Expression of the oxytocin gene, but not the vasopressin gene, in the rat uterus during pregnancy: influence of oestradiol and progesterone

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Abstract

Oxytocin (OT) and vasopressin (VP) are neurohypophyseal hormones with potent stimulatory actions on the uterus. In order to determine whether these hormones may have a paracrine action on the uterus, OT and VP gene expression was studied in myometrium from pregnant rats at gestational ages of 14 and 20 days, and from ovariectomized animals treated with oestradiol and progesterone. OT and VP mRNA concentrations were measured using real-time quantitative reverse transcription-PCR, and OT- and VP-like immuno-reactivities were determined using RIA. OT mRNA was detected in the uterus from pregnant rats, but did not differ between the groups of different gestational ages. Oestradiol significantly (P<0.05) stimulated OT gene expression in ovariectomized rats. Progesterone alone was without effect on OT mRNA concentrations, but significantly (P<0.05) reduced the oestradiol-induced OT mRNA accumulation. The OT-like immunoreactivity in an extract of myometrium from pregnant rats was eluted from a reverse-phase HPLC column with a retention time identical to that of synthetic OT. Neither VP mRNA nor VP-like immunoreactivity was detected in the myometrium from pregnant or ovariectomized rats. The study demonstrates steroid-dependent expression of the OT gene in the rat uterus and processing of uterine preprooxytocin to the mature nonapeptide. The data support the theory that this peptide may act in a paracrine pathway. No evidence was found for the presence of VP in the uterus so that, if the hormone is involved in a stimulatory action on this tissue, it probably acts via an endocrine mechanism.

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Introduction

The neurohypophyseal peptide hormones, oxytocin (OT) and vasopressin (VP), have potent uterotonie effects in both pregnant and non-pregnant humans and rats (Bossmar et al. 1994, 1995, Chan et al. 1996). Although OT is traditionally regarded as the more important of the two hormones in uterine physiology, it has been shown that the human myometrium is more sensitive to VP than to OT (Bossmar et al. 1995, Thornton et al. 2002). The density of OT and VP receptors in the myometrium is very high during late stages of pregnancy and the sensitivity to these peptides is increased (Fuchs et al. 1982, Bossmar et al. 1994). However, there is no firm evidence for an up-regulation of these receptors at the onset of labour (Bossmar et al. 1994, Helmer et al. 1999, Wathes et al. 1999). OT is well known for its involvement in parturition, but its precise physiological role in the initiation and progress of labour is poorly understood (Fuchs et al. 1982, Higuchi & Negoro 1988) and parturition can take place in the absence of circulating OT in cases of experimental or clinical pituitary gland dysfunction (Chard 1989). Several studies have demonstrated OT mRNA, as well as OT-like immunoreactivity, in rat uterus and human amnion, chorion and decidua (Lundin et al. 1989, Lefebvre et al. 1992, Chibbar et al. 1993). These findings indicate a uterine origin and synthesis of OT. A possible paracrine action of this hormone may contribute to both physiological and pathophysiological processes in the uterus during pregnancy. It has been suggested that, with respect to parturition, OT acts as a paracrine or autocrine mediator rather than as a circulating hormone (Lefebvre et al. 1992). Sex steroids may regulate such paracrine actions as the OT mRNA concentration in the uterus is altered under the influence of oestradiol and progesterone (Lefebvre et al. 1994, Chibbar et al. 1995). Although VP has been much less studied than OT in pregnancy, the foetus secretes VP during labour (Chard et al. 1971) and the peptide may also be involved in stimulating uterine contractions. VP has been identified in the human myometrium during pregnancy (Thornton et al. 2001), but local presence of VP mRNA has not been reported.

In the non-pregnant condition, VP is also a potent uterotonic agent, but in this situation the effect of OT is reduced by the presence of OT.
less pronounced (Bossmar et al. 1995). A recent study has shown the presence of OT mRNA in the endometrium of non-pregnant women and its concentration was ovarian hormone dependent (Steinwall et al. 2004). There is substantial evidence for the involvement of VP in conditions of uterine hyperactivity, such as primary dysmenorrhoea (Åkerlund et al. 1976). However, presence of VP mRNA in the uterus during the non-pregnant condition has never been demonstrated, and it remains to be shown whether locally produced or the neurohypophyseal hormone is responsible for the VP actions on the uterus. Clinical studies using OT and VP receptor antagonists strongly support the theory that one or both of these hormones are involved in the pathophysiology of preterm labour and primary dysmenorrhoea (Åkerlund et al. 1987, Brouard et al. 2000).

In the present article, we report on the quantitative transcription of the OT gene and the identification of OT in the uterus of pregnant rats and describe the dependence of OT gene expression on sex steroids in ovariectomized rats.

Materials and Methods

Animals

Female Wistar rats aged approximately 6 months (body weight 190–250 g) were obtained from the animal house at the Faculty of Medicine and Health Sciences, UAE University. Ten animals were mated and kept under standardized conditions of light, temperature, food and water intake. They were killed on days 14 and 21 of pregnancy. The uterus was removed and dissected on chilled plates. The placental tissue was separated from the myometrium and the samples were stored at −80 °C until use. Twenty animals were ovariectomized by laparotomy under ketamine and xylazine anaesthesia and kept under the same conditions as the pregnant animals for a period of 30 days. The ovariectomized rats were divided into four groups and injected subcutaneously once daily for 6 days with 17β-oestradiol (E; 0.001 μg/g body weight), progesterone (P; 20 μg/g body weight), 17β-oestradiol (0.001 μg/g body weight) + progesterone (20 μg/g body weight) (EP) and vehicle only. On the day after the last injection, the animals were killed and the uterus was dissected in the same manner as for the pregnant animals. Tissue specimens were stored at −80 °C until use. The study was approved by the Local Ethical Committee for Animal Research and it conformed to the UK guidelines for use of laboratory animals.

Total RNA isolation and digestion of genomic DNA, cDNA synthesis

Three cubic millimetres of myometrium were cut from the frozen samples. This was performed on a chilled plate in order to prevent possible RNA degradation. The dissected tissues were homogenized in lysis buffer until the samples were uniformly homogeneous. Total RNA was extracted using an RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. Concentration and purity of the RNA samples were determined by measuring the absorbance at 260 nm (A260) and calculating the ratio of absorbance at 260 and 280 nm (A260/A280) using a Beckman DU 70 Spectrophotometer. RNA sample of 1·0 μg was treated with 1·0 μl RNase-free DNase I and 10× DNase I buffer at 37 °C for 15 min in a thermocycler in order to remove any contaminating genomic DNA before reverse transcription (RT). All DNase I-treated RNA samples were reverse transcribed using a One-step RT-PCR kit in a total reaction volume of 40 μl incubated at 50 °C for 30 min. The reaction was terminated by heating at 95 °C for 15 min. The cDNA samples were stored at −20 °C until used.

Real-time quantitative PCR

The target genes for real-time RT-PCR analyses were OT and arginine vasopressin (Avp). Pre-designed and labelled primer/probe sets (RN00566449-M1 and RN00564446-G1, Applied Biosystems, Foster City, CA, USA) were selected from the Applied Biosystems’ Assays-on-Demand product line (http://appliedbiosystems.com). Rat β-actin, ACTB (4352340E, Applied Biosystems), was used as endogenous control in the experiments.

The PCRs were carried out in 20 μl reaction volumes containing TaqMan Universal Master Mix (2×, 4331182, Applied Biosystems), TaqMan Gene Expression Assay for the target gene (OT or Avp or ACTB, 20×) and optimized quantities of cDNA samples. The TaqMan Universal Master Mix contained AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, passive reference and optimized buffer components. Real-time PCR was performed in an ABI Prism 7500 Sequence Detection System (Applied Biosystems), which was set to detect both FAM and VIC reporter dyes simultaneously. Reactions were set up in triplicate. Thermal cycling was initiated by incubation at 50 °C for 2 min and 95 °C for 10 min for optimal AmpErase UNG activity and activation of AmpliTaq Gold DNA polymerase respectively. After this initial step, 50 cycles of PCR were performed. Each PCR cycle consisted of heating to 95 °C for 15 s, followed by annealing and extension at 60 °C for 1 min. ACTB was included as endogenous control in a separate well.

For optimization of the real-time PCR assay, different quantities of RNA converted to cDNA (0·01, 0·05, 0·2, 0·5, 1·0 and 10·0 ng) were tested. Amplification was clearly detectable up to 1·0 ng RNA converted to cDNA. Following the validation experiments, multiplex reaction mixtures, which contained two primer pairs, were composed for the target gene expression assay. Each primer pair amplified either a target sequence (OT or Avp) and/or the ACTB endogenous control sequence. The composition of the PCR mixture was the same as mentioned earlier with 40 ng RNA converted to cDNA. Also the thermal profile and the number of cycles were identical to the aforementioned protocol.
Relative quantification

Relative quantification (RQ) determines the change in expression of a nucleic acid sequence (target) in a test sample relative to the same sequence in a calibration sample. RQ provides accurate comparison between the initial levels of template in each sample, without requiring the exact copy number of the template. Relative levels of templates in the samples can be determined without the use of standard curves.

Data were collected throughout the real-time PCR runs. Calculations for RQ were performed as outlined in User Bulletin no. 2 ABI Prism 7500 Sequence Detection System (Applied Biosystems). The SDS software mathematically transforms the raw fluorescence data to establish a comparative relationship between the spectral changes in the passive reference dye and those of the reporter dyes. Based on these comparisons, the software generates Ct and gene expression analyses compared with the calibrator sample. Results are presented as relative values.

RIA and HPLC purification

Frozen tissue from each animal was boiled with 1 M acetic acid (10 ml/g wet weight) for 10 min. The supernatant was removed, centrifuged (4000 g for 20 min at 4 °C) and lyophilized using a Savant Speed-Vac freeze-drier. The lyophilized tissue extracts were redissolved in 0.1% trifluoroacetic acid/water (70.0:29.9:0.1, v/v/v) and freeze-dried. The lyophilized tissue extracts were redissolved in 0.1 M sodium phosphate buffer (pH 7.4), and OT-like immunoreactivity (OT-LI) and VP-like immunoreactivity (VP-LI) were measured by RIA using commercially available kits supplied by Peninsula Laboratories, Inc (San Carlos, CA, USA). The antibody to OT showed <0.1% cross-reactivity with VP and the antibody to VP showed <0.1% cross-reactivity with OT. The detection limit for the OT and the VP assays was 30 fmol/ml.

The tissue extracts from 20-day pregnant rats were pooled and pumped into two Sep-Pak C-18 cartridges (Waters Associates, Milford, MA, USA) connected in series at a flow rate of 2 ml/min. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70:0.29:9:0.1, v/v/v) and freeze-dried. The extract was redissolved in 0.1% trifluoroacetic acid/water (2 ml) and injected into a 46×25 cm Vydac 218TP54 (C-18) column equilibrated with 0.1% trifluoroacetic acid at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 42% (v/v) over 60 min using a linear gradient. Fractions (1 min) were collected, lyophilized and reconstituted in 0.1 M sodium phosphate buffer (pH 7.4; 1 ml). OT-LI and VP-LI were determined using RIA. The retention times of synthetic OT and VP were determined under the same conditions of chromatography.

Statistical analysis

Software SPSS 13.0 (SPSS, Chicago, IL, USA) for Windows was used and the non-parametric Mann–Whitney U-test is applied for comparisons between the groups, taking into account that multiple comparisons were not performed. The exact P values are given and P<0.05 is considered as statistically significant.

Results

OT mRNA was detected in the uterine specimens from pregnant rats in gestational weeks 14 and 20, but there was no significant difference in concentration between the two groups (Fig. 1). OT mRNA was also found in lower concentrations in the tissues from the ovariectomized animals. There was a statistically significant difference between both groups of pregnant rats and progesterone-primed animals, and the control group (P=0.008 and 0.014 respectively). The corresponding difference from animals primed with a combination of oestradiol and progesterone showed a trend to increase (P=0.055), while there was no difference between the pregnant group and the oestradiol-primed animals (P=0.075). The content of OT mRNA differed significantly in the ovariectomized rats, which had been primed with oestradiol compared with the
progesterone-primed ($P=0.016$) and control groups ($P=0.036$). There was also a difference in the OT mRNA content between the group primed with the combination of oestrogen and progesterone and the controls ($P=0.031$), VP mRNA was not detected in any uterine tissue sample from the pregnant or ovariectomized animals. In a control study, VP mRNA was detected in brain tissue from a healthy rat employing the PCR probes and RT-PCR methodology used for the uterine tissues.

OT-like immunoreactivity was measured only in uterine tissue from pregnant rats (Table 1). The OT-like immunoreactivity in a pooled extract of uterine tissue from pregnant rats was eluted from a reverse-phase HPLC column as a single fraction with the same retention time as synthetic OT (Fig. 2). The antibody used in RIA does not recognize the COOH-terminally extended forms of OT, previously shown to be present in high concentration in late-gestational rat uterus (Mitchell et al. 1998). VP-like immunoreactivity was below the detection limit of the assay in extracts of all tissues consistent with the assertion that the VP gene is not expressed in uterine tissue.

**Discussion**

RT-PCR is a powerful tool to detect specific gene expression in a small amount of tissue and is more sensitive than the traditional RNA assays such as northern blotting and *in situ* hybridization. Similarly, real-time quantitative RT-PCR is considered to be more accurate in assessing the changes in gene expression following treatment with a particular hormone or drug.

This study demonstrates the presence of OT mRNA but absence of VP mRNA in the rat uterus. The results in the present study confirm the previous findings that OT mRNA is expressed in the rat uterus during pregnancy (Lefebvre et al. 1992, Higuchi et al. 1995). We also found an influence of ovarian steroids on the expression of OT mRNA in ovariectomized rats. Progesterone significantly counteracted the stimulatory action by oestradiol on *OT* gene expression and there was no difference between the progesterone- and vehicle-treated animals. This result contrasts markedly with an earlier study using similar doses of the steroids in which progesterone was found to produce a sevenfold potentiation of the stimulatory action of oestrogen on OT gene expression (Lefebvre et al. 1994). The reason for the contradictory observations is not entirely clear. The earlier study was carried out on rats ovariectomized at 28 days compared with 180 days in the present study, progesterone was delivered via silastic implants rather than by daily injection, and northern blot analysis rather than real-time RT-PCR was used for quantitation. Our results are in accordance with the general effects of these hormones on the uterus, where progesterone is known to down-regulate oestrogen receptors (Hsueh et al. 1975, Lessey et al. 1988).

RIA showed that endogenous OT was produced in the myometrium of pregnant rats. Biologically inactive carboxy-extended forms of the peptide would not be detected with the antiserum used in the assay. Our results support the hypothesis that the OT gene is transcribed in the uterus and that the primary gene product (preprooxytocin) is processed to the biologically active nonapeptide. The fact that we could not demonstrate OT-LI in the samples from the ovariectomized rats in which OT mRNA was present may be due to the relatively low amount of myometrial tissue especially from the progesterone-treated animals. Another explanation could be that unknown factors operate during pregnancy, which translate translation of OT mRNA so that OT is more likely to be detected in the uterine tissue from pregnant animals. In contrast, our study does not indicate that the VP gene is expressed in the rat uterus and no evidence for the presence of the peptide in uterine extracts was obtained. Local quantification of VP mRNA in the uterus has not been reported before, but there have been a few indications of a possible local

**Table 1** Weight of the uterine tissue and the content of oxytocin-like immunoreactivity measured by RIA. Data indicate means ± S.E.M.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Weight of tissue (g)</th>
<th>Oxytocin (fmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age 20 days</td>
<td>1.30±0.29</td>
<td>680±130</td>
</tr>
<tr>
<td>Gestational age 14 days</td>
<td>0.90±0.24</td>
<td>450±110</td>
</tr>
<tr>
<td>Oestradiol treated</td>
<td>0.11±0.05</td>
<td>ND</td>
</tr>
<tr>
<td>Progesterone treated</td>
<td>0.03±0.01</td>
<td>ND</td>
</tr>
<tr>
<td>Oestradiol+progesterone treated</td>
<td>0.07±0.02</td>
<td>ND</td>
</tr>
<tr>
<td>Controls (vehicle)</td>
<td>0.03±0.01</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not detected.
presence of this peptide in non-pregnant as well as pregnant human myometrium (Lundin et al. 1989). We were unable to demonstrate VP gene expression or measure VP-LI in myometrium from either pregnant or non-pregnant rats. The available data support the theory that local production and paracrine pathways of OT may be of importance in the physiology and pathophysiology of uterine contraction in the pregnant and non-pregnant conditions. In contrast, if VP contributes to the regulation of uterine contractility, this is probably an endocrine effect. The role of OT and VP in the mechanisms of labour, both preterm and at term, is still not fully understood (Thornton et al. 2001). It is unclear whether the onset of labour is caused by an increased amount of OT in circulating blood in both human and rat (Leake et al. 1981, Higuchi et al. 1985, Thornton et al. 1992). A possible contribution of OT and VP from the foetus itself has been proposed (Chard et al. 1971, Baldwin et al. 2000). These peptides are synthesized by the foetus, but no clear correlation to onset of labour has been demonstrated. Receptors for OT and VP in the uterus are present in both non-pregnant and pregnant women, but it is not clear whether there is an increase of any of these receptors in preterm or term labour (Fuchs et al. 1982, Bossmar et al. 1994, 1995). It seems more likely that other factors involving paracrine pathways rather than an instant up-regulation of these receptors may be the explanation for the regulatory effects OT and/or VP have in the initiation of labour. In conclusion, the present study supports the theory that OT is locally synthesized in the uterus and suggests that OT is much more important than VP in acting as a paracrine regulator of uterine function. Further evidence for an important involvement of OT and/or VP in preterm labour is confirmed by the beneficial effect of the OT- and VP-receptor antagonist, atosiban (Tractocile, Ferring Pharmaceuticals; Worldwide Atosiban versus β-Agonist Study Group 2001), in attenuating uterine contractions. This is an unselective antagonist that binds to both the OT and VP receptors (Åkerlund et al. 1999). Our data demonstrating local production of OT but not VP emphasize the value of developing novel receptor antagonists with greater selectivity for the OT receptor. In fact, two highly selective OT receptor antagonists designed for treatment of preterm labour have recently been described (Nilsson et al. 2003, Serradeil-Le-Gal et al. 2004).

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References


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