Human endometrium expresses urocortin II and III messenger RNA and peptides

Urocortin II and urocortin III are recently identified corticotropin-releasing factor–related neuropeptides, and their endometrial expression throughout the menstrual cycle and in early pregnancy was evaluated in the present study by semiquantitative reverse transcriptase polymerase chain reaction and immunohistochemistry. The endometrial expression of urocortin II mRNA was significantly \( (P < 0.01) \) higher in the early proliferative phase of the menstrual cycle than in other phases and maternal decidua (MD), whereas that of urocortin III mRNA was higher \( (P < 0.01) \) in MD than in all other endometrial samples. Both peptides were immunolocalized in epithelial, stromal, and endothelial cells. (Fertil Steril 2006;86:1766–70. ©2006 by American Society for Reproductive Medicine.)

Corticotropin-releasing factor (CRF) is a neuropeptide of 41 amino acids and has a key role in diverse aspects of the stress regulation of the hypothalamus-pituitary-adrenal (HPA) axis (1). The family of CRF comprises recently identified neuropeptides, namely urocortin (UCN) I, II, and III (2–4). Urocortin I is a 40-amino-acid peptide that shares 45% sequence homology with CRF (2); UCN II shows moderate homology to human CRF (34%) and human UCN I (43%) and UCN III (37%–40%), which in turn shares 32% homology to human CRF and 26% to human UCN I (3, 4). The UCNs and CRF bind with different affinity to two distinct receptors, respectively, CRF-type 1 (CRF-R1) and -type 2 (CRF-R2). Urocortin I displays high affinity for CRF-R2, whereas CRF has approximately ten-fold lower affinity to this receptor; UCN II and III specifically bind only to CRF-R2 (5).

Corticotropin-releasing factor (6, 7) and UCN I (8) are expressed by the human endometrium (epithelial and stromal cells) (6, 7), and immunoreactive CRF concentrations in endometrial biopsies are higher in the secretory than in the proliferative phase (9). These findings, together with the evidence that the human endometrium also expresses both CRF-R1 (10) and CRF-R2 (11), suggest that the endometrium is the source but also the target of both CRF and UCNs. In this regard, the activation of CRF receptor subtypes has been demonstrated to have important roles in several aspects of endometrial physiology, as in the case of decidualization of endometrial stromal cells (12, 13), the induction of blastocyst implantation and early maternal tolerance (14), and the inhibition of tumor cell growth and proliferation of endometrial cells derived from a tumor cell line (15).

Therefore, the present study evaluated whether UCN II and III messenger (m) RNA and peptides are expressed by human endometrium and whether their expression changes through the endometrial cycle and in early pregnant decidua.

MATERIALS AND METHODS

Samples of nonpregnant endometrium were obtained from healthy fertile subjects \( (n = 34; \) age range 37 to 43 years) with normal menstrual cycle and actually ovulatory, as assessed by transvaginal ultrasonography (16), who were medical students or laboratory staff and who volunteered for the study, underwent hysteroscopy, and were found to be free of endometrial pathologies. Subjects who had received steroid treatment during the previous 6 months were not included in the study. Specimens were classified as early (EP; \( n = 10 \)) and late (LP; \( n = 9 \)) proliferative, and early (ES; \( n = 8 \)) and late (LS; \( n = 7 \)) secretory endometrium according to the last day of menstruation and confirmed by both transvaginal ultrasonography (16) and by the histologic criteria of Noyes et al. (17).

We also collected samples of maternal decidua (MD; \( n = 7 \), gestational age ranging from 8 to 12 weeks from pregnant women who underwent a voluntary termination of pregnancy) and pregnant myometrium \( (n = 3) \) obtained from women undergoing elective cesarean section at term, which were used as positive control for UCN II (18). Informed written consent was obtained from all subjects before inclusion in the study, for which approval was obtained from the local Institutional Review Board. Frozen kidney tissues (about 1 g) obtained within 4 h postmortem from two male patients were used as positive control for UCN III (19).
Samples were disrupted and homogenized using Mixer Mill MM 300 (Qiagen, Milan, Italy), and total RNA was extracted with RNeasy Protect Mini Kit and then treated with RNase-free DNase according to the instructions of the manufacturer (Qiagen). RNA was quantified by UV absorption, and 1 µg was reverse transcribed to prepare complementary DNA (cDNA). Reaction conditions for reverse transcriptions were as previously described (20). Two microliters of reaction product was used for polymerase chain reaction (PCR). The PCR conditions were as follows: 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.25 mmol/L dNTPs, 1 U Taq DNA polymerase recombinant (Invitrogen, Milan, Italy), and 0.4 µmol/L (final concentration) UCN II (sense 5′-gtgtaggcaatgtgagctgagaga-3′ and antisense 5′-atctgatatgacagtacgtgctgt-3′) and UCN III (sense 5′-tgtagctgctctgtgctgtc-3′ and antisense 5′-tgtagctgctctgtgctgtc-3′) primers in 50 µL total volume. Sequence homology among the different oligomers used in the present study was also avoided, excluding possible cross-reactions. The expected size of the amplified fragment was 195 base pairs (bp) for UCN II and 310 bp for UCN III. Blank for each reaction, consisting of amplifications performed in the absence of reverse transcriptase enzyme (RT), was done.

To estimate differences in mRNA expression of UCN II and UCN III, different phases of the menstrual cycle were compared by semiquantitative RT-PCR, adjusted with glyceraldehyde-3-phosphate dehydrogenase (GAPDH; primers: sense 5′-gaagctgtaagcctgagactg-3′ and antisense 5′-cttggagagggagaagccgttc-3′) as the internal standard. The number of cycles needed to perform the PCR in linear phase was selected after a number of calibration experiments in the range of 28–36 runs. The UCN II amplification was carried out at 94°C for 1 minute, at 60°C for 1 minute, and at 72°C for 1 minute for 30 thermed cycle steps, followed by a final step at 72°C for 10 minutes. The UCN III amplification was carried out at 94°C for 1 minute, at 63°C for 1 minute, and at 72°C for 1 minute for 32 thermed cycle steps, followed by a final step at 72°C for 10 minutes. The GAPDH amplification was carried out at 94°C for 30 seconds, at 52 °C for 30 seconds, and at 72°C for 30 seconds for 28 cycles, followed by a final step at 72°C for 10 minutes. Amplification products were visualized on 2% agarose gel and stained with 3% ethidium bromide. The expected bands were quantified by densitometric analysis performed using Image J software (National Institutes of Health) by an employee of the federal government. Relative amount of UCN II and UCN III mRNA was calculated as UCN II/GAPDH and UCN III/GAPDH mRNA ratios.

To evaluate the localization of UCN II and UCN III, immunohistochemistry was carried out on 5-µm thick sections, obtained from paraffin-embedded samples, mounted on electrostatically charged slides, and dried overnight at 37°C. Sections were dewaxed, rehydrated, and washed in Tris-buffered saline [TBS: 20 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 7.6)]. Tissue solutions were heated in a microwave oven twice for 5 minutes at 750 W and rinsed in 3% H2O2 to block endogenous peroxidase. Slides were incubated overnight at room temperature with primary antibody. Antibodies used for UCN II (21) and UCN III (22) were rabbit antihuman polyclonal diluted 1:1000, provided by Professor W. Vale (Salk Institute for Peptide Biology, La Jolla, CA). Anti-UCN II and anti-UCN III are affinity-purified goat polyclonal antibodies, raised against peptide mappings at the carboxy terminus of UCN II and UCN III of human origin. These antibodies, identical to corresponding mouse and rat sequences, recognize UCN II and III of mouse, rat, and human origin with no cross-reactivity, and human or rat CRF, sheep CRF, PACAP38, human ACTH, and sauvagine (21, 22).

The reactions were developed by successive incubations with antirabbit immunoglobulins labeled with biotin, the avidin-biotin peroxidase complex (Vector Lab, Burlingame, CA), and 1 mg/mL 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) as chromogen substrate in TBS containing 0.3% H2O2. Harris hematoxylin was used for nuclear counterstaining. A positive reaction was characterized by the presence of granular brown staining in the cytoplasm. For each case, a negative control was obtained by using the antibody preadsorbed with the corresponding peptide at the concentration of 20 µg/mL diluted antibody.

After confirming a normal distribution, the data were summarized as mean ± standard error (SE). Between-group differences were evaluated by using the analysis of variance test, followed by the post-hoc Tukey test for multiple comparisons. Statistical significance was assumed when P<.05.

RESULTS
Total RNA extracted from endometrium was analyzed by RT-PCR. Predicted bands corresponding in size to UCN II (196 bp) and to UCN III (310 bp) products were obtained, and no amplified fragment caused by DNA contamination was detected in any experiment. When evaluated by semiquantitative RT-PCR, the expression of UCN II mRNA (expressed as UCN II/GAPDH mRNA ratio) was highest in the early proliferative endometrium, significantly (P<.01) higher than in other samples and decidua, and the highest expression of UCN III mRNA was in maternal decidua, significantly (P<.01) higher than in endometrial phases (data not shown).

In proliferative endometrium, immunoreactive UCN II was found in luminal and glandular epithelial and vascular endothelial cells, and UCN II-positive stromal cells were
most abundant close to the superficial layers (Fig. 1A). In
secretory endometrium, luminal epithelial cells were
strongly immunostained, whereas staining was weaker in
glandular cells (Fig. 1B). Maternal decidua cells showed
positive UCN II stain, with immunoreactivity found in
epithelial stromal and endothelial cells from spiral arte-
rioles (Figs. 1C and 1D).

Urocortin III intensely immunostained superficial and
glandular cells as well as vascular endothelial and stromal
cells of the proliferative endometrium (Fig. 1F). Secretory
endometrium (Fig. 1G) and maternal decidua (Figs. 1H and
I) showed intense UCN III immunostaining in superficial
and glandular epithelial, stromal, and vascular cells.

DISCUSSION
The present study is the first to refer to the expression of
UCN II and III into human endometrium and maternal
decidua and to their immunolocalization in epithelial, stro-
mal and endothelial cells. The mRNA expression of UCN
II was higher in the early proliferative endometrium, and
that of UCN III in pregnant decidua. The facts that UCN II
and UCN III are members of the CRF family of peptides
(2–4) and that CRF (6, 7), UCN I (8, 23) and CRF
receptors (10,11) are expressed throughout the endometrial
cycle together reinforce the notion that the human endo-
metrium possesses the ability to synthesize peptides/hor-
mones that are normally expressed into the brain. More-

UCN II (A–E) and III (F–J) immunolocalization in human endometrium and pregnant decidua. (A) Proliferative
endometrium. Intense UCN II immunostaining was mainly localized in epithelial luminal (black arrows) and
glandular (white arrows) cells, whereas a weaker staining was found in stromal cells. Endothelial cells of the
vessel walls (dotted circles) showed an intense staining. (B) Secretory endometrium. Epithelial glandular (white
arrows) and luminal (black arrows) cells showed intense UCN II staining. Again, UCN II immunostaining was less
intense in stromal cells. (C, D) Early pregnant endometrium. UCN II was localized in epithelial stromal (black
arrows) and endothelial (dotted circles) cells from spiral arterioles. (E) Negative control obtained by using the
antibody preadsorbed with UCN II (20 μg). (F) Proliferative endometrium. Intense UCN III immunostaining was
mainly localized in epithelial luminal (black arrows) and glandular (white arrows) cells, whereas a weaker staining
was found in stromal cells. Endothelial cells of the vessel walls (dotted circles) showed an intense staining. (G)
Secretory endometrium. Epithelial glandular (white arrows) and luminal (black arrows) cells showed intense UCN
III staining. Again, UCN III immunostaining was less intense in stromal cells. (H, I) Early pregnant endometrium.
UCN III was localized in epithelial (black arrows), stromal, and endothelial (dotted circles) cells. (J) Negative
control obtained by using the antibody preadsorbed with UCN III (20 μg).
over, the findings of the local expression of UCN II and UCN III open the question of the possible roles played by these neuropeptides in the human endometrium.

Urocortins II and III specifically bind to CRF-R2 (5). The fact that the coupling of CRF-R1 and -R2 activates different intracellular signalling cascades that mediate different effects of CRF and UCNs leads us to suggest that distinct signaling networks exist in the human endometrium, involving UCN II and III and CRF-R2 receptors as well as involving CRF and UCN I with CRF-R1.

Vascular endothelial cells express CRF-R2 (24, 25), the binding of UCNs to CRF-R2 mediates vasodilatation (26), and both UCN II and III have shown to exert cytprotective effect in myocardiocytes (27). The endometrium is a tissue with strong remodeling, and a potential involvement of both UCNs in endometrial cell survival and apoptosis during the menstrual cycle and early in pregnancy may be hypothesized. Therefore, we may propose that UCN II and III regulate endometrial angiogenesis and/or vascular endothelial tone that are key events in the mechanisms of menstruation. Prostaglandins (PGs) are involved in menstruation (28), and the activation of CRF-Rs modulates its synthesis (12) through the modulation of cyclooxygenase-2 (COX-2) activity, the enzyme that locally synthesizes PGs (29, 30). The inhibition of PG release is also fundamental for pregnancy to occur, and a marked inhibition of decidual PGs synthetase activity occurs very soon during early pregnancy (27).

These findings and the evidence that CRF suppresses the production of PGs from human endometrial cells in time- and dose-dependent fashion, probably through the inhibition of COX-2 (12, 31), lead us to suggest that UCNs may be involved in regulating the endometrial PG pathway. Finally, the recent studies on the potent effects of UCNs on the immune system (32), together with the effect of CRF on early maternal tolerance (14), lead us to suggest that UCNs may also have important local roles on early maternal tolerance.

In conclusion, UCN II and III are expressed by human endometrium and early decidua. Their different secretory patterns, together with their selective binding to CRF-R2, suggest that they may have different actions in endometrial physiology.

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