et al. (1991) and provides evidence that the antisense probe to mRNA encoding oxytocin derived from ovine oxytocin-neurophysin 1 cDNA gene was specific in hybridizing to regions that are encoding oxytocin mRNA in pig tissue.

As reproductive status changed during the oestrous cycle, pregnancy and into lactational anoestrus, the concentration of mRNA encoding oxytocin in the pig uterus varied significantly ($P < 0.05$ ANOVA). Tissue type was a significant factor determining the signal strength ($P < 0.001$ ANOVA). Autoradiographic sections of uteri taken from representative animals at oestrus, day 7 and 14 of the luteal phase, during pregnancy (early and late), at parturition and in lactational anoestrus and hybridized with the oligonucleotide oxytocin mRNA probes are shown in Fig. 2 (a–h). The mean concentrations of mRNA encoding oxytocin calculated against $^{14}$C standards from the autoradiographs are shown in Fig. 3 (a–d). Luminal epithelial expression of mRNA encoding oxytocin at oestrus (Fig. 4a) was greater than during day 14 of the luteal phase (Fig. 3a, $P < 0.01$) and at any stage of pregnancy ($P < 0.05$). On day 7, concentrations of mRNA encoding oxytocin in luminal epithelial tissue were intermediate and not significantly different.
From those of day 0 and day 14 of the luteal phase (Fig. 3a), luminal epithelial mRNA encoding oxytocin during lactational anoestrus was not significantly different from that during the oestrous cycle or pregnancy. The concentrations of mRNA encoding oxytocin in the luminal epithelia obtained from parturient animals (Fig. 2g) were below the minimum standard (8.0 nCi g⁻¹).

mRNA encoding oxytocin in circular muscle was significantly greater at oestrus (Fig. 4b) than at day 14 (P < 0.01) of the oestrous cycle, and concentrations at day 7 were intermediate (Fig. 3b). Oestrus concentrations of mRNA encoding oxytocin in circular muscle were also significantly greater than concentrations obtained at mid- or late pregnancy (P < 0.05). At parturition, concentrations of mRNA encoding oxytocin in uterine circular muscle were minimal (Fig. 3g) and concentrations were significantly lower (P < 0.05) than those found on day 0, day 7 and in lactational anoestrus (Fig. 3b).

In longitudinal muscle, the concentration of mRNA encoding oxytocin was significantly lower during late pregnancy than at oestrus (P < 0.05) or during the luteal phase (P < 0.05 Fig. 3c). At parturition, mRNA encoding oxytocin in uterine longitudinal muscle was minimal compared with other stages (Fig. 3c).

Uterine stromal tissue had the lowest concentration of mRNA encoding oxytocin (Fig. 3d). However, concentrations were significantly greater (P < 0.05) on day 7 than on day 14 of the luteal phase (Fig. 3d). The concentrations of mRNA encoding oxytocin in stromal tissue did not vary significantly as pregnancy progressed and values obtained from parturient animals were below the minimum standard (8.0 nCi g⁻¹).

**Oxytocin measurements in endometrial and myometrial extracts**

The mean (± SEM) extraction efficiency was 42.9 ± 1.1%. Regression analysis showed a significant quadratic relationship with time (endometrium = 312 − 4.34 day + 0.0311 days², P = 0.027).
Fig. 6. Oxytocin content in (a) the endometrial tissue and (b) musculature of the pig uterus during the oestrous cycle, throughout pregnancy and in lactational anoestrus. Values (pg g⁻¹ of tissue) are means ± SEM of 2–7 animals at each time point. Significant differences: P < 0.05 a versus b.

Discussion

Oxytocin receptors have been characterized on the endometrium of cyclic pigs (Whiteaker et al., 1994; Okano et al., 1996) and on the myometrium from a late pregnant sow (Soloff and Swartz, 1974). There are, as yet, no reports of the binding characteristics of the oxytocin receptors in the pig uterus as pregnancy progresses, although our previous work has shown that mRNA coding for an oxytocin receptor is present throughout pregnancy and in lactational anoestrus (Boulton et al., 1995). The source of the oxytocin which is capable of binding to these receptors has previously been thought to be primarily of hypothalamic origin, although some ovarian production of oxytocin occurs in the pig (Pitzel et al., 1984; Choy and Watkins, 1988; Jarry et al., 1990). Our study is the first to report the presence of mRNA encoding oxytocin in the pig uterus and shows that the concentration varies according to reproductive status. These observed changes in mRNA could be due to changes in transcription rates or to changes in the stability of the mRNA. Both mechanisms have been shown to be operative in the control of gene expression (Darnell, 1982). Whilst caution must be exercised in making the assumption that an increase in the concentration of mRNA encoding oxytocin would result in any difference in the amount of peptide produced, recent studies in sheep and humans indicate that control is at the transcriptional rather than translational level (Takemura et al., 1994; Wathes et al., 1996).
Our study suggests that local uterine synthesis of oxytocin may be more important in events during the oestrus cycle than in pregnancy or at parturition in the pig since the luminal epithelium and circular musculature of animals at oestrus was particularly rich in mRNA encoding oxytocin. It is interesting to note that this maximal concentration of mRNA encoding oxytocin in the early part of the oestrous cycle occurs at a time when the concentration of oxytocin receptors is also highest (Okano et al., 1996). Possible roles for uterine oxytocin at this time include uterine motility during sperm transport (Gilbert et al., 1992). Maternal recognition of pregnancy in pigs occurs at day 11 to 12 (Dhindia and Dzulik, 1968) and is thought to be dependent on production of oestrogen by the blastocyst which acts in an anti-luteolytic way to cause a re-orientation of uterine PGF₂α secretion away from the vasculature and into the uterine lumen (see Flint 1995 for review). However, the mechanisms that lead to episodic secretion of prostaglandin B2 by the uterus in the pig are uncertain. Circulating concentrations of oxytocin in pigs increase during luteolysis on days 13–15 after oestrus and this increase is temporally associated with increased uterine secretion of PGF₂α (Kotwica et al., 1990). There is now growing evidence that oxytocin may be involved in the regulation of prostaglandin secretion (Gross et al., 1988; Kieborz et al., 1991; Printz et al., 1994). In the study reported here, all tissue types showed a significant reduction in the concentration of mRNA encoding oxytocin on day 14 compared with concentrations obtained earlier in the oestrous cycle. This pattern of oxytocin synthesis is similar to that observed in the ovaries of pigs (Pitzel et al., 1984; Choy and Watkins, 1988). Extra-hypothalamic sites of oxytocin production in the pig would allow autocrine and/or paracrine mechanisms to operate as seen in humans and rats (Chibbar et al., 1993; Lefebvre et al., 1992b, 1993); however, this paracrine mechanism does not occur in sheep (Wathes et al., 1996).

Previous reports in the rat have shown an increase in uterine mRNA encoding oxytocin as gestation progresses, such that concentrations peak at term (Lefebvre et al., 1992b), whilst in the sheep no such message exists (Wathes et al., 1996). In the study reported here, as pregnancy proceeded in the pig, no significant increase in uterine concentration of mRNA encoding oxytocin was seen and furthermore, at term, these concentrations were minimal. It may be that artificially catagorising pregnancy into trimesters was insufficient to detect subtle changes in mRNA encoding oxytocin as pregnancy proceeded. However, the lack of an increase in the concentration of mRNA encoding oxytocin at parturition implies that the increase in peripheral oxytocin concentrations observed at this time (Forsling et al., 1979; Gilbert et al., 1994; Lawrence et al., 1995) is of hypothalamic or possibly placental origin.

In addition to the measurable changes in the concentrations of mRNA encoding oxytocin observed by in situ hybridization as reproductive status changed, there were significant differences in the immunoreactive oxytocin concentrations obtained from tissue extracts. Immunoreactive oxytocin tissue concentrations tended to be highest at oestrus and lowest during late pregnancy, a trend which was confirmed for endometrial tissue by quadratic regression analyses. The variations in peptide concentrations with time show a close match with mRNA signal and support the hypothesis of local uterine synthesis of oxytocin. Although other workers have demonstrated a lag phase between the variation in concentration of message in the ovary and appearance of the change in mature peptide in the periphery (Ivell et al. 1985; Jones and Flint, 1988), this study has been unable to confirm such a delay. In addition, although our data demonstrates the presence of a uterine oxytocin synthetic apparatus, there is as yet no evidence that the product has local bioactivity.

Flint and Sheldrick (1983) showed the presence of oxytocin in uterine tissue extracts of sheep obtained from the mid-luteal phase, with these figures being in the nanogram range. However, the presence of oxytocin in ovine uterine tissue extracts could not be detected in pregnancy (Wathes et al., 1996). In the present study, in pigs, oxytocin was present throughout the oestrous cycle and pregnancy, although the concentrations were in the picogram range. These values are expressed as pg g⁻¹ of tissue and make no allowance for the large difference in uterine mass between nonpregnant and pregnant pigs.

Further work is needed to localize oxytocin to specific cell types in the pig uterus using immunocytochemical techniques. In addition, work is required to determine the underlying mechanisms controlling the synthesis of mRNA encoding oxytocin in the different tissue types and how this variation in concentrations of mRNA encoding oxytocin relates to the function of the mature peptide in the uterus. As yet, the synthesis of mRNA encoding the oxytocin receptor, the production of peptide and the binding characteristics of the oxytocin receptor in the pig uterus as reproductive status changes throughout the oestrous cycle and pregnancy are not fully understood.

This work is supported by a MAFF commission. The authors thank P. Byrd and his staff for expert and thoughtful care of the animals. Statistical analyses were performed with the patient assistance of D. Brown.

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Concentrations of mRNA encoding oxytocin in the pig uterus


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