Changes in Placental CRH, Urocortins, and CRH-Receptor mRNA Expression Associated with Preterm Delivery and Chorioamnionitis

Michela Torricelli, Romina Novembri, Errico Bloise, Maria De Bonis, John R. Challis, and Felice Petraglia

Obstetrics and Gynecology, Department of Pediatrics, Obstetrics, and Reproductive Medicine (M.T., R.N., E.B., M.D.B., F.P.), University of Siena, 53100, Siena, Italy; and Departments of Physiology, Obstetrics & Gynecology, and Medicine (J.R.C.), University of Toronto, Toronto, Ontario, Canada M5S 1A81

Context: The pathogenesis of preterm delivery (PTD) is not clear, although inflammation/infection play a major role. Corticotropin releasing-hormone (CRH) and Urocortins (Ucns) are involved in the pathophysiology of PTD.

Objective: This study evaluates trophoblast mRNA expression of CRH, Ucn, Ucn2, Ucn3, and their receptors [CRH-type 1 receptor (CRH-R1), CRH-R2] in infective conditions. To determine whether infection or glucocorticoids contribute to change their placental mRNA expression, the effects of lipopolysaccharide or dexamethasone was evaluated.

Design: Placentas were obtained from spontaneous PTD; premature rupture of membranes (pPROM) and pPROM with chorioamnionitis.

Setting: Placental specimens were collected from women receiving perinatal care at our Division of Obstetrics and Gynecology.

Patients or Other Participants: Pregnant women delivered preterm were enrolled.

Interventions: mRNA expression was evaluated by RT-PCR.

Main Outcome Measure: Because CRH and Ucns are involved in immunological functions we evaluated their involvement in PTD with or without infection.

Results: CRH, Ucn2, and CRH-R1 mRNA expression were higher, while Ucn and CRHR-2 were lower in pPROM with chorioamnionitis than in PTD and pPROM. Ucn3 mRNA expression was lower in pPROM with and without chorioamnionitis than in PTD. The addition of lipopolysaccharide in trophoblast explants decreased Ucn, Ucn3, and CRH-R2 and increased CRH, Ucn2, and CRH-R1 mRNA expression in a dose-dependent manner. Dexamethasone increased CRH and decreased Ucn2 mRNA expression in a dose dependent manner.

Conclusions: Our findings showed a significant impact of pPROM with chorioamnionitis on placental CRH peptides and receptors, suggesting that placental expression of stress-related pathways is activated in infective process. **(*J Clin Endocrinol Metab* 96: 534–540, 2011)**

Preterm delivery (PTD) occurs at less than 37 weeks gestational age, but the low gestational age cut-off still remains controversial (1). PTD may be related to physician-initiated delivery (indicated PTD) or spontaneous PTD. Indicated PTD may result from maternal or fetal risks perceived to be greater than the neonatal risks of PTD. Spontaneous PTD results from two clinical conditions: i) spontaneous preterm labor (PTL) leading to PTD

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Abbreviations: cDNA, Complementary DNA; CRH, corticotropin-releasing hormone; CRH-R1, CRH-type 1 receptor; Dxm, dexamethasone; pPROM, preterm premature rupture of membranes; PTD, preterm delivery; PTL, spontaneous preterm labor; Ucn, urocortin.

(idiopathic), and ii) preterm premature rupture of membranes (pPROM) (2). These two clinical conditions are represented in approximately equal proportions (3, 4).

The pathogenesis of PTD is not yet clear, although PTL might result from an early idiopathic activation of the normal labor process or as a result of various pathological insults (1). In pPROM, focal infection and inflammation play a major role in its pathogenesis (5, 6). The most severe complication associated with pPROM is the chorioamnionitis, defined as inflammation of the amniochorionic (fetal) membranes of the placenta in response to microbial invasion or due to other pathological process. A strong association exists between infection and earlier PTD (7): intermembrane cultures in women who delivered at less than 30 weeks are at least two times more likely to be positive than after 30 weeks, with the highest incidence of subclinical histologic chorioamnionitis in early PTD (8). In this context, placenta and fetal membranes are key tissues in the response to infection and in activating the inflammatory pathways leading to PTD through the upregulation of chemokines, cytokines, and corticotropin releasing hormone (CRH) (9).

Urocortins (Ucns) are peptides showing sequence homology with CRH; CRH and Ucn are ligands for CRH-type 1 (CRH-R1) and type 2 (CRH-R2) receptors, whereas Ucn2 and Ucn3 specifically bind only CRH-R2 (10). Ucns are expressed by gestational tissues such as trophoblast and fetal membranes (11) and may be involved in some biological functions during pregnancy (9) as well as modulating immune and placental endocrine function (12). A complex cross-talk exists between these placental peptides and the pathways involved in the onset of PTD. Indeed, both CRH and Ucn stimulate ACTH (13, 14), prostaglandin (15, 16), and oxytocin (17) release by placental cells in culture, and also exert different effects on myometrial contractility. Moreover, CRH also stimulates uterine contractility when the myometrial intracellular pathways have been already primed by uterotonic agents (oxytocin; prostaglandins) (18). On the contrary Ucn directly (14) and indirectly (18) triggers myometrial contractility. Recently, we found that a main antiinflammatory role of Ucn is mediated by CRH-R2 in trophoblast culture cells. We demonstrated that Ucn treatment modulates lipopolysaccharide (LPS)-induced TNF-α secretion and IL-4 and IL-10 release in trophoblast cultured cells, suggesting an immunomodulatory role of this neuropeptide (19). However, no data exist about the immune function of Ucn2 and Ucn3 in placental tissues. Because infection exerts a key role in the pathogenesis of PTD the aim of the present study was to evaluate trophoblast mRNA expression of CRH, Ucn, Ucn2, Ucn3 as well as that of CRH-R1 and CRH-R2 in trophoblasts collected from women who had experienced early PTD after PTL with intact membranes in the absence of histological chorioamnionitis, as well as in those with pPROM with and without histologic chorioamnionitis.

To determine the extent to which infection or glucocorticoids might be causal factors leading to changes in placental mRNA expression, the effects of LPS or dexamethasone on expression of CRH, Ucns, and CRH-Rs mRNA by trophoblast explants was evaluated.

Materials and Methods

Definitions

PTL was defined by the presence of regular uterine contractions occurring at a frequency of at least two every 10 min associated with cervical change before 37 completed weeks of gestation that required hospitalization.

pPROM was defined as spontaneous rupture of the membranes at less than 37 weeks gestation at least 1 h before the onset of contractions.

Histologic chorioamnionitis was diagnosed based on the presence of inflammatory cells in the chorionic plate and/or chorioamniotic membranes.

Sample collection

Placental specimens were collected from a group of women (n = 26) who received perinatal care at our Division of Obstetrics and Gynecology from January 2008 to April 2010 and were included in the present study. Pregnant women experiencing early PTD were first divided into three groups according to the following diagnoses:

i) with PTL with intact membranes without histologic chorioamnionitis (n = 8);
ii) with pPROM without histologic chorioamnionitis (n = 12);
iii) with pPROM with histologic chorioamnionitis (n = 6).

Cervical incompetence, uterine malformations, polyhydramnios, multiple gestation, and fetal-maternal complications (thyroid disease, asthma, cardiovascular diseases, diabetes, hypertension, preeclampsia, abruptio placentae, fetal growth retardation, and fetal malformation) were excluded. Among women experiencing PTD, the gestational age at admission was between 23 and 34 weeks, assigned on the basis of the last menstrual period on ultrasound before 20 weeks of gestational age. All patients received intramuscular dexamethasone (Dxm, 12 mg administered twice with an interval of 12 h) to induce fetal lung maturity. All women experienced PTD within 24 h after the end of the Dxm treatment. All women with pPROM received antibiotic therapy. Informed written consent was obtained from all patients before their inclusion in the study, for which approval was obtained from the local Human Investigation Committee. Tissues were collected and immediately submerged in an RNA stabilization reagent (RNA late, Quiagen, Milan, Italy) and frozen at −80 C until assay.

Placental explants and treatments

To investigate the effect of LPS and Dxm on mRNA expression for CRH, Ucn, Ucn2, and Ucn3 and on CRH-Rs, placental villi were collected from elective cesarean section at term (n =

10). Placental explants were extensively washed and dissected under sterile conditions in ice-cold Hanks' balanced salt solution (HBSS) supplemented with penicillin and streptomycin. The explants (50 mg/wet weight) were placed in 24-well plastic plates and cultured in DMEM supplemented with 10% fetal bovine serum, 2 mmol/liter L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 C under 5% CO_2 and 95% air. The cultured medium was replaced with fresh DMEM after 1 d, the explants were treated with different concentrations of LPS from *Escherichia coli* serotype 0111:B4 (Sigma-Aldrich, Steinheim, Germany) (10,100,1000 ng/ml); placental villi clusters were collected after 24 h and supernatants and cells were collected and kept frozen at −20 C and −80 C, respectively until used. Dxm (Sigma-Aldrich 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} M) treatment with or without the antagonist RU486 (10^{-5} M), was added according to previous studies. As control, vehicle was dissolved in fresh DMEM. Placental villi and the supernatants were collected after 3 and 24 h of treatment and kept frozen at −20 C and −80 C respectively until used.

RNA extraction and complementary DNA (cDNA) preparation

Frozen samples were disrupted and homogenized using Mixer Mill MM 300 (Quiagen, Milan, Italy). Total RNA was extracted with RNeasy Protect Mini Kit and then treated with RNase-free DNase according to the instructions of the manufacturer (RNase protect Mini Kit Qiagen, Hilden, Germany). RNA was quantified by UV absorption (OD260) using Nanodrop (Celbio, Milan, Italy), and RNA purity was determined from the OD260:OD280. The purified RNA was stored at −80 C until cDNA preparation. About 200 μg of RNA were reverse transcribed to prepare cDNA. Reversion was carried out in a reaction volume of 20 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 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, Milan, Italy). The negative control consisted of retrotranscription performed in the absence of reverse transcriptase enzyme (RT) or in the absence of RNA samples (H_2ORT). RNA was initially denatured at 85 C for 5 min. The reaction mixture was then added, and RT was performed at 50 C for 40 min. The reaction was stopped by denaturing the enzyme at 85 C for 15 min. The cDNA was subsequently subjected to RT-PCR.

RT-PCR

Differences in mRNA expression of Ucns and CRH-Rs were compared by RT-PCR (TaqMan PCR, Applied Biosystems, Weiterstadt, Germany), using an Opticon 2 thermal cycler (MJ Research, Bio-Rad Laboratories, Waltham, MA). The housekeeping gene 18S (assay identification no. Hs 99999901_s1) was used as internal standard. All samples were run 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). The TaqMan probes for CRH (assay identification No. Hs00384289_g1), Ucn (assay identification No. Hs00175020_m1), Ucn2 (assay identification No. Hs00264218_s1), Ucn3 (assay identification No. Hs00846499_s1), CRH-R1 (assay identification No. Hs01062290_m1) and CRH-R2 (assay identification No. Hs00266401_m1) were obtained from the commercially available Assays on Demand (Applied Biosystems). After an initial denaturation for 10 min at 95 C, denaturation for the subsequent 40 cycles was performed for 15 sec at 95 C, followed by primer annealing and elongation at 60 C for 1 min. The CT method was applied as a comparative method of quantification.

Statistical analysis

All data were assessed for normality of distribution using a computer program (Prism 4; Graphpad Software, La Jolla, CA). Where the data were normally distributed, differences among three or more groups were analyzed by ANOVA with Tukey's multiple comparison test. Two groups were analyzed using a *t* test. χ^2 test was used to compare proportions. Statistical significance was achieved when $P < 0.05$.

Results

Clinical findings

Clinical data of women enrolled in this study are summarized in Table 1. There were no significant differences between maternal age, parity, gestational age at delivery, and fetal weight.

CRH, Ucns, and CRH-Rs mRNA expression in trophoblast tissues

CRH (Fig. 1A) and Ucn2 (Fig. 1C) mRNA expression was significantly higher ($P < 0.001$) in pPROM with chorioamnionitis than in PTD and pPROM, both without chorioamnionitis. Ucn (Fig. 1B) mRNA expression in trophoblast tissues was significantly lower ($P < 0.01$) in pPROM with chorioamnionitis than in PTD and pPROM, both without chorioamnionitis. Ucn3 (Fig. 1D) mRNA expression was significantly lower in pPROM with and without chioamnionitis ($P < 0.001$ and < 0.01, respec-

TABLE 1. Characteristics of patient groups

	PTD with intact membranes without chorioamnionitis (n = 8)	pPROM without chorioamnionitis (n = 12)	pPROM without chorioamnionitis (n = 6)	*P*
Maternal age, y	30.62 ± 3.8	31.83 ± 4.4	29.00 ± 3.7	ns
Parity	0.25 ± 0.46	0.50 ± 0.5	0.33 ± 0.5	ns
Gestational age at delivery, weeks	27.25 ± 2.9	28.00 ± 2.8	28.33 ± 2.8	ns
Fetal weights, g	1715.00 ± 30	1833.33 ± 17	1766.66 ± 48	ns

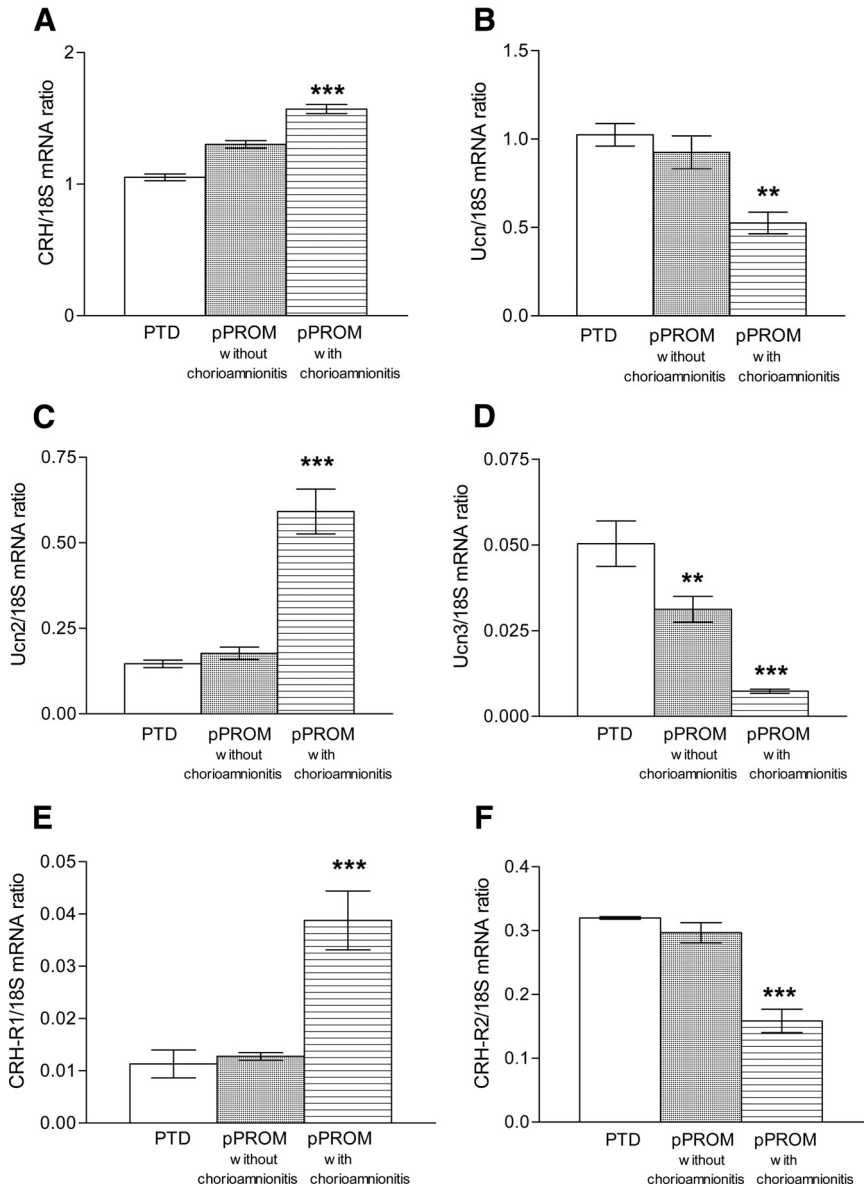

FIG. 1. Expression of mRNA for CRH-family members and their receptors in trophoblast tissues collected from women with early PTD with PTL with intact membranes in absence of histological chorioamnionitis, in those with pPROM with and without histologic chorioamnionitis. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.001$.

tively) than in PTD. Additionally, Ucn3 mRNA expression was significantly lower with pPROM than without chorioamnionitis ($P < 0.01$). With respect to CRH-receptors, while CRH-R1 (Fig. 1E) mRNA expression was significantly higher in pPROM with chorioamnionitis than in PTD and pPROM in absence of chorioamnionitis ($P < 0.05$), CRH-R2 (Fig. 1F) expression was significantly lower in pPROM with chorioamnionitis than in PTD and pPROM in the absence of chorioamnionitis ($P < 0.05$).

Effects of LPS on CRH, Ucns, and CRH-Rs mRNA expression in placental explants

The addition of LPS to the trophoblast explants significantly ($P < 0.001$) decreased Ucn and Ucn3 mRNA expression in a dose-dependent manner with the lowest expression of Ucn at LPS concentrations of 100 ng/ml and 1000 ng/ml. LPS significantly ($P < 0.01$) increased CRH and Ucn2 mRNA expression in a dose-dependent manner. LPS significantly ($P < 0.01$) increased CRH-R1 mRNA expression, but significantly ($P < 0.01$) decreased CRH-R2 expression, both in a dose-dependent manner (Fig. 2).

Effects of Dexamethasone on CRH family members and CRH-Rs mRNA expression in placental explants

Dexamethasone did not induce any differences of mRNA expression in all CRH-related peptides after 3 h of treatment (data not shown). However, at 24 h, it did not induce any significant changes in Ucn and Ucn3 mRNA expression in placental explants (Fig. 3), while it is able to increase CRH mRNA expression (Fig. 3) and reduce Ucn2 mRNA expression in a dose-dependent manner (Fig. 3). The specific GR antagonist RU 486 reversed the effect of Dxm on CRH and on Ucn2 (Fig. 3).

Discussion

The present study showed that trophoblast collected from women delivering preterm with pPROM and associated with histological chorioamnionitis have significantly higher expression of CRH, Ucn2 and CRH-R1 and significantly lower expression of Ucn, Ucn3 and CRH-R2 mRNA in comparison to women with PTD or pPROM without chorioamnionitis. These findings suggest that infective pathways leading to chorioamnionitis activate placental CRH pathways. Furthermore, these changes were completely reproduced by treating placental trophoblasts with LPS *in vitro*.

The role of these neuropeptides in the response to infection has been unclear. Previous studies have suggested a possible role of Ucn as an antiinflammatory mediator through the inhibition of i) experimental autoimmune encephalomyelitis by a glucocorticoid-independent mechanism (20); ii) the release of TNF-α in mouse LPS-activated

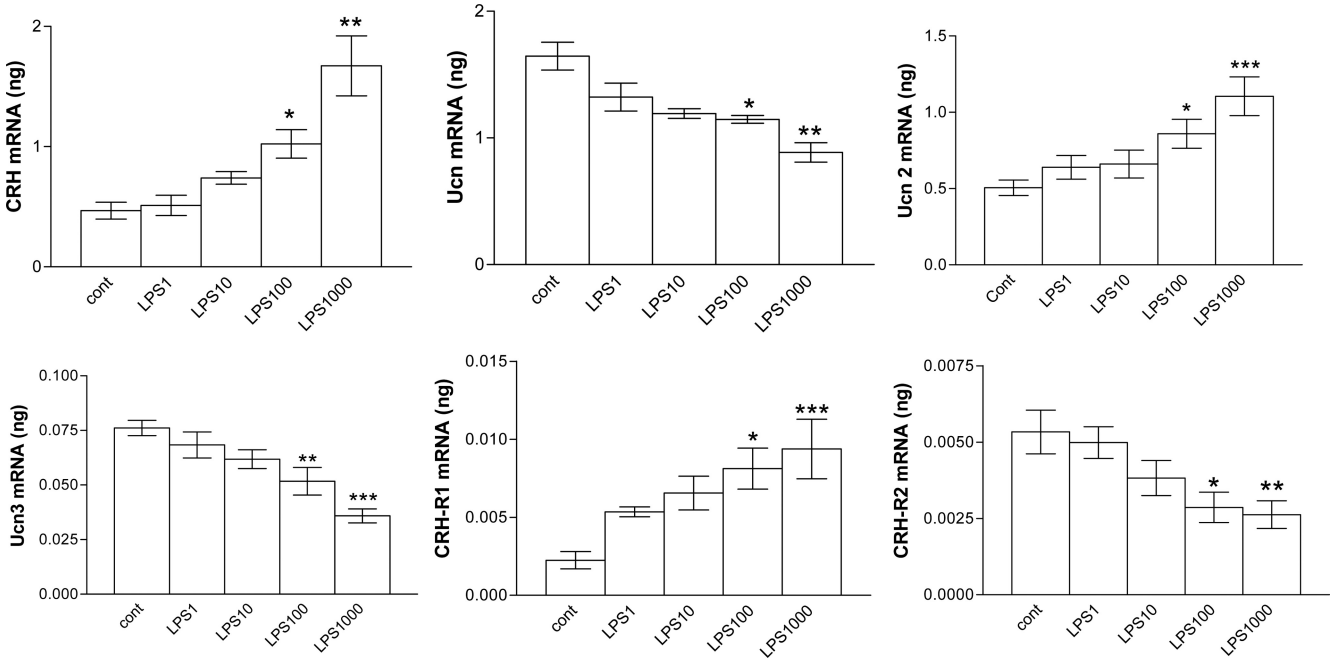

FIG. 2. Effect of LPS treatment on CRH, Ucns, and CRH-receptor mRNA expression in term trophoblast cells. *, $P < 0.05$; **, $P < 0.01$.

macrophages (21); and iii) LPS-induced TNF-α production in cultured microglia (22). In addition, our previous study demonstrated that Ucn exerts an antiinflammatory effect in trophoblast cultures treated with infective stimuli, such as LPS, via CRH-R2 (19). Moreover, it is important to consider that CRH-R2 represents a target for all Ucns, establishing a complex cross-talk between these ligands and their receptors. The finding that CRH-R2 is down-regulated with infection and after LPS treatment suggests fine regulation of this effect. Our observation of increased CRH-R1 mRNA expression under inflammatory/infective conditions is in agreement with previous studies showing that, in an animal model, CRH-R1 expression is up-regulated by LPS stimulation (23), because proinflammatory effects of CRH via CRH-R1 receptor were shown (24), while Ucn exerts an antiinflammatory action

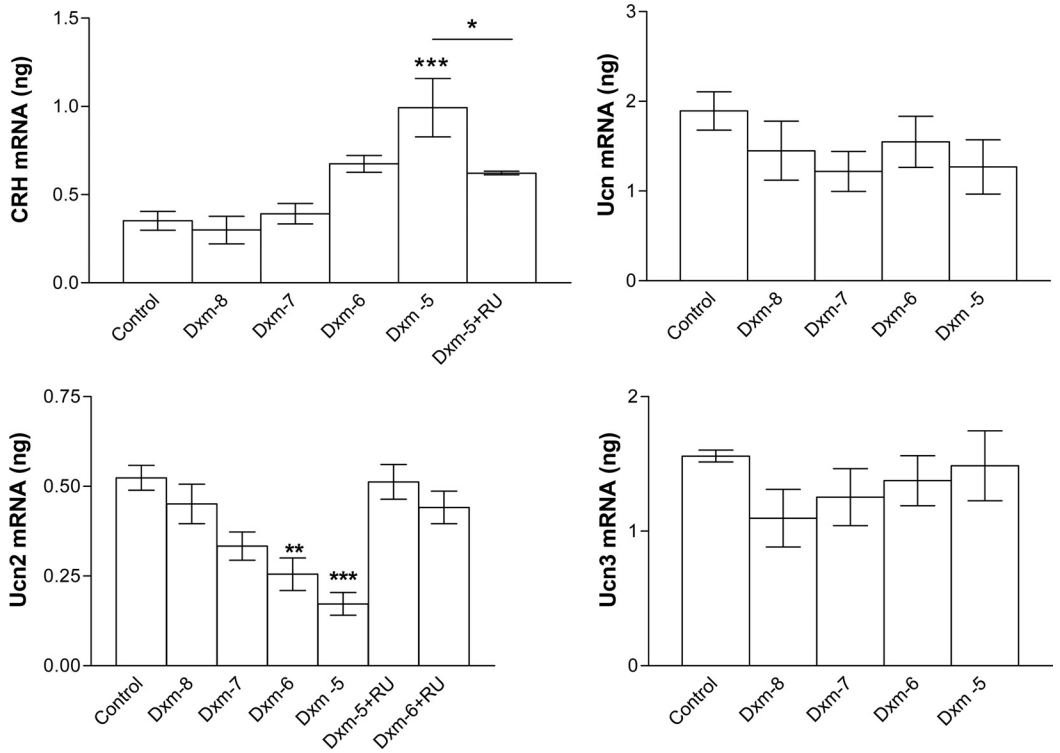

FIG. 3. Effect of dexamethasone (Dxm) treatment on CRH, Ucns, mRNA expression in term trophoblast cells. *, $P < 0.05$; **, $P < 0.01$.

via CRH-R2, the mRNA increase of CRH-R1 may be suggested as a CRH-related event. To corroborate our findings, it is well known that women with PTL showed higher plasma CRH concentration than healthy women at the same gestational age or at term not in labor, and those with microbial invasion of the amniotic cavity had higher plasma CRH concentrations than the ones without microbial invasion of the amniotic cavity (25).

Moreover, several lines of evidence demonstrate that a correlation exists between proinflammatory cytokines and CRH. It is well known that CRH enhances the LPS-induced IL-1β secretion through activation and it is able to activate transcription of both IL-1β and TNF-α, moreover these effects are mediated by CRH-R1 (23). *Vice versa*, in cultured human placental cells, IL-1β increases CRH production (26) and treatment with CRH, LPS, or CRH plus LPS increases proinflammatory cytokine secretion such as TNF-α and IL-8 in trophoblast cells (27).

There is much evidence to indicate that proinflammatory cytokines, such as IL-1β and TNF-α, play a central role in the mechanisms of inflammation/infection-induced PTD (28, 29). The involvement of these cytokines in PTD is supported by the following observations: i) IL-1β and TNF-α stimulate prostaglandin production by amnion, deciduas, and myometrium (30, 31); ii) human decidua is able to produce IL-1β and TNF-α in response to bacterial products (30); iii) (3) amniotic fluid IL-1β and TNF-α concentrations are elevated in women with PTL and intraamniotic infection (30–32); iv) in women with pPROM and intraamniotic infection, IL-1β, and TNF-α concentrations are higher in the presence of labor (30, 31); and v) placental tissue obtained from patients with labor, particularly those with chorioamnionitis, produces larger amounts of IL-1β than that obtained from women not in labor (33).

To determine effects of glucocorticoids themselves on Ucn output, mRNA expression of CRH/Ucn peptides was also evaluated in placental explants stimulated with Dxm. We found that CRH mRNA was increased, confirming earlier studies (34, 35), while Ucn2 mRNA was decreased. The precocious activation of the hypothalamic pituitary adrenal axis at PTD results in increased fetal cortisol simulating increases in placental CRH then increasing prostaglandin output (36). Ucns are abundantly expressed in early and late gestational tissues and levels of mRNA for Ucn2 and Ucn3 are increased at lower oxygen tensions (37). Previous studies also showed that CRH and Ucn2 stimulated aromatization of androgen into enhanced estradiol output by placental trophoblasts (38, 39). Moreover, it was found that conversion of these precursors into estrogen is stimulated by Ucn2 in a time- and dose-dependent manner.

Taken together, these data suggest that Ucn and Ucn3 may be strictly regulated by infective conditions leading to early PTD in the presence of histological chorioamnionitis, while CRH and Ucn2 could have a broader influence on a multiple pathways, being activated not only by infective mechanisms but also by paracrine/endocrine regulation.

In conclusion these data provide novel information concerning the relationship between CRH-related peptides and their receptors with infective pathways in chorioamnionitis at PTD. Our observations open a new avenue of investigation about the role of these neuropeptides in pathogenetic mechanisms leading to PTD in which inflammatory and infective pathways represent key events. The finding that these responses can be reproduced by treating trophoblast cells with LPS suggests their potential importance in the process of infection, although further studies are required to understand the exact role of Ucns in this process.

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Address all correspondence and requests for reprints to: Felice Petraglia, M.D., Obstetrics and Gynecology, University of Siena, Policlinico "Le Scotte," Viale Bracci, 53100 Siena, Italy. E-mail: petraglia@unisi.it.

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