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ORIGINAL RESEARCH

Urocortin 2 and urocortin 3 in endometriosis: evidence for a possible role in inflammatory response

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ABSTRACT: Urocortin 2 (Ucn 2) and urocortin 3 (Ucn 3) are neuropeptides expressed by human endometrium. This study evaluated (i) the expression of Ucn 2 and Ucn 3 mRNA in endometriotic lesions and in endometrium of women with endometriosis; (ii) the effect of Ucn 2 and Ucn 3 on cytokines secretion from cultured endometrial stromal cells. Endometriotic tissue was collected from endometrioma ($n = 39$); endometrial specimens were obtained from women with ($n = 39$) and without ($n = 41$) endometriosis throughout menstrual cycle. Tissue specimens were analysed for Ucn 2 and Ucn 3 mRNA expression and peptide localization; the effects of Ucn 2 or Ucn 3 on tumour necrosis factor (TNF-α) and interleukin (IL-4) secretion from cultured endometrial stromal cells was studied. Ucn 2 and Ucn 3 mRNA expression and localization were assessed by RT–PCR and by immuohistochemistry, respectively; cytokines secretion were measured by ELISA. Results showed that endometriotic tissue expressed both Ucn 2 and Ucn 3, with Ucn 3 expression higher in ectopic than in eutopic endometrium. Endometrial Ucn 2 mRNA expression in controls showed peak values at early proliferative phase, while in endometriotic patients low expression and no significant changes throughout menstrual cycle were observed. Endometrial Ucn 3 mRNA expression was highest in late secretory phase in controls, while in endometriotic patients low levels and no menstrual-cycle-related changes were found. When added to cultured endometrial cell cultures, Ucn 2 significantly increased TNF-α ($P < 0.01$) and IL-4 ($P < 0.001$), while Ucn 3 induced an increase of IL-4 secretion ($P < 0.01$). In conclusion, endometriotic tissue expressed and localized Ucn 2 and Ucn 3; patients with endometriosis showed Ucn 2 and Ucn 3 mRNA expression in eutopic endometrium lower than in control group, with no endometrial cycle-related changes. Ucn 2 and Ucn 3-modulated TNF-α and IL-4 secretion from culture endometrial cells. These data suggest a possible involvement of Ucn 2 and Ucn 3 in the mechanisms of endometriosis.

Key words: endometriosis / urocortin 2 / urocortin 3 / endometrium / inflammation

Introduction

Human endometrium undergoes morphological and functional changes throughout menstrual cycle; sex steroid hormones modulate proliferation and differentiation of endometrial stromal cells interacting with local growth factors and cytokines (King and Critchley, 2010; Maybin *et al.*, 2011). Endocrine, paracrine and immunological influences regulate cellular events leading to embryo implantation, or alternatively menstruation when conception does not occur (Maybin *et al.*, 2011). Proliferative/secretory phases and decidualization are endometrial processes that prepare endometrium for blastocyst implantation and establishment of pregnancy, and for regulating trophoblast invasion when pregnancy is achieved (Strowitzki *et al.*, 2006).

Corticotrophin-releasing hormone (CRH), urocortin (Ucn), urocortin 2 (Ucn 2) and urocortin 3 (Ucn 3) are neuropeptides, originally described in central nervous system, exerting endocrine, immune and vasoactive effects (Zoumakis *et al.*, 2009; Kalantaridoua *et al.*, 2010). Reproductive organs also express and localize these neuropeptides and their specific receptors CRH-R1 and CRH-R2, encoded by two different genes (Grigoriadis *et al.*, 1996). Human endometrial epithelial and stromal cells as well as vascular endothelial cells localize and express CRH (Mastorakos *et al.*, 1996), Ucn (Florio *et al.*, 2002), Ucn 2 and Ucn 3 (Florio *et al.*, 2006) as well as CRH-R1 and CRH-R2

mRNAs (Di Blasio *et al.*, 1997; Karteris *et al.*, 2004). A critical role in endometrial decidualization has been described for CRH (Ferrari *et al.*, 1995; Zoumakis *et al.*, 2009) and Ucn (Torricelli *et al.*, 2007), suggesting that the CRH pathway may also be critical for preparing endometrium for embryo implantation (Zoumakis *et al.*, 2009).

Endometriosis is a sex steroid hormone dependent disease, characterized by inflammation and neoangiogenesis, which affects women during reproductive age (Giudice, 2010). The presence of neuroendocrine cells in eutopic endometrium of women with endometriosis has been shown (Wang *et al.*, 2010). The evidence of CRH (Kempuraj *et al.*, 2004) and Ucn (Florio *et al.*, 2007) in endometriotic tissue and their deranged expression in endometrium of endometriotic patients (Novembri *et al.*, 2011) suggests that these neuropeptides may also participate in the pathogenesis of endometriosis. Because Ucn 2 and Ucn 3 are involved in inflammation, vascularization and apoptosis (Tsatsanis *et al.*, 2005; Im *et al.*, 2010), the present study aimed to evaluate: (i) the possible expression and localization of Ucn 2 and Ucn 3 in endometriotic tissues; (ii) the changes of Ucn 2 and Ucn 3 mRNA expression in eutopic endometrium of endometriotic women during menstrual cycle; (iii) the effect of Ucn 2 and Ucn 3 on cytokines secretion from cultured human endometrial cells.

Materials and Methods

Samples collection

The study group included non-pregnant women with endometriosis ($n = 39$) (age range between 23 and 40 years) who underwent laparoscopic excision of endometrioma (cyst diameter measured was in the range of 40–72 mm). All patients of the study group were classified as having stage III or IV endometriosis according to the American Society for Reproductive Medicine classification of endometriosis (The American Fertility Society, 1985).

The control group was represented by 'healthy' non-pregnant women ($n = 41$) (age range between 25 and 41 years), undergoing laparoscopic sterilization or diagnostic hysteroscopy.

All women showed a regular menstrual cycle (28–30 days); endometrial specimens were classified as early and late proliferative or secretive phase according to the last menstrual period confirmed by ultrasound scans by transvaginal probe at 4.5–7.0 MHz (Esaote, Italy) (Severi *et al.*, 2003) and by the histological criteria (Noyes *et al.*, 1975). The number of samples per cycle phases are shown in Table I.

Informed consent was obtained from all women prior to inclusion in the study, which was approved by the local Human Investigation Committee. Women with endocrine disorders, uterine fibroma or other clinic disorders were excluded. Subjects who had received steroid treatment during the previous 3 months were excluded. Endometriotic and endometrial tissues were collected through biopsy under anaesthesia during the endoscopic procedure. Specimens were separated in two aliquots: one aliquot was immediately submerged in liquid nitrogen to allow subsequent RNA extraction and real-time PCR, while the other was fixed by immersion in 10% buffered formalin to allow diagnosis confirmation and peptide localization by immunohistochemistry.

Immunohistochemistry

To evaluate the localization of Ucn 2 and Ucn 3, 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 sections were rinsed in 3% hydrogen peroxide to block endogenous peroxidise and heated in microwave oven for 15 min at 750 W in citrate buffer pH 6 (Ucn 2) or EDTA (Ucn 3). Slides were incubated overnight at room temperature with primary antibody. For Ucn 2 and Ucn 3, we used polyclonal antisera kindly donated by Dr Joan Vaughan from the Salk Institute (La Jolla, CA, USA) diluted 1:100. Anti-Ucn 2 (PBL #6488, 10/26/00 bleed) was raised in rabbit against synthetic GlyTyr-human Ucn 2 conjugated to human α-globulins via bis-diazotized benzidine. Anti-Ucn 3 (PBL #6570, 08/23/01 bleed) was raised in rabbit against synthetic GlyTyr-human Ucn 3 conjugated to human α-globulins via bisdiazotized benzidine. Methods used for generation of Ucn 3 antisera were similar to those described in detail for inhibin subunits (Vaughan *et al.*, 1989). Characterization and specificity of Ucn 2 (Chen *et al.*, 2003) and Ucn 3 (Li *et al.*, 2002) antisera was described previously. The reaction was developed by successive incubations with anti-rabbit immunoglobulins labelled with biotin, the avidin–biotin peroxidise complex (Vector Laboratories, Burlingame, CA), and 1 mg/ml 3,3′-diamonobenzidine tetrahydrocloride (Sigma, St Louis, MO) in TBS containing 0.3% H_2O_2, as chromogen substrate. Harris haematoxylin was used for nuclear counterstaining. A positive reaction was characterized by the presence of glandular brown staining in the cytoplasm. For each case, a negative control was obtained by using the antibody pre adsorbed with the corresponding peptide at the concentration of 20 μg/ml of diluted antibody.

Endometrial cultures

In order to evaluate the effect of Ucn 2 and Ucn 3 on cytokines secretion, endometrial cells from controls were cultured ($n = 8$). All samples collected were in proliferative phase (according to the last menstrual period, assessed by vaginal ultrasound scans and histology).

Human endometrial stromal cells (HESCs) were isolated immediately after collection as previously described (Ferrari *et al.*, 1995). Briefly, tissue samples were gently minced into small pieces and incubated for

Table I

Characteristics	Controls				Endometriosis			
Phases of menstrual cycle	Proliferative		Secretory		Proliferative		Secretory	
	Early	Late	Early	Late	Early	Late	Early	Late
Patients *n*°	9	10	11	9	9	11	10	11
Age (mean years)	30	29.30	28.82	28.56	30.89	30.55	29.60	30.45
SD	(6.10)	(6.20)	(6.09)	(6.76)	(6.75)	(6.15)	(5.58)	(6.00)

Early proliferative (Days 5–10); late proliferative (Days 11–14); early secretory (Days 14–22); late secretory (23 onwards).

1–2 h at 37°C in a shaking water bath, in 10 ml of Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Italy) containing 0.1% collagenase. Stromal cells were then separated by filtration and centrifuged for 10 min at 1200 rpm at room temperature. Then, the pellet was resuspended in DMEM/F12 medium supplemented with 10% charcoal-stripped calf serum (Sigma, Italy) and antibiotics (Invitrogen, Italy) and cultured at 37°C in a 95% air and 5% CO_2 incubator. The purity of stromal cells obtained by this method was usually >95%, as determined by immunohistochemical staining for vimentin (stromal cell marker) and cytokeratin (epithelial cell marker). Macrophages contamination of cultures was <2%, as assessed by flow cytometric analysis. After confluence, conditioned medium was changed to a serum-free DMEM-F12 and after 16 h of starvation, stromal cells were treated with: (i) vehicle alone—dimethylsulphoxide (Euroclone, Milan, Italy) (control) or (ii) Ucn 2 or Ucn 3 (at 10^{-6}, 10^{-7} or 10^{-8} M), and the cytokines secretion analysed after 24 h of treatment. The pretreatment with a CRH-R2 selective antagonist, Ast2b (10^{-7} M), was also studied. Each treatment was performed in duplicate.

RNA extraction and cDNA preparation

Frozen endometrial specimens 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 (RNeasy protect Mini Kit Qiagen, Hilden, Germany). RNA was quantified by UV absorption (OD260), and 300 ng were reverse-transcribed to prepare complementary DNA (cDNA). cDNA synthesis from total RNA was carried out in a reaction volume of 20 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 5 MM random hexamer primer, 2.7 mM deoxynucleoside triphosphate and 10 U/Ml SuperScript II reverse transcriptase (all reagents obtained from Invitrogen Life Technologies, Inc.). RNA was initially denatured at 85°C for 5 min. The reaction mixture was then added, and RT was performed at 42°C for 90 min. The reaction was stopped by denaturing the enzyme at 85°C for 15 min. The cDNA was subsequently subjected to RT–PCR.

Real-time polymerase chain reaction

Differences in mRNA expression of Ucn 2 and Ucn 3, between controls and endometriosis patients were measured by RT–PCR (TaqMan PCR, Applied Biosystems, Weiterstadt, Germany), using an Opticon 2 thermal cycler (MJ Research, Bio-Rad Laboratories, Waltham, MA). We used TaqMan gene expression assays (Applied Biosystems): housekeeping gene 18s (assay identification no. Hs Hs03003631_g1) was used as internal standard, Ucn 2 (assay identification no. Hs00264218_s1) and Ucn 3 (assay identification no. Hs00846499_s1). The following thermal cycle protocol was applied: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and primer annealing and elongation at 60°C for 1 min using 100 ng cDNA in a final reaction volume of 20 μl. Negative control for each reaction consisted of amplification performed in the absence of reverse transcriptase enzyme (RT) and blank samples in absence of RNA samples (H_2O RT). All experiments were done in triplicate on 96-well optical PCR plates (Applied Biosystems), optimized to the universal PCR protocol of the manufacturer, with a TaqMan Universal PCR Master Mix (Applied Biosystems) and standard curves were constructed for all target genes by serial dilution of a standard sample starting from 200 ng to cDNA. The results were then normalized to the housekeeping gene 18S in order to correct differences in concentration of the starting template. The two computerized tomography (CT) method was applied as a comparative method of quantification (Livak and Schmittgen, 2001).

Cytokines assay

Cell culture media were assayed for cytokines secretion using commercially available ELISA kits, according to the manufacturer's instructions: Interleukin (IL)-4 (range: 1.1–58 pg/ml) (Abcam, UK) and tumour necrosis factor (TNF)-α (range: 39.0–250 pg/ml) (Phoenix Peptides, USA) Results are expressed as pg/ml.

Statistical analysis

The real-time PCR results were analysed based on the CT methods, which is the primary source of data variability (Livak and Schmittgen, 2001). The CT values were normally distributed and therefore they were summarized as mean ± standard error. Two-way analysis of variance (ANOVA) was used to estimate the overall effects of group (control versus endometriosis) and menstrual cycle phase (proliferative versus secretory) on the relative expression of Ucns mRNA in the endometrium. When significant at the $P < 0.05$ level, the ANOVA was followed by Student–Newman–Keuls test for multiple comparison. Two group comparisons between eutopic and ectopic endometrium from endometriosis were performed with Student's *t*-test, considering $P < 0.05$ as statistically significant.

Results

Ucn 2 and Ucn 3 immunolocalization in endometriotic tissue

The tissue distribution of Ucns in endometrioma and in endometrium of patients with endometriosis was analysed by immunohistochemistry. Figure 1 shows the images of sections of these tissues stained with antibody anti-Ucn 2 or anti-Ucn 3.

Ucn 2 was localized in the surface and glandular epithelia of eutopic endometrium from women with endometriosis, as well as in the epithelial layer of endometriotic cystis (Fig. 1). Ucn 2 was also localized in some stromal cells and vascular endothelium of endometrial specimens evaluated (Fig. 1).

Ucn 3 was detected in superficial and glandular cells, as well as vascular endothelial, inflammatory and stromal cells of eutopic endometrium from women with endometriosis and of endometriotic tissue (Fig. 1). The staining in endometriotic tissue appeared more intense than in eutopic endometrium (Fig. 1).

Staining was abolished by absorbing the antibodies with the blocking peptides (Fig. 1; NC, negative controls).

Ucn 2 and Ucn 3 mRNA expression in endometrioma

Ucn 2 and Ucn 3 were evaluated and found to be expressed in all endometrioma tissue (Fig. 2). While no differences were observed in Ucn 2 mRNA expression between eutopic endometrium and endometriomas, the mRNA expression of Ucn 3 was significantly higher in endometriomas than in eutopic endometrium ($P < 0.05$) (Fig. 2B). Neither Ucn 2 nor Ucn 3 expression in endometriomas changed throughout endometrial cycle.

Changes of Ucn 2 and Ucn 3 mRNA expression in eutopic endometrium throughout menstrual cycle

mRNA encoding Ucn 2 and Ucn 3 were detectable in eutopic endometrium from women with or without endometriosis (Fig. 3). In

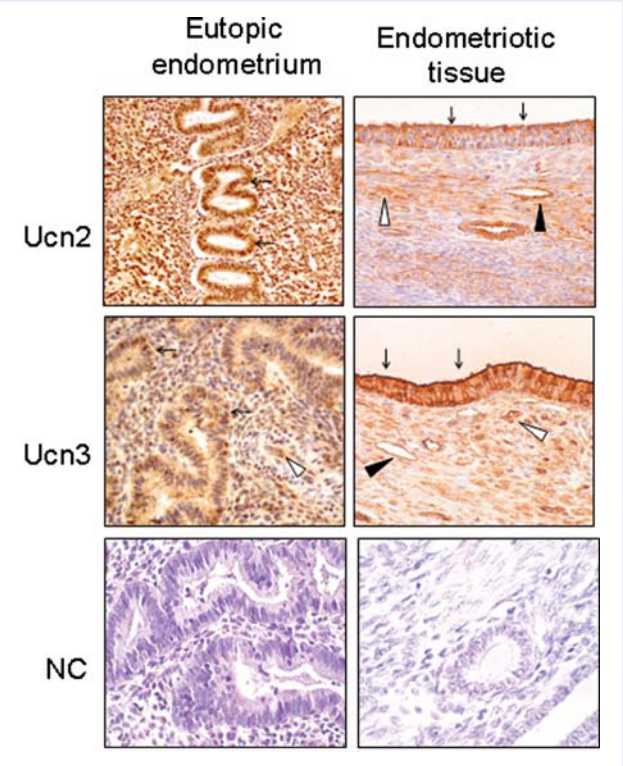

Figure 1 Representative examples of endometriotic tissue and eutopic proliferative endometrium immunostained for Ucn 2 and Ucn 3 in endometriotic patients. The thin arrows (↑) show an intense staining in glandular and surface epithelia both in eutopic and in endometriotic tissue; the black arrows (▲) show a specific staining in vascular endothelial cells and the white arrows (△) show staining in stromal cells. (NC, negative control).

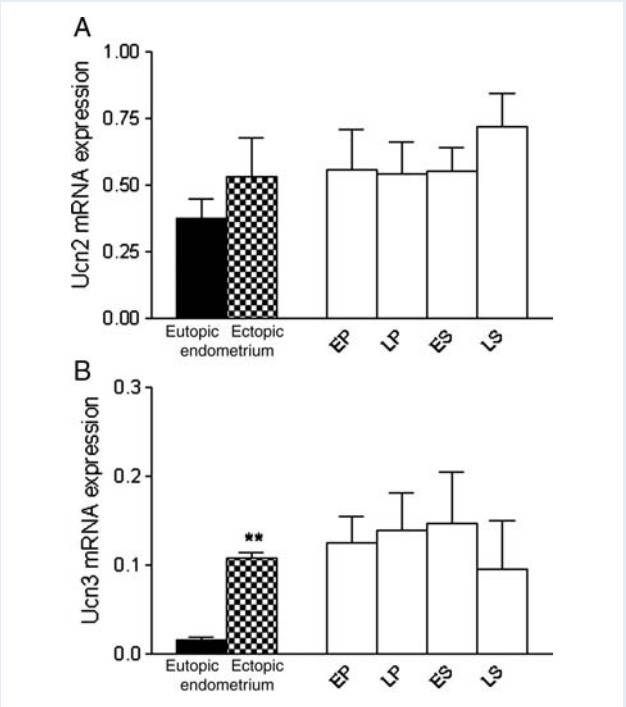

Figure 2 mRNA expression of Ucn 2 and Ucn 3 in eutopic versus ectopic endometrium. While no significant difference was shown for Ucn 2 mRNA expression (**A**) Ucn 3 mRNA expression (**B**) in endometriotic lesions was higher than in eutopic endometrium (**$P < 0.01$). No cycle-related changes were observed either for Ucn 2 (A) or for Ucn 3 (B) mRNA expression in endometrioma.

control group, endometrial Ucn 2 mRNA expression in early proliferative phase resulted higher ($P < 0.05$) than in the other phases of the menstrual cycle (Fig. 3A) while Ucn 3 mRNA had peak values at late secretory phase ($P < 0.05$; Fig. 3B). In endometriotic group, this pattern of expression was totally altered: expression of both Ucn 2 and Ucn 3 mRNA were significantly lower expressed than in control group, and remained unchanged throughout menstrual cycle (Fig. 3).

Ucn 2 and Ucn 3 effect on cytokines secretion from HESC

When cultured endometrial stromal cells were treated with increasing doses of Ucn 2, a dose-dependent increase of TNF-α secretion was shown ($P < 0.01$), an effect counteracted by the addition of ast2b, a CRH-R2 synthetic antagonist (Fig. 4A). A significant increase of IL-4 was also evident (Fig. 4B).

The addition of Ucn 3 did not significantly modify TNF-α secretion (Fig. 4A) but increased IL-4 secretion, in a dose-dependent manner ($P < 0.05$), an effect counteracted by the addition of ast2b (Fig. 4B).

Discussion

The present study showed for the first time that Ucn 2 and Ucn 3 mRNAs and peptides are expressed and localized by endometriotic tissue and by endometrium of patients with endometriosis. In eutopic endometrium of endometriotic women, these neuropeptides showed a lower expression than in the control group, and unlike controls no menstrual cycle-related changes in Ucn 2 or Ucn 3 mRNA were detected in either ectopic or endometriotic endometrium. Finally, a possible biological significance of Ucn 2 and Ucn 3 in endometrium and in endometriosis may be related to their effect on local cytokines secretion via CRH-R2.

It was recently described that endometrium and endometriotic cells express neuropeptides, neurohormones and neuroendocrine factors. Endometrial neuroendocrine cells in porcine (Stroband *et al.*, 1986; Vittoria *et al.*, 1989) uterus may be involved in the regulation of uterine functions. CRH (Mastorakos *et al.*, 1996), Ucn (Florio *et al.*, 2002), substance P, vasoactive intestinal peptide and somatostatin (Bokor *et al.*, 2009) are neuropeptides/neurohormones produced by human endometrial cells; they act locally on various functions, and their expression is modulated by progesterone (Ferrari *et al.*, 1995). Endometrium of endometriotic women shows staining for neuroendocrine cells more intensely than in healthy controls, and this has been related to endometriotic symptoms and pathogenesis (Wang *et al.*, 2010). Also endometriotic lesion express somatostatin receptors (Fasciani

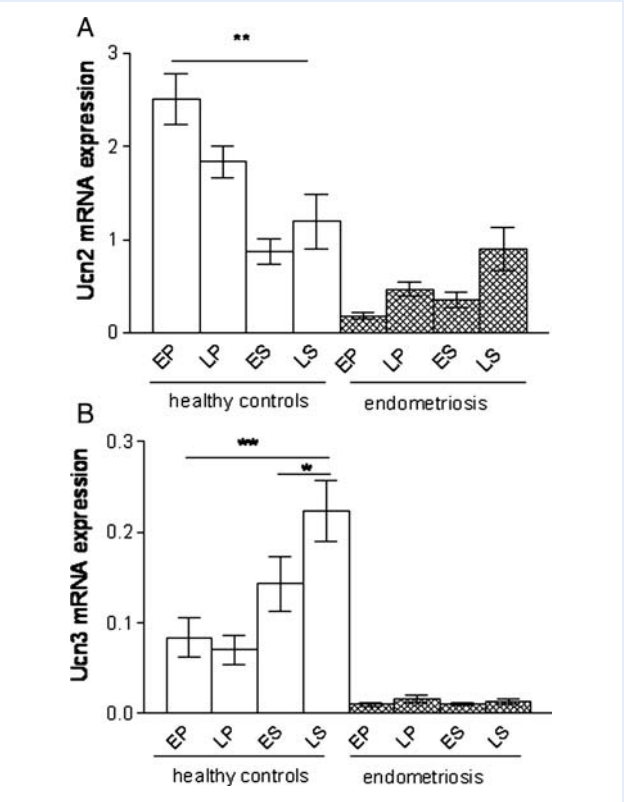

Figure 3 Expression of Ucn 2 (**A**) and Ucn 3 (**B**) in endometrial biopsies of healthy women (control) and endometriotic patients. In control group, Ucn 2 mRNA expression was significantly higher in early proliferative than in other phases of the menstrual cycle, while no changes were observed in endometriotic patients. In control group, Ucn 3 mRNA expression was highest in late secretory phase and remained unchanged in endometriotic patients ($^{*}P < 0.05$, $^{**}P < 0.01$).

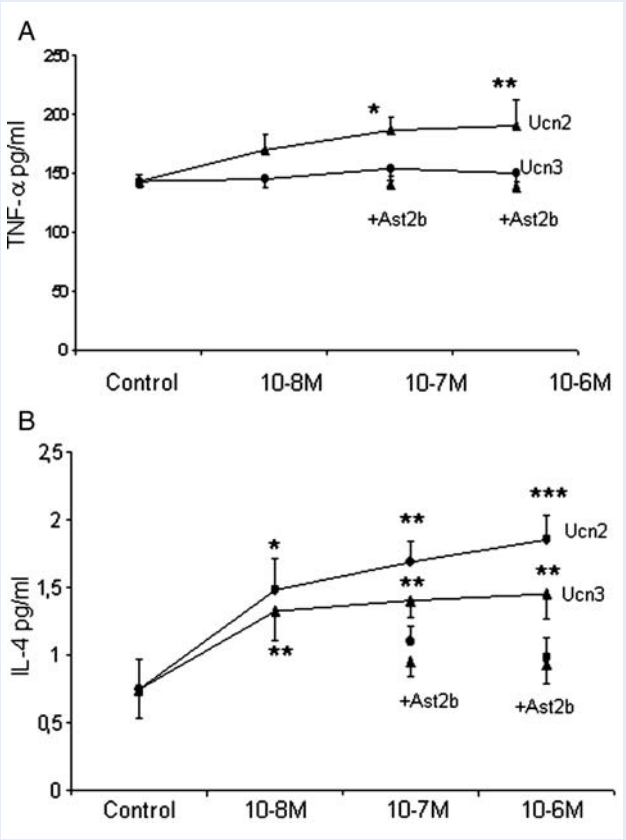

Figure 4 Effect of Ucn 2 and Ucn 3 on TNF-α (**A**) and on IL-4 (**B**) secretion from HESC. Ucn 2 induced in a significant increase of TNF-α and IL-4 release, Ucn 3 increased the release of IL-4 ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Effects were counteracted by the addition of CRH-R2 antagonist, Ast2b. (• Ucn 3 + ast2b, ▼ Ucn 2 + ast2b).

et *al*., 2010) supporting neuroendocrine characteristics of these cells.

The present descriptive evidence of two new neuropeptides expressed and localized in endometrial and endometriotic tissue raises the question of the possible biological action of Ucn 2 and Ucn 3. These neuropeptides are related to the CRH family and therefore effects on endocrine, immune and vascular systems are hypothesized. Indeed, studies conducted on other organs and tissues revealed that Ucn 2 inhibits apoptosis in cardiomyocites (Chanalaris *et al.,* 2003; Buhler *et al.,* 2009) and in arterial smooth muscle (Tao *et al*., 2006), and additionally has also a potent vasodilator effect on aortic cells (Kageyama *et al.,* 2003). Studies on endometrial or endometriotic cells may be useful for understanding whether Ucn 2 and Ucn 3 have a similar neuroactive effect in those two tissues. The present results showing a decreased mRNA expression in endometrium of endometriosis patients suggest a possible significance in the development of the disease.

Endometriosis has an endocrine/immune pathogenesis (Bulun, 2009; Giudice, 2010) and Ucn 2 is active in stimulating aromatases activity and oestradiol secretion in trophoblast culture (Imperatore *et al.,* 2009) as well as increases the expression of the potent chemoattractant IL-8 via nuclear factor-κB (NF-κB) and mitogen-activated protein kinase through the CRH-R2 in human colonocytes (Moss *et al*., 2007). Ucn 3 is able to decrease progesterone secretion from human cultured granulosa-lutein cells (Yata *et al*., 2009) as well as having an anti-inflammatory function in human colon during stress conditions (Saruta *et al*., 2005) and in cultured placental tissue (Torricelli *et al*., 2011). Further support for the possible role of Ucn 2 and Ucn 3 in the development of endometriosis derives from the present data showing an increased secretion of TNF-α and IL-4 regulated by Ucn 2 and Ucn 3, respectively. Indeed, TNF-α and IL-4 are two cytokines involved in the pathogenesis of endometriosis (Yang *et al.,* 2008; Haider and Knofler, 2009) and their endometrial modulation by CRH-R2 is supported by the present data. Physiological alterations in endometrium may in part be a causal factor for symptoms associated with endometriosis such as infertility and pain.

The factors modulating endometrial Ucn 2 and Ucn 3 are still unknown, but an effect of steroid hormones and cytokines in regulating the expression and synthesis of neuropeptides throughout endometrial cycle has been shown (Jabbour *et al.,* 2006), suggesting that these peptides may participate in paracrine signalling to modulate endometrial functions. The high levels of Ucn 2 during proliferative

phase in healthy women confirm the previous investigation (Florio *et al.*, 2007) and suggest a possible influence of estrogens on Ucn 2 expression. Additionally, the high levels of Ucn 3 during secretory phase in healthy endometrium may be probably related to a progesterone action. The lack of the Ucn 3-cycle-related changes in endometriosis may be associated with the progesterone resistance, a characteristic of this pathology (Burney *et al.*, 2007).

Several studies suggest an effect of inflammatory state on Ucn 2 and Ucn 3: Ucn 2 mRNA expression is increased in mucosal samples of patients with inflammatory bowel disease, in a rat model of gastrointestinal colitis (Chang *et al.*, 2007), in intestinal human cells after exposure to *Clostridium difficile* toxin A (Moss *et al.*, 2007) and in placental tissue in the presence of infective/inflammatory condition (Torricelli *et al.*, 2011).

Finally, the present study also showed that endometriotic tissue and eutopic endometrium of endometriotic women were similar in lacking menstrual cycle variation in Ucn 2/Ucn 3 mRNA expression, suggesting common biological characteristics for eutopic and ectopic endometrium in endometriosis. However, it is unclear whether these changes in eutopic endometrium are consequences of the development of endometriosis or are primary defects subsequently transferred to ectopic implants.

In conclusion, the present descriptive observations on Ucn 2 and Ucn 3 expression in endometriotic tissue and the lack of their cycle-related changes in endometrium of endometriotic patients suggest that these two neuropeptides may be involved in the development of endometriosis. Further proteomic and mechanistic analyses are needed to investigate this possibility.

Authors' roles

R.N. was involved in conducting experimental work and drafted the manuscript. P.C., A.L.R. and L.B. carried out experimental work. P.T. conducted immunohistochemistry. F.M.R. and P.F. played a role in project planning. F.P. coordinated the project and revised the manuscript.

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