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Oxytocin mRNA content in the endometrium of non-pregnant women

Margareta Steinwalla, Stefan Hansson, Thomas Bossmar, Iréne Larsson, Radovan Pilka, Mats Åkerlund

Objective To study oxytocin mRNA in the human endometrium at different phases of the menstrual cycle.

Design An exploratory study in non-pregnant women.

Setting The Department of Obstetrics and Gynecology, Lund University Hospital, Sweden.

Participants Thirty-three women of fertile age undergoing hysterectomy or endometrial curettage on routine benign gynaecologic indications.

Methods Endometrial tissue was obtained throughout the menstrual cycle. The presence of oxytocin mRNA was investigated by in situ hybridisation and by real time PCR.

Main outcome measures Oxytocin mRNA signalling intensity found by in situ hybridisation of tissue obtained at different times of the menstrual cycle. Relative amounts of oxytocin mRNA measured by real time PCR.

Results The signal for oxytocin mRNA obtained by in situ hybridisation was more pronounced in glandular epithelial cells than in stromal cells. Furthermore, it was most marked around mid-cycle. The expression of oxytocin mRNA was confirmed by real time PCR.

Conclusions The results indicate that oxytocin may be synthesised in the endometrium of non-pregnant women, particularly in the glandular epithelial cells. Hormone released from these sources may have a paracrine action on the uterus. Oxytocin mRNA expression seems to be ovarian hormone dependent with the highest concentration around mid-cycle.

INTRODUCTION

In the pregnant human uterus, oxytocin mRNA has been demonstrated in amnion, chorion and decidua. In non-pregnant conditions, immunoreaction to oxytocin has previously been shown, particularly in the isthmus part of the cervix. This peptide has also been detected in human follicular fluid. These findings may indicate a uterine origin of oxytocin. The possible synthesis of oxytocin in the non-pregnant endometrium has, however, not to our knowledge been studied previously. In a pilot study, we demonstrated oxytocin mRNA in the endometrium of non-pregnant women (unpublished). Oxytocin of endometrial origin may stimulate myometrial activity and, thereby, be involved in sperm and egg transport, implantation and menstruation. We investigated the content of endometrial oxytocin mRNA by in situ hybridisation and real time PCR in samples obtained throughout the menstrual cycle.

METHODS

Endometrial tissue was obtained from a total of 33 regularly menstruating, parous women, with a median age of 44 years (range 34–50 years). Sampling was performed at diagnostic curettage or hysterectomy. The diagnoses were leiomyoma in 13 patients, menorrhagia in 10, ovarian cyst in 4, adenomyosis in 3, cervical dysplasia in 2 and uterine prolapse in 1 patient. Only women without hormonal treatment or intrauterine device were included in the study. All subjects were well informed about the purpose and procedure of the investigation and gave their written consent to the sampling. The study was approved by the Ethics Committee at Lund University.

Tissue aliquots measuring approximately 3 × 3 × 3 mm were snap frozen on dry ice and stored at −80°C. The samples were also examined by a histopathologist for exclusion of endometrial pathology and for identification of the menstrual phase. Samples were classified as belonging to early, mid and late proliferative phases, and early, mid and late secretory phases of menstruation. Tissue sections measuring 12 μm were cut on a cryostat, thaw-mounted on to silanised slides, and stored at −80°C prior to hybridisation. Fresh frozen tissue, rather than fixative-treated material, was used to maximise sensitivity for mRNA detection. Thawing of tissue did not occur prior to sectioning to ensure the best possible tissue integrity.
For the human oxytocin mRNA, a probe was used corresponding to 194 NT (3'-125), Genbank accession No. M25650.\textsuperscript{6} DNA template was generated by PCR amplification, using bipartite primers consisting of either a T7 RNA promoter and a downstream gene-specific sequence or a T3 RNA promoter and upstream, gene-specific sequence. PCR reactions using 1 ng cDNA, 0.5 \( \mu \text{M} \) primers, 200 \( \mu \text{M} \) dNTPs, 3 mM MgCl\textsubscript{2}, 10 mM Tris, pH 8.3, 50 mM KCl, and 5 units Taq polymerase (Roche, Basel) were amplified at 95\(^\circ\)C for 1 min, 62\(^\circ\)C for 1 min and 72\(^\circ\)C for 1 min for 30 cycles with a final extension at 72\(^\circ\)C for 10 min. DNA templates were purified from agarose gels using GeneClean (Bio101) and thereafter sequenced using a cycle sequencing reaction kit (ABI PRISM, Big dye). cRNA probes were transcribed from 40 ng of gel-purified DNA template using 800 Ci/mmol of \( ^{35}\text{S}-\text{UTP} \) (Dupont NEN, Paris) and either T3 or T7 RNA polymerase according to manufacturer’s instructions (Ambion MAXIscript, Ambion Europe, Cambridge, UK) to generate sense and antisense probes.

Tissue sections were fixed, dehydrated and delipidated as previously described.\textsuperscript{7} Sections were hybridised for 20–24 hours at 55\(^\circ\)C with 2 \( \times \) 10\textsuperscript{6} cpm of denatured \( ^{35}\text{S}-\text{cRNA} \) probe per 50 \( \mu \text{L} \) hybridisation buffer consisting of 20 mM Tris–HCl (pH 7.4), 1 mM EDTA (pH 8.0), 300 mM NaCl, 50\% formamide, 10\% dextran sulphate, 1 \( \times \) Denhardt’s, 250 \( \mu \text{g/mL} \) yeast tRNA, 100 \( \mu \text{g/mL} \) salmon sperm DNA, 250 \( \mu \text{g/mL} \) yeast total RNA (fraction XI, Sigma-Aldrich, St Louis, Missouri), 150 mM dithiothreitol (DTT), 0.15\% sodium thiosulphate (NTS), and 0.15\% sodium dodecyl sulphate (SDS).

Following washing to remove excess probe, slides were opposed to Kodak Hyperfilm Biomax MR for three days and then coated with nuclear track emulsion (NTB-3, Kodak, New York). After four weeks of exposure at 4\(^\circ\)C,
slides were developed in Dektol (Kodak), fixed and counterstained with a Giemsa stain. All slides were examined by two independent investigators blinded for the experimental conditions (SH and MS) and the signal intensity was graded in five steps from negative to maximal intensity.

Microphotographs were prepared using an Axiophot microscope (Olympus, Tokyo, Japan) equipped for darkfield and brightfield microscopy and a digital camera (Olympus O50-CU). Captured images were assembled electronically using Adobe Photoshop 5.0. Figures were printed on matte-finished paper by a Fujix Pictography 3000 (Fuji Photo Film, New York) printer at 400 dpi resolution.

Total RNA was extracted using TRIzol reagent (Invitrogen, Invitrogen AB, Lidingo, Sweden) according to manufacturer’s instructions. RNA integrity was confirmed on a denaturing formaldehyde gel. RNA was reversely transcribed according to protocols from Applera (Stockholm, Sweden) in a 50 µL reaction containing: 0.5 µg total RNA, and a final concentrations of 1 × TaqMan RT buffer, 5.5 mM MgCl2, 500 µM dNTPs, 2.5 µM random hexamers, 0.4 U/µL RNase inhibitor, and 1.25 U/µL MultiScribe Reverse Transcriptase. The samples were incubated at 25°C for 10 min, at 48°C for 30 min and then 5 min of inactivation at 95°C. They were then stored at −20°C until further used.

Gene transcripts were quantified using real time PCR on ABI PRISM 7000 sequence detection system (Applera). Primers and Fam-labeled probes were obtained from Assays on-Design (Applera).

PCR reactions were carried out in a 25 µL final volume containing final concentrations: 1 × Universal PCR Master Mix (Applera), 1 × Assaymix (Applera), 0.25 µM probe, 0.9 µM of forward and reverse primers, respectively, and 1 µL of 10 ng/µL of a DNA aliquot. The thermal cycling conditions were initiated by UNG activation at 50°C for 2 min and an initial denaturation at 95°C for 10 min, then 40 cycles at 95°C for 15 seconds, annealing at 60°C for 1 min. Two negative controls without template were included in every amplification. RNA samples were tested for genomic DNA contamination prior to further investigation. For each reaction, triplicate or duplicate assay was carried out. Transcript of β-actin as a housekeeping gene, with Gene bank accession number NM_001101, was quantified as endogenous RNA of reference to normalise each sample. Quantification was achieved through a calibration curve obtained by serial fourfold dilutions of the template DNA (0.08–80 ng). Results are expressed as relative values.

**Table 1.** Oxytocin mRNA content in endometrial samples obtained from women in different phases of the menstrual cycle. The oxytocin mRNA signalling intensity in average for the respective menstrual phase was graded from negative (−), equal to background (+), distinguishable from background (++), less than half of maximum (+++) and maximal signalling (++++). Results are presented in a box-plot diagram. The relative amounts of oxytocin mRNA obtained by real time PCR at different times of the menstrual cycle were compared using non-parametric Mann-Whitney U test.

**Figure 2.** Real time PCR quantification of oxytocin mRNA. The amount of oxytocin mRNA normalised to the amount of β-actin mRNA. The relative values are presented in a box-plot diagram. No significant changes were obtained between the proliferative and the secretory phases. Early proliferative (EP, number of observations n = 4), mid proliferative (MP, n = 6), late proliferative (LP, n = 4), early secretory (ES, n = 4), mid secretory (MS, n = 1) and late secretory phases (LS, n = 6).
endometrial stromal cells, the signal was weaker, but a
cyclical variation in expression of oxytocin mRNA was
again observed (Table 1). In the adjacent myometrium, the
signal for oxytocin mRNA was absent or scarce.

In samples other than those used for in situ hybridisation,
but from the same women, the presence of oxytocin mRNA
in the endometrium was confirmed (Fig. 2). The expression
levels were relatively low, but oxytocin mRNA expression
was found in all menstrual phases. No statistically signif-
icant difference in expression was found between the
different phases (Fig. 2).

DISCUSSION

By in situ hybridisation, we demonstrated marked ex-
pression of oxytocin mRNA in endometrial samples
obtained from non-pregnant women around mid-cycle.
The signal was more obvious in glandular epithelial cells
than in stromal cells. In endometrium from women at
menstruation and in the early proliferative phase, the
signal intensity was equal to background. The pattern of
oxytocin expression was so unique that an alternative
analysis using real time PCR was used for further valida-
tion. This method confirmed the presence of oxytocin
mRNA, although the expression was comparatively low
in all phases. Whether or not the low gene expression level
also reflects a low level of functional peptide remains to be
determined.

The cyclical variation in oxytocin expression observed
by in situ hybridisation could not be verified by quantitative
PCR. Instead, real time PCR showed expression of low
intensity throughout all the examined phases without sig-
nificant differences. Although this lack of difference may
be related to a low number of observations in some groups,
a more likely explanation is the difficulty to quantify low
transcript gene from heterogenous tissue. Indeed, the ap-
pearance of endometrial glands and stroma differs mark-
elly between the proliferative and secretory phases of the
menstrual cycle.

Throughout the menstrual cycle there is a proliferation
of the endometrial stroma in addition to the morphological
changes of the endometrial glands. Therefore, an expres-
sion in glandular cells will appear as weaker when mea-
sured in the midst of a large amount of negative stromal
cells. Both methods used can detect low levels of RNA in
tissue, but only in situ hybridisation allows identification of
low intensity expression in individual cells.

The cyclical variation in endometrial content of oxytocin
mRNA could be due to a stimulation by oestadiol in the
proliferative phase of oxytocin production, an effect which
in the luteal phase is counteracted by progesterone. This
concept is in agreement with the finding that oestrogen
receptors are well expressed already in the early luteal phase
of the menstrual cycle, whereas those for progesterone are
developed somewhat later.8 The effects of ovarian steroids

on oxytocin mRNA in the endometrium would therefore
be similar to those seen in the hypothalamus, regulating
the release of oxytocin into the blood. In previous studies
in postmenopausal women, we observed a stimulatory
influence of oestradiol on oxytocin release, an effect
which was counteracted by progesterone.9,10 This effect
of oestradiol is also in agreement with the finding that the
human oxytocin promoter gene has an oestrogen respon-
sive element.11 The endometrial content of vasopressin
mRNA was not studied here, but regarding the circulating
level of this closely related peptide, a variation with peak
plasma concentration at mid-cycle has also been observed
by our group.12 We also observed high vasopressin peptide
levels after unopposed oestradiol treatment of post-
menopausal women and that addition of progestogen
counteracted this effect.10,12,13

Myometrial and endometrial contents oxytocin and
vasopressin V1a receptors as well as the in vivo sensitivity
of the myometrium to these hormones vary in a way, which
is opposite to that presently observed for oxytocin mRNA
in the endometrium. Thus, maximal density of these recep-
tors and the highest myometrial sensitivity are found at the
onset of menstruation.14–16 Our results, with high oxytocin
mRNA levels in the endometrium at mid-cycle but low
oxytocin receptor concentration in the myometrium,
could imply different physiological functions of oxytocin
in these two tissues. However, regarding receptors it must
be kept in mind that individual cells can show a great
heterogeneity and rapid changes in their expression of
oxytocin receptor.17

Oxytocin has since long been ascribed a significant role
in the start of labour preterm and at term. An important
proof of the involvement of oxytocin and vasopressin in
mechanisms of preterm labour is the therapeutic effect of
atosiban, an oxytocin and vasopressin V1a receptor block-
ing agent.20,21 However, any marked rise in plasma con-
centration of oxytocin or in uterine receptors at the onset
of labour has not been demonstrated.18,19 Indeed, data
supporting a local synthesis of oxytocin in the pregnant
uterus, not reflected in plasma levels, and a paracrine action
have accumulated during the latest years.1,22,23 The present
results suggest a uterine synthesis of oxytocin also in non-
pregnant condition. Oxytocin of endometrial origin could
possibly induce myometrial contractions indirectly by an
effect over endometrial receptors stimulating the synthesis
of PGF2α, which would mediate contractions in parallel to
the situation in pregnancy.24,25 Locally released oxyto-
cin could also directly stimulate contractions of the non-
pregnant uterus via oxytocin receptors in adjacent myome-
 trium, as in pregnancy.14 In fact, the uterine contractility
in vivo in non-pregnant women in the late follicular phase
was shown by ultrasound technique to involve only the
subendometrial layer of the myometrium.26 This observa-
tion is in agreement with the previous in vitro finding of
a variation in uterine contractility between different layers
of the myometrium in non-pregnant condition.27 In that
study, myometrium closest to the endometrial cavity had the most pronounced activity. It may be that the retrograde transport of sperms towards the fallopian tubes at this time of the menstrual cycle is facilitated by the selective contractility of myometrium close to the endometrium. An involvement of endometrial oxytocin in the uterine hyperactivity of primary dysmenorrhoea is less probable, in view of the lack of observed endometrial oxytocin mRNA around the onset of menstruation.

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References


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