RAPID COMMUNICATION

Identification of urocortin mRNA and peptide in the human endometrium

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Abstract

Urocortin is a 40-amino acid peptide belonging to the corticotropin-releasing factor (CRF) family. In human reproductive tissues, urocortin expression has been previously demonstrated in the ovary, in the placenta and fetal membranes and in pregnant uterine tissues, while no data are available on the expression of the peptide in the nonpregnant uterus. In this study, urocortin expression was evaluated by both immunohistochemistry and reverse transcription-polymerase chain reaction, in human uterine tissues and cells at different phases of the menstrual cycle.

Urocortin was immunolocalized in endometrial epithelial and stromal cells, as well as in the myometrium, and in vascular smooth muscle cells. No differences between proliferative and secretory phase were observed. These results were confirmed by reverse transcription-polymerase chain reaction analysis of isolated endometrial epithelial and stromal cells, and myometrial specimens. These findings open new questions on the roles played by urocortin in the human uterus.

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Introduction

Recent evidences suggest that the human endometrium is a neuroendocrine organ, being able to produce several brain peptides (Zoumakis *et al.* 1997). Among the hypothalamic factors, corticotropin-releasing factor (CRF) is one of the most studied and well characterized. In fact, CRF has been localized by different techniques in the human endometrium (Petraglia *et al.* 1992, Mastorakos *et al.* 1996, Di Blasio *et al.* 1997, Zoumakis *et al.* 1997) and expression of its R1 receptor has been provided in human endometrial stromal cells (Di Blasio *et al.* 1997).

Urocortin is a 40-amino acid peptide belonging to the CRF family, sharing a high sequence homology with CRF (Vaughan *et al.* 1995), binding CRF receptor with high affinity and increasing the release of ACTH from dispersed rat anterior pituitary cells (Asaba *et al.* 1998). To date, it is well known that, among human reproductive tissues, urocortin is expressed in the ovary (Muramatsu *et al.* 2001), in the placenta and fetal membranes (Petraglia *et al.* 1996, Florio *et al.* 1999), and in pregnant uterine tissues (Clifton *et al.* 2000), while there are no indications

available on the expression of the peptide in the non-pregnant uterus. The aim of this study was to evaluate urocortin localization and gene expression in uterine specimens at different stages of the menstrual cycle, and in isolated endometrial stromal cells and glands, by immunohistochemistry and reverse transcription-polymerase chain reaction. The results demonstrate that the nonpregnant human uterus is a site of urocortin expression.

Materials and Methods

Tissue samples

All patients gave their informed consent and the permission of the Human Investigation Committee was granted. Samples of nonpregnant endometrium ($n=27$) were obtained from patients with normal menstrual cycles undergoing either hysteroscopy for evaluation of the morphology of the uterine cavity or hysterectomy for leiomyoma. Subjects who had received steroid treatment during the past 6 months were not included in the study. All specimens were histologically examined by an

experienced gynecological pathologist (M.C.) and only normal endometria were selected for further analysis. Endometrial tissues were classified according to the last menstrual period and by the histological criteria of Noyes *et al.* (1975). Endometrial biopsies from hysteroscopy ($n=12$; age range 21–39 years; 4 proliferative phase; 8 secretory phase) were rinsed in sterile Hank's balanced salt solution (HBSS) at room temperature to remove excess blood and fixed in 10% buffered neutral formalin and embedded in paraffin for immunohistochemistry. Uteri at different phases of the menstrual cycle ($n=15$; age range 29–55 years), were either fixed in 10% buffered neutral formalin and portions embedded in paraffin for immunohistochemistry (5 proliferative phase; 4 secretory phase), or used for endometrial glands isolation (3 proliferative phase and 3 secretory phase) and myometrium sampling ($n=2$). Trophoblasts from placentas collected from health pregnant women ($n=3$) at term pregnancy (39 weeks of gestation) were used as positive control (Petraglia *et al.* 1996, Florio *et al.* 1999).

Immunohistochemistry

Immunohistochemistry was performed as described (Arcuri *et al.* 2001), using the streptavidin–biotin method. Sections were dewaxed, rehydrated and washed in Tris–buffered saline [TBS; 20 mM Tris–HCl, 150 mM NaCl (pH 7.6)]. Antigen retrieval was carried-out by incubating sections in sodium citrate buffer (10 mM, pH 6.0) in a microwave oven at 750 Watts for 5 min. Slides were incubated overnight at 4 °C with anti-rat urocortin polyclonal antibody (Santa Cruz biotechnology, Santa Cruz, CA, USA), diluted 1:50 in TBS and then incubated with a rabbit anti-goat antibody labeled with biotin (DAKO, Copenhagen, Denmark) at the dilution of 1:500 for 30 min. The reaction was revealed using the streptavidin–biotin complex (DAKO). Sections were mounted and examined under a light microscope. For each case, a negative control was obtained by using the antibody pre-adsorbed with the blocking peptide (Santa Cruz biotechnology) at the concentration of 20 µg per mL of diluted antibody.

Endometrial glands and stromal cells isolation

Endometrial glands were isolated from cycling endometria as described by Schatz and Gurpide (1987). Briefly, tissues were trimmed, minced and digested with type I collagenase (Worthington Biochemical Corp., Freehold, NJ, USA). Glands and stromal cells were then separated by filtration through a 20-µm disposable filter (DAKO). The glands, trapped on the filter, were backwashed with HBSS, and seeded on a tissue culture plastic Petri dish. The dish was then placed for 30 min in a standard incubator at 37 °C with a humidified atmosphere of 5% CO₂–95% air. This procedure was repeated twice to

purify the glands to virtual homogeneity. The gland-containing medium was then centrifuged for 10 min at $2000 \times g$ at 4 °C, the supernatant discarded and the pellet stored at –80 °C. The stromal cell-enriched fraction passed through the filter, was placed overnight in a standard incubator, washed with HBSS to remove contaminating bone marrow-derived elements and harvested with a cell scraper. Cells were recovered by centrifugation (10 min at $2000 \times g$ at 4 °C), and the pellet stored at –80 °C. To confirm the purity of cell preparations, aliquots of both glands and stromal cells were centrifuged on slides and analyzed by IHC for cytokeratin and vimentin expression.

Oligodeoxynucleotides

Urocortin primers were used for amplification by polymerase chain reaction (PCR). The 5' primer was 5'–CAG GCGAGCGGCCGCG–3', the 3' primer was 5'–CTTG CCCACCGAGTCGAAT–3'. The expected size of the amplified fragment was 145 base pairs.

Detection of urocortin mRNA

Urocortin mRNA was detected by reverse transcriptase–PCR (RT–PCR). Total RNA was extracted using the method of Chomczynski and Sacchi (1987). Samples were digested with a RNase-free DNase (Promega Corporation, Madison, WI, USA) following the manufacture's suggested conditions, and RNA quantified by UV absorption. RT–PCR analysis was carried out as previously described (Arcuri *et al.* 2001). In brief, one µg of total RNA was diluted in 10 mM Tris–HCl, 50 mM KCl, 5 mM MgCl₂ (pH 8.3), containing 50 U of M–MLV reverse transcriptase (GeneAmp Kit, Perkin Elmer, Norwalk, CT), 20 U of placental RNase inhibitor, deoxynucleotides (dNTPs; 1 mM each of dGTP, dATP, dTTP and dCTP), 2.5 µM oligo d(T) primers in 20 µl of volume. The mixture was incubated at 42 °C for 15 min, 99 °C for 5 min, and 5 °C for 5 min in a programmable thermal cycler (MJ Research, Watertown, MA, USA). For each specimen, a blank was prepared by omitting the reverse transcriptase.

Two µl of RT reaction product were added to a mix containing 5X reaction buffer [300 mM Tris–HCl, 75 mM (NH₄)₂SO₄, 7.5 mM MgCl₂ (pH 8.5)], dNTP mixture (final concentration 0.25 mM) 1.0 U of cloned *Thermus aquaticus* DNA polymerase (Life Technologies, Grand Island, NY, USA), and urocortin primers (final concentration 0.4 µM) in a volume of 50 µl. PCR was carried out in a programmable thermal cycler (Bio–Rad, Hercules, CA, USA). Amplifications were carried out for 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C for 30 cycles followed by a final 10 min at 72 °C. For each specimen, a blank was prepared using 2 µl of the

corresponding RT blank. One-fifth of each PCR solution was fractionated by electrophoresis in a 1.8% agarose gel.

Assessment of PCR product

PCR product identity was confirmed by restriction analysis. In brief, PCR products were extracted with phenol:chloroform and precipitated with ethanol. The amplified fragment was digested with *Av*II (Sigma Chemical Co.) following the manufacturer's suggested conditions. The products were separated by 12% polyacrylamide gel electrophoresis, visualized by ethidium bromide staining and photographed.

Results

Immunohistochemical localization

Tissue distribution of urocortin in the human endometrium was first analyzed by immunohistochemistry. Figure 1 presents images of sections of cycling endometrium stained with the anti-urocortin antibody. Immunoreactivity was depicted in all human uterine specimens examined. Strong staining was observed in endometrial luminal and glandular epithelial cells, and moderate immunoreactivity was present in stromal cells of both proliferative and secretory phase specimens (Figure 1A and 1B). Intense immunostaining was also evident in the myometrium (Figure 1C), as well as in the myometrial vascular smooth muscle cells (Figure 1D). Abolishing of staining was obtained by absorbing the antibody with the blocking peptide (negative control, Figure 1E).

Expression of urocortin mRNA

To evaluate the steady-state levels of urocortin mRNA in the human uterus, isolated endometrial epithelial and stromal cells, and specimens of myometrial tissue, were analyzed by RT-PCR. When amplification was carried out in the presence of urocortin primers, a band of 145 bp, corresponding to the urocortin product, was generated from the cDNA of endometrial epithelial ($n=6$) and stromal ($n=6$) cells from both proliferative and secretory phase tissues, and as well as from that of myometrial specimens ($n=2$) (Figure 2). The identity of urocortin PCR product was then confirmed by restriction analysis with *Av*II. As shown in Figure 3, in all samples examined the 145 bp PCR fragment contained an *Av*II site yielding products of the expected size.

Discussion

The human endometrium is a site of synthesis and action of neuroendocrine peptides (Zoumakis *et al.* 1997). In

particular, endometrial CRF expression and functions have been extensively evaluated, as the peptide plays important roles in decidualization (Ferrari *et al.* 1995, Zoumakis *et al.* 2000), and in cell proliferation of human endometrial cells (Graziani *et al.* 2002). CRF mRNA and peptide are detectable in the epithelial cells of human endometrium (Gravanis *et al.* 2001), and CRF mRNA is expressed, and the peptide secreted, by human endometrial stromal cells throughout the menstrual cycle (Di Blasio *et al.* 1997). Finally, stromal cells express CRF receptor type 1 mRNA (Di Blasio *et al.* 1997).

The present study represents the first demonstration of urocortin expression in the human uterus during the menstrual cycle. The peptide was immunolocalized in the endometrial epithelial and stromal cells. In addition, urocortin was identified in the myometrium, as well as in vascular smooth muscle cells. Finally, urocortin mRNA was detected by RT-PCR in both stromal and epithelial endometrial cells and in the myometrium. These findings open the question of the possible roles played by urocortin in the human endometrium. Indeed, the high homology in sequence and biological activity with CRF (Vaughan *et al.* 1995), together with the high affinity of the peptide for CRF receptor type 1 (Vaughan *et al.* 1995, Perrin and Vale 1999), suggest an involvement of urocortin in endometrial physiology. Thus, as CRF modulates cell growth of human endometrial cells via CRF-receptor1-mediated activation of cAMP-PKA pathway, a participation of urocortin on this process may be hypothesized (Graziani *et al.* 2002). Moreover, a role of urocortin in modulating endometrial hormonal secretion by a paracrine/autocrine mechanism may also be postulated. In fact, CRF and urocortin stimulate pituitary proopiomelanocortin (POMC) gene expression (Vaughan *et al.* 1995, Turnbull and Rivier 1997, Asaba *et al.* 1998, Hsu and Hsueh 2001). It is also well known that human endometrium expresses the POMC gene and synthesizes its peptides (Zoumakis *et al.* 1997), and that β -endorphin is detectable in human endometrium and in uterine secretions (Petraglia *et al.* 1986). The expression of urocortin in the human endometrium shown in the present study therefore suggest an effect of the peptide on endometrial POMC-derived peptide secretion, as in the placenta (Petraglia *et al.* 1999) and in the pituitary (Vaughan *et al.* 1995, Turnbull and Rivier 1997, Asaba *et al.* 1998, Hsu and Hsueh 2001).

Urocortin may also play a role in the regulation of myometrial tone, as suggested for CRF and β -endorphin. This is supported by the presence of β -endorphin, CRF (Clifton *et al.* 1998) and its type 1 receptor (Grammatopoulos *et al.* 1998) in the human myometrium, and by the known effect of urocortin on myometrial contractility (Petraglia *et al.* 1999, Grammatopoulos *et al.* 2000).

Finally, urocortin may be involved in the modulation of endometrial inflammatory and/or vascular changes, as

Figure 2 Representative results of RT-PCR analysis of uterine urocortin mRNA. Lane 1: endometrial epithelial cells (Epith); lane 3: endometrial stromal cells (Strom); lane 5: myometrium (Myom); lane 7: trophoblasts (Troph, positive control). Lane 2, 4, 6, 8: blanks. The size of molecular weight marker (lane M, bp) is indicated.

Figure 3 Uterine urocortin PCR product verification by restriction analysis. Fragments digested with Avall and undigested, of endometrial epithelial cells (Epith, lanes 1 and 2); endometrial stromal cells (Strom, lanes 3 and 4); myometrium (Myom, lanes 5 and 6); trophoblasts (Troph, lanes 7 and 8), were analyzed by polyacrylamide gel electrophoresis. Predicted sizes of fragments are 61 and 84 bp. The size of molecular weight markers (lanes M; bp) is indicated.

already proposed in the placenta (Leitch *et al.* 1998). Indeed, while endometrial vascular changes have been reported during the menstrual cycle, the urocortin expression by myometrial and vascular smooth muscle cells, described in the present study, supports a participation of urocortin in the process of endometrial vascularization.

In conclusion, the present study showed that urocortin is expressed by both epithelial and stromal cells of the human endometrium, in the myometrium, as well as in vascular smooth muscle cells. The distribution of urocortin

in the endometrium suggests the existence of multiple sites of action of the peptide and its involvement in several aspects of endometrial physiology.

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Figure 1 Immunolocalization of urocortin in the human endometrium. A: proliferative phase endometrium, original magnification (200 ×). B: secretory phase endometrium, original magnification (200 ×). C: myometrium, original magnification (100 ×). D: vascular smooth muscle cells, original magnification (200 ×). E: negative control, original magnification (200 ×).

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