

Corticotropin-releasing hormone inhibits progesterone production in cultured human placental trophoblasts

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Abstract

Placental-derived corticotropin-releasing hormone (CRH) seems to play a major role in the mechanisms controlling human pregnancy and parturition. It has been suggested that CRH directly modulates the endocrine function of placental trophoblasts, including the production of estrogen, ACTH, and prostaglandin. In this study, we sought to investigate the effect of CRH, locally produced by placenta, on progesterone production. Percoll-purified placental trophoblasts were obtained from uncomplicated term pregnancies and cultured for 72 h. Progesterone concentration in culture media was measured by RIA. The mRNA transcripts encoding *CYP11A1* and *HSD3B1*, the enzymes for progesterone synthesis, were determined by quantitative real-time reverse transcription (RT)-PCR. Results showed that CRH (10^{-8} – 10^{-6} mol/l) caused a significant decrease in progesterone levels in a dose-dependent manner. The CRH antagonist, α -helical CRH 9-41, at 10^{-7} – 10^{-5} mol/l stimulated progesterone secretion. Consistent with this thesis, CRH decreased, whereas α -helical CRH increased, the mRNA levels of *CYP11A1* and *HSD3B1*. Since CRH has been shown to activate the phospholipase C–protein kinase C (PKC) signal pathway in placenta, we examined whether the effect of CRH on progesterone synthesis was dependent on PKC signal pathway. Treatment of cells with PKC inhibitor, Gö6976, resulted in a significant increase in progesterone production, and exogenous CRH restored progesterone production. In conclusion, placental CRH exhibits a tonic inhibitory effect on progesterone production in a PKC-dependent fashion.

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Introduction

Progesterone, as its name implies is a pro-gestation hormone. Early in human pregnancy, progesterone is produced mainly by the persistent corpus luteum. After about 6–8 weeks of pregnancy, luteal production of progesterone declines as a result of diminished human chorionic gonadotropin (HCG) production by the placenta, and the placental trophoblasts take over as the main source of progesterone production (Tuckey 2005). Placental progesterone continues to increase up to term when the placenta is lost following parturition (Mesiano 2001). The essential role of progesterone in maintaining pregnancy is unequivocal and remarkably universal. In all mammals so far, it has been shown that pregnancy cannot exist in the complete absence of progesterone. It has been shown that disruption of progesterone synthesis initiates myometrial contractions or labor and delivery at all stages of pregnancy (Selinger *et al.* 1987, Haluska *et al.* 1997). Progesterone can be synthesized from cholesterol by the syncytiotrophoblast (Tuckey 2005). A variety of endogenous factors such as estrogen, HCG, and cyclic AMP are known to regulate placental progesterone production (Feinman *et al.* 1986, Rodway *et al.* 1988, Chaudhary *et al.* 1992). Thus, further study of the mechanism by which the

hormone network controls progesterone production in placenta is a prerequisite to gain insight into the mechanism of pregnancy and parturition.

Corticotropin-releasing hormone (CRH) appears to be a key element in the control of the maintenance of human pregnancy and parturition. During human pregnancy, CRH is expressed by fetomaternal tissues and the concentration of CRH peptide and mRNA in the placenta increases with advancing gestation, in parallel with exponential increase in maternal plasma CRH concentrations (Sasaki *et al.* 1987, Riley *et al.* 1991). The rise in maternal CRH level occurs earlier and more rapidly in women who deliver preterm (McLean *et al.* 1995, Wadhwa *et al.* 1998), and more slowly in women who deliver postterm, than in women who deliver at term (McLean *et al.* 1995). Previous *in vitro* studies showed that CRH stimulated adrenocorticotrophic hormone (ACTH) and prostaglandin (PG) production in placenta and fetal membrane and enhanced $\text{PGF}_{2\alpha}$ and oxytocin-mediated myometrial contractility (Petraglia *et al.* 1987, Quartero & Fry 1989, Jones & Challis 1990, Florio *et al.* 1996). CRH may directly stimulate cortisol and dehydroepiandrosterone sulfate (DHEAS) production from fetal adrenals at the end of pregnancy, which promotes the

myometrial activation at this time (Smith *et al.* 1998, Sirianni *et al.* 2005a,b). Thus, it has been suggested that CRH is involved in the mechanisms that determine the duration of gestation and the onset of parturition.

Two major CRH receptor subtypes are recognized, CRH-R1 and CRH-R2, which belong to the class II G-protein-coupled receptor superfamily. The human placenta has been shown to express both subtypes of CRH receptors (Florio *et al.* 2000). More recently, Jeschke and colleagues showed that the addition of CRH to the placental trophoblast cultures resulted in a decrease in progesterone release (Jeschke *et al.* 2005). However, the effect of CRH produced locally in the placental trophoblasts on progesterone biosynthesis, and a detailed and quantitative analysis of the effects of CRH on all the enzymes needed for progesterone production as well as the specific CRH receptors responsible for the actions of CRH have not been performed. Therefore, in the present study, we conducted experiments to determine the effects of exogenous and endogenous CRH on progesterone production in placental cells and, quantitatively, the effect of CRH on mRNA levels of all the enzymes needed for progesterone production.

Materials and methods

Human placental cell culture

Human term placentae were obtained from normal term pregnancies after elective cesarean section. None of the patients had received prostaglandins, glucocorticoids, or oxytocin. Collections of placentae were performed with the approval of Changhai Hospital human ethics committee. Cytotrophoblasts were isolated and cultured according to a slightly modified Kliman's method as described previously (Kliman *et al.* 1986, Ni *et al.* 1997). Briefly, approximately 60 g chorionic villi tissue were obtained from the maternal side of the placenta, digested with 0.125% trypsin (Life Technologies, Inc.) and 0.02% DNase-I (Sigma) in phenol red-free DMEM (Sigma), thrice for 30 min each. The dispersed cells were filtered with 200 µm nylon gauze and loaded onto a discontinued Percoll (Amersham Biosciences) gradient (5–70%), then centrifuged at 2300 *g* for 20 min. The cytotrophoblast cells between a density of 1.049 and 1.062 g/ml were collected, then plated onto 12-well plates at a density of 1.2×10^6 /well and grown in a phenol red-free DMEM with 10% charcoal-stripped fetal calf serum at 37 °C in 5% CO₂–95% air for 72 h. The DMEM serum-free medium was then used to replace the culture medium. The hormones and antagonists were added and incubated for 24 h or for different time periods. At the end of the experiment, representative wells of cells

were fixed and immunostained for cytokeratin and vimentin using primary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to assess cell purity (Kliman *et al.* 1986).

CRH and α -helical CRH 9-41 were purchased from Sigma Chemical Co. (Sigma). Substances were used at final concentrations ranging from 10⁻⁹ to 10⁻⁵ M, in order to embrace the maternal or intrauterine tissue concentrations found in human pregnancy at term. Vehicle-treated wells (controls) were present in each experiment. Each treatment was performed in quadruplicate for each preparation of cells. After incubation, the medium was harvested and stored at -20 °C until progesterone assay. Cells were used for RNA extraction. A well of cells in each treatment was used to assess the cell viability by MTT assay (Mosmann 1983). The assay depends on the reduction of the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; Sigma Chemical Co.) by functional mitochondria to formazan. After a 2-h incubation at 37 °C with MTT, the cells were lysed with dimethyl sulfoxide in Sorensen's glycine buffer and the formazan crystals solubilized. Absorbance was read at 550 nm using a spectrophotometric microplate reader.

Hormone assays

Progesterone was assayed using commercially available RIA kits (Shanghai Institute of Biological Product, Shanghai, China). The mean intra- and interassay coefficients of variation were 4.4 and 6.9% respectively (manufacturer's data).

CRH immunoreactivity in the culture media was assayed by RIA performed as previously described (Ni *et al.* 1997).

Total RNA extraction and quantitative real-time RT-PCR

Total RNA was isolated by using TRIzol reagent according to the manufacturer's instructions (Invitrogen). First-strand cDNA was synthesized from 1 µg RNA with oligo(dT)12–18 primer using superscript first-strand synthesis kit (Invitrogen) according to the manufacturer's instruction. For every RT reaction set, one RNA sample was set up without reverse transcriptase enzyme to provide a negative control. Quantitative real-time PCR (QT-RT-PCR) analysis was carried out using Rotor Gene 3000 (Corbett Research, Sydney, Australia). Reverse-transcription products (cDNA) were diluted for subsequent QT-RT-PCR. QT-RT-PCR solution consisted of 2.0 µl diluted cDNA product, 0.2 µM of each paired primer, 2.0 mM Mg²⁺, 100 µM deoxynucleotide triphosphates, 2 U Taq DNA polymerase, and 1 × PCR buffer. SYBR green I (BMA, Rockland, ME, USA) was used as

detection dye. The nucleotide sequences of the primers are shown in Table 1. The specificity of the primers was verified by examining the melting curve as well as subsequent sequencing of the QT-RT-PCR products.

To determine the relative quantitation of gene expression for both target and housekeeping genes, the relative standard curves method was used (Corbett Research). Briefly, standard curves for *GAPDH*, *CYP11A1*, and *HSD3B1* were constructed by performing a dilution series of the untreated control cDNA. For each RNA sample, the relative amounts of *GAPDH*, *CYP11A1*, and *HSD3B1* mRNA were obtained, and the ratios of *CYP11A1* mRNA to *GAPDH* mRNA and *HSD3B1* mRNA to *GAPDH* mRNA were calculated. For each experiment, the amount of *CYP11A1* or *HSD3B1* mRNA under various treatment conditions is expressed relative to the amount of transcript present in the untreated control.

Statistical analyses

The values are expressed as means \pm s.d. Statistical analyses were carried out using one-way ANOVA followed by Student–Newman–Keul test. Significance was set at $P < 0.05$.

Results

Cell morphology

At the end of the culture period, placental trophoblast cells tended to form aggregates, thought to correspond to syncytium formation *in vivo*. These cells were predominately cyokeratin-positive (>90%) and vimentin-negative.

Concentration and time-dependent effects of CRH on progesterone production

After placenta trophoblast cells were cultured for 72 h, they were treated with increasing concentrations of CRH (10^{-9} – 10^{-6} M) for 24 h; media content of progesterone was determined by RIA. CRH caused a concentration-dependent decrease in progesterone

production, with significant inhibition seen at the dose of 10^{-8} – 10^{-6} mol/l (Fig. 1A). The maximal effect was achieved at 10^{-6} mol/l, which caused a 76% decrease in progesterone production. The time course of placental cell response to treatment with CRH (10^{-7} M) is shown in Fig. 1B. A significant decrease was seen after 6 h treatment ($P < 0.05$) and maximal inhibition was obtained after 24 h treatment ($P < 0.01$; Fig. 1B).

In human placenta, the synthesis of progesterone depends principally on *CYP11A1* and *HSD3B1* (Tuckey 2005). In order to investigate the effect of CRH on the expression of *CYP11A1* and *HSD3B1*, we measured the mRNA encoding *CYP11A1* and *HSD3B1* in placental cells by quantitative real-time RT-PCR. The melting curve of quantitative real-time PCR showed a single peak of melting temperature value for PCR products of *CYP11A1* and *HSD3B1* respectively (data not shown). Sequence analysis of *CYP11A1* and *HSD3B1* PCR products showed complete alignment with the corresponding sequences of human *CYP11A1* and *HSD3B1* gene in the gene bank (data not shown).

As shown in Fig. 2, CRH (10^{-8} – 10^{-6} mol/l) induced a significant decrease in *CYP11A1* and *HSD3B1* mRNA levels in a dose-dependent manner. Maximal effect was achieved at a concentration of 10^{-6} mol/l, which caused about 69 and 68% reduction of *CYP11A1* and *HSD3B1* mRNA expression respectively. Time-course analysis revealed that the significant decreases in *CYP11A1* and *HSD3B1* mRNA were achieved by 10^{-7} mol/l CRH at 6 h. At 24 h, 10^{-7} mol/l CRH produced a 50% decrease in *CYP11A1* mRNA level and a 52% decrease in *HSD3B1* mRNA level.

At the end of each experiment, cell viability was assessed by MTT assay. There were no differences between the cells of each treatment (data not shown).

Effect of CRH receptor antagonist on progesterone production

It is known that placental trophoblast cells can produce CRH endogenously (Ni *et al.* 1997). We measured the level of CRH in the culture media of control cells by RIA. The contents of CRH in culture media were 297.67

Table 1 RT-PCR oligonucleotide primers used for the study

Gene	Primer sequence
<i>HSD3B1</i> (299 bp)	Sense: 5'-CGTCTCGGTCATCATCCACA-3' Antisense: 5'-TCAGATTCCACCCGTTAGCC-3'
<i>CYP11A1</i> (344 bp)	Sense: 5'-TGGCTGAGCAAAGACAAGAACA-3' Antisense: 5'-GAGCAGGACTTGGGACAGACGA-3'
<i>GAPDH</i> (189 bp)	Sense: 5'-GACACCCACTCCTCCACCTTTGA-3' Antisense: 5'-CTCTCTTCTCTTGTGCTCTTGC-3'

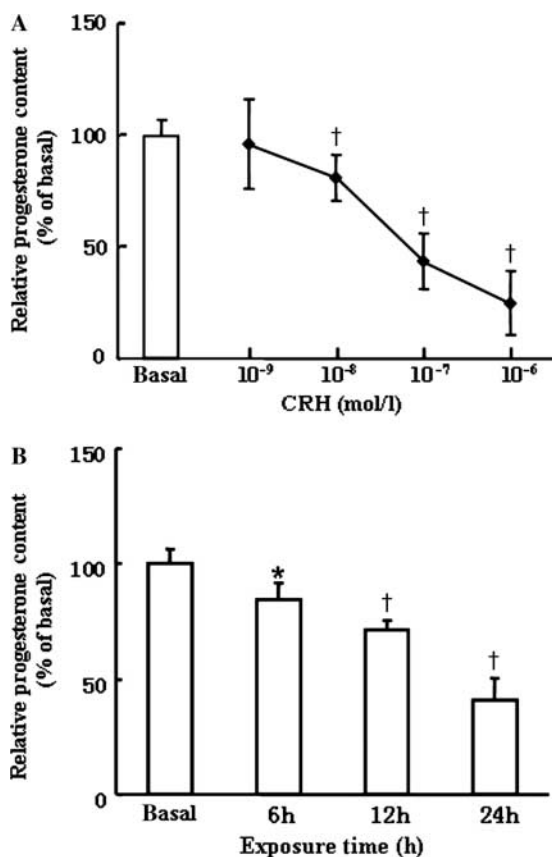


Figure 1 Concentration- and time-dependent effects of CRH on progesterone production in placental trophoblast cells. Placental cells were treated with indicated concentrations of CRH for 24 h (A). Cells were incubated for the indicated times with CRH (10^{-7} mol/l) (B). Progesterone levels in culture media were assayed by RIA. Values are presented as mean percentage control \pm s.d. for total of seven experiments ($n=6$) performed in triplicate. * $P<0.05$, † $P<0.01$ compared with vehicle controls.

(64 pM) \pm 40.47 pg/ml after a 3-h incubation period. To block the effects of endogenous CRH, cells were treated with increasing concentrations of the CRH receptor antagonist α -helical CRH 9-41, which, at 10^{-7} – 10^{-5} mol/l, resulted in a dose-dependent increase in progesterone production, to approximately 196% of the control value at a concentration of 10^{-5} mol/l after a 24-h treatment period (Fig. 3A). Addition of exogenous CRH reversed the increase in progesterone production caused by the addition of α -helical CRH 9-41, to a value that was decreased significantly, compared with cells treated with α -helical CRH 9-41 alone (Fig. 3B).

As shown in Fig. 4, α -helical CRH 9-41 (10^{-7} – 10^{-5} mol/l) induced a significant increase in *CYP11A1* and *HSD3B1* mRNA levels in a dose-dependent manner, to approximately 91% increase in *CYP11A1* mRNA and 92% increase in *HSD3B1* mRNA at a concentration of

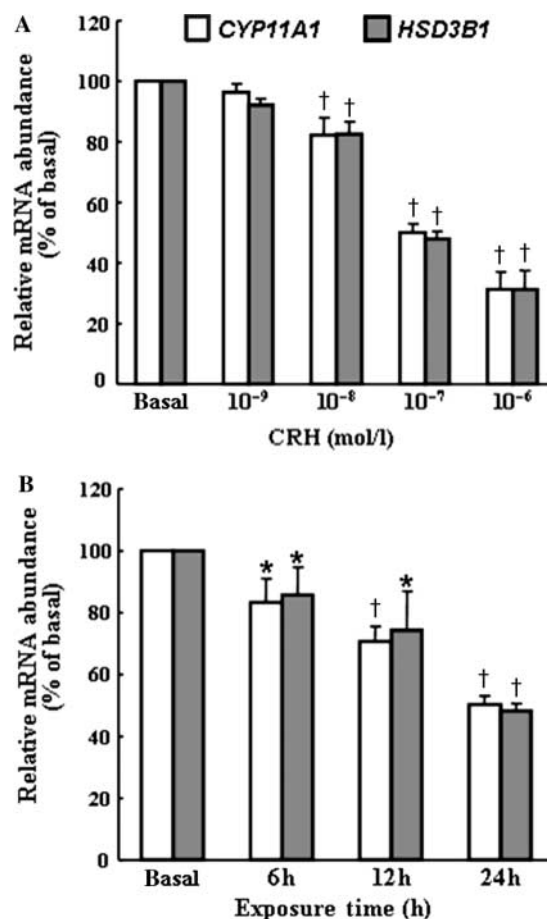


Figure 2 Concentration- and time-dependent effects of CRH on *CYP11A1* and *HSD3B1* transcript levels in placental trophoblast cells. Real-time RT-PCR was used to quantify mRNA levels of *CYP11A1* and *HSD3B1* in placental trophoblasts. (A) Cells were treated with the indicated concentrations of CRH for 24 h. (B) Cells were incubated for the indicated times with CRH (10^{-7} mol/l). Data points are the values calculated as described in Materials and methods and are presented as mean percent control \pm s.d. for a total of six experiments ($n=6$) performed in triplicate. * $P<0.05$, † $P<0.01$ compared with basal.

10^{-5} mol/l. These effects can also be reversed by the addition of exogenous CRH.

Effect of PKC inhibitor on progesterone production

Treatment of placental cells with a protein kinase C (PKC) inhibitor Gö6976 (10^{-7} – 10^{-9} mol/l) significantly increased progesterone production in a dose-dependent manner. Maximal effect was obtained at the concentration of 10^{-7} mol/l, which caused 84% increase in progesterone production. The concentration of 10^{-7} mol/l CRH reversed the stimulatory effects of 10^{-8} or 10^{-9} mol/l Gö69769 on progesterone production (Fig. 5A).

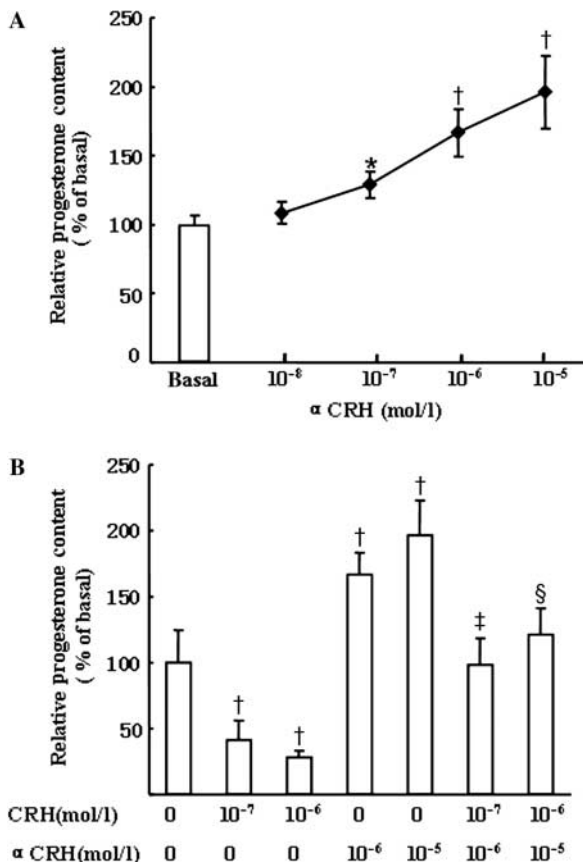


Figure 3 Effects of α -helical CRH 9-41 on progesterone production in placental trophoblasts. (A) Cells were incubated with increasing concentrations of α -helical CRH 9-41 for 24 h. (B) Cells were treated with indicated concentrations of CRH, α -helical CRH 9-41, and CRH plus α -helical CRH 9-41 for 24 h. Values are presented as mean percent control \pm s.d. for a total of five experiments performed in triplicate. α CRH, α -helical CRH 9-41; * P < 0.05, † P < 0.01 compared with vehicle controls; ‡ P < 0.01 vs CRH 10^{-7} mol/l; § P < 0.01 vs CRH 10^{-6} mol/l.

Consistently, Gö6976 (10^{-7} – 10^{-9} mol/l) significantly increased *CYP11A1* and *HSD3B1* mRNA levels. The maximal effect was achieved at a dose of 10^{-7} mol/l Gö6976, which caused about a 71% increase in *CYP11A1* mRNA and a 69% increase in *HSD3B1* mRNA. Addition of exogenous CRH (10^{-7} mol/l) reversed the increases in *CYP11A1* and *HSD3B1* mRNA caused by the addition of Gö6976 (10^{-8} – 10^{-9} mol/l), to a value that was decreased significantly, compared with cells treated with Gö6976 alone (Fig. 5B).

Discussions

In this study, we have demonstrated that treatment of cultured syncytiotrophoblast with CRH significantly decreased progesterone production in a dose-dependent pattern. When CRH receptors were blocked by the

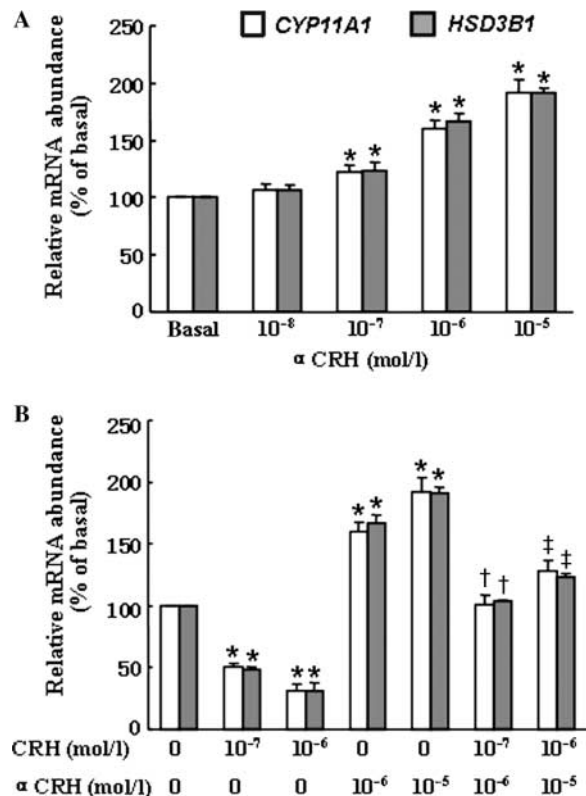


Figure 4 Effects of α -helical CRH 9-41 on *CYP11A1* and *HSD3B1* transcript levels in placental trophoblasts. (A) Cells were incubated with increasing concentrations of α -helical CRH 9-41 (α CRH) for 24 h. (B) Cells were treated with indicated concentrations of CRH, α -helical CRH 9-41 (α CRH), and CRH plus α -helical CRH 9-41 (α CRH) for 24 h. Data points are the values calculated as described in Materials and methods and are presented as mean percent control \pm s.d. for a total of five experiments performed in triplicate. * P < 0.01 compared with vehicle controls. † P < 0.01 vs CRH 10^{-7} mol/l; ‡ P < 0.01 vs CRH 10^{-6} mol/l.

administration of CRH receptor antagonist, there was a significant increase in progesterone concentration in the culture media. Progesterone production in placental cells was significantly increased when PKC activity was inhibited, and this increase could be reversed by the addition of exogenous CRH. We have also found that CRH inhibited the expression of the mRNA encoding the enzymes that comprise the progesterone biosynthesis pathway. These data support the hypothesis that CRH may act through a PKC-dependent pathway to inhibit progesterone production in placental cells throughout gestation.

The placental cells endogenously produced not only CRH (Sun *et al.* 1994, Ni *et al.* 1997), but also CRH-related peptides including urocortin, urocortin II, and urocortin III (Clifton *et al.* 2000, Karteris *et al.* 2005). These peptides bind to the CRH receptors within the placenta. Our results showed that the significant effect

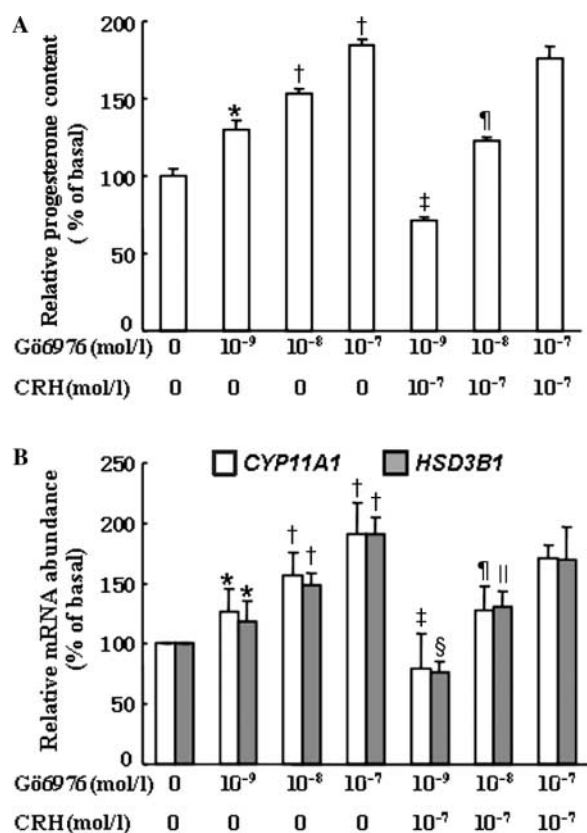


Figure 5 Effects of G66976 treatment on progesterone production and expression of *CYP11A1* and *HSD3B1* mRNA. Cells were treated with indicated concentrations of G66976, and CRH plus G66976 for 24 h. (A) Progesterone levels in culture media were assayed by RIA. (B) Real-time RT-PCR was used to quantify mRNA levels of *CYP11A1* and *HSD3B1* in placental trophoblasts. Data points are the values calculated as described in Materials and methods and are presented as mean percent control \pm s.d. for a total of five experiments ($n=5$) performed in triplicates. * $P<0.05$, † $P<0.01$ compared with vehicle controls. ‡ $P<0.05$, § $P<0.01$ vs G66976 10⁻⁹ mol/l; † $P<0.05$, ‡ $P<0.01$ vs G66976 10⁻⁸ mol/l.

of CRH on progesterone production occurred at the concentration of 10⁻⁸ mol/l, which is higher than CRH level in maternal plasma at term (McLean *et al.* 1995), most probably because the placental cells secrete CRH and CRH-related peptides. Therefore, we administered a specific CRH receptor antagonist, α -helical CRH 9-41, to block the CRH receptors in placental cells. It resulted in an increase in progesterone production in a dose-dependent manner, and this increase could be reversed by the addition of exogenous CRH. The human placental trophoblasts have been shown to express CRH-R1 α , CRH-R1 β , variant CRH-R1c, and CRH-R2 β (Grammatopoulos *et al.* 1999, Florio *et al.* 2000, Karteris *et al.* 2000). In the current study, we were unable to determine which receptor subtype was responsible for these effects on progesterone production. Our evidence, combined with the demonstrated expression of

CRH by placenta (Sasaki *et al.* 1987, Riley *et al.* 1991) and of CRH receptor subtypes in these tissues (Grammatopoulos *et al.* 1999, Florio *et al.* 2000, Karteris *et al.* 2000), leads us to suggest a paracrine/autocrine role for CRH in the regulation of progesterone production *in vivo*. Study conducted on primary cultures of granulosa-lutein cells of human ovary revealed that CRH significantly inhibited progesterone production through a paracrine/autocrine pathway (Ghizzoni *et al.* 1997).

Karteris *et al.* have demonstrated that, within the placenta, CRH receptors may be coupled to the α -subunit of Gq and can also activate Go and Gz, but not Gs and Gi. However, the way in which CRH activates Go and Gz in the placenta is not very clear. Activation of the Gq subunit by CRH can then go on to activate the phospholipase C β pathway, which could activate the PKC pathway (Karteris *et al.* 2000). Our results demonstrated that progesterone production was up-regulated by G66976, a PKC inhibitor, and this increase could be reversed by the addition of exogenous CRH. These data support the hypothesis that CRH may act through a PKC-dependent pathway to inhibit progesterone production in placental trophoblast cells. Other signal pathways involved in CRH actions remained to be further investigated.

In human placenta, progesterone is formed by two steps. First, cholesterol is converted to pregnenolone by cytochrome P450scc (*CYP11A1*). Pregnenolone is then converted to progesterone by type 1 β -hydroxysteroid dehydrogenase (*HSD3B1*; Tuckey 2005). We found that CRH significantly inhibited *CYP11A1* and *HSD3B1* mRNA expression in the cultured trophoblasts, which is consistent with a decrease in the concentration of progesterone, the product of these enzymes, in the culture media. These results suggest that CRH inhibits progesterone production by down-regulating these enzymes' gene expression. More recently, CRH has been shown to stimulate cortisol and DHEAS production by up-regulating the gene expression of the enzymes responsible for the synthesis of these steroids in fetal adrenal (Sirianni *et al.* 2005a,b).

Increasing evidence strongly suggests that placental CRH production is linked to the length of gestation in human (McLean *et al.* 1995). The mechanism by which placental CRH might precipitate parturition has remained unclear. Recent research has supported the view that parturition is a cascade of events that commences early in pregnancy and involves the mother, fetus, and placenta. Placental production of CRH is proposed as an early event regulating the cascade of the events (Smith *et al.* 2001). In most mammals, a pivotal event for the onset of parturition is changes in the maternal plasma concentrations of estrogens (which increase) and progesterone (which decrease) at the end of pregnancy (Norwitz *et al.* 1999). Although human

parturition is not preceded by these systemic changes in estrogens and progesterone, changes in progesterone/17 β -estradiol ratios (which decrease) occur during human parturition at term (Romero *et al.* 1988). Mazor *et al.* (1994) showed that progesterone/17 β -estradiol ratios decreased in preterm birth. More recently, we found that placental CRH stimulates 17 β -estradiol production by placental trophoblasts (You *et al.* 2006). In this study, we demonstrated that CRH locally produced in placenta inhibits progesterone production. Taken together, these results show that the effects of placental CRH on the production of sex steroid hormones might contribute to the changes in progesterone/17 β -estradiol ratios at term.

In conclusion, our results suggest that CRH locally produced in placenta exhibits a tonic inhibitory effect on progesterone production and progesterone biosynthesis pathway through a PKC-dependent pathway, which might be associated with changes in progesterone/17 β -estradiol ratios at term.

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