Regulation of Estradiol and Progesterone Production by CRH-R1 and -R2 Is through Divergent Signaling Pathways in Cultured Human Placental Trophoblasts

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CRH and its related peptides urocortins (UCN) have been identified in placenta and implicated to play pivotal roles in the regulation of pregnancy and parturition in humans. The objectives of present study were to investigate the effects of endogenous CRH and its related peptides in the regulation of steroid production in placenta. Placental trophoblasts were isolated from term placenta tissues and cultured for 72 h. Estradiol (E_2) and progesterone (P_4) contents in culture media were determined by radioimmunoassay. Treatment of cultured trophoblasts with CRH or UCNI antibody showed decreased E_2, whereas increased P_4 production. Treatment of cells with CRH receptor type 1 antagonist antalarmin or CRH receptor type 2 (CRH-R2) antagonist astressin-2b also decreased E_2 but increased P_4 production. Knockdown of CRH receptor type 1 or CRH-R2 cells showed a decrease in E_2 production and an increase in P_4 production. In CRH-R2 knockdown cells, CRH stimulated GTP-bound Gαs protein and phosphorylated phospholipase C-β3. Adenylyl cyclase and protein kinase A inhibitors blocked CRH-induced increased E_2 production but not decreased P_4 production. PLC inhibitor U73122 and protein kinase C inhibitor chelerythrine blocked the effects of CRH on E_2 and P_4 production in CRH-R2 knockdown cells. UCNIII, the specific CRH-R2 agonist, stimulated GTP-bound Gαi protein and phosphorylated phospholipase C-β3 expression. Both U73122 and chelerythrine blocked UCNIII-induced increased E_2 production and decreased P_4 production. We suggest that CRH and its related peptides might be involved in changes in the progesterone to estrogen ratio during human pregnancy. **(*Endocrinology* 153: 4918–4928, 2012)**

Estrogen and progesterone play pivotal roles in the regulation of pregnancy and parturition. They play important roles in the control of essential maternal endocrine functions (1) and contribute to the normal differentiation and maturation of many fetal organs (2). In the context of parturition, it is well known that progesterone is essential for the establishment and maintenance of pregnancy, whereas estrogen promotes changes in the myometrium and cervix, which facilitate labor and delivery (2, 3). After approximately 9 wk of pregnancy, the human placenta becomes the main source of maternal estrogen and progesterone, and maternal serum estrogen and progesterone levels increase with advancing gestation (4, 5). In placenta, estrogen and progesterone are mainly synthesized in syncytiotrophoblasts. Progesterone synthesis depends on maternal lipoprotein-cholesterol delivery, cytochrome P450 cholesterol side-chain cleavage (CYP)-11A, and 3β-hydroxysteroid dehydrogenase (5). Estrogen is believed to be synthesized using the C19 androgen dehydroepiandrosterone and dehydroepiandrosterone sulfate (DHEAS) as precursors due to lack of CYP17 (6); however, it has recently been demonstrated that human placenta expresses

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Abbreviations: AC, Adenylyl cyclase; CRH-R1, CRH receptor subtype 1; CRH-R2, CRH receptor subtype 2; CYP, cytochrome P450 cholesterol side-chain cleavage; DHEAS, dehydroepiandrosterone sulfate; E_2, estradiol; GP, G protein; P_4, progesterone; PLC-β3, phospholipase C-β3; pPLC-β3, phosphorylated PLC-β3; PKA, protein kinase A; PKC, protein kinase C; siRNA, small interfering RNA; UCNI, urocortin I; UCNII, urocortin II; UCNIII, urocortin III.

CYP17 and is able to generate androgen *de novo* (7). Various factors, such as human chorionic gonadotropin, IGF, and prostaglandins are found to regulate estrogen and progesterone production in human placenta (8, 9).

An increasing body of evidence suggests that CRH, a 41-amino acid peptide hormone, plays a pivotal role in the control of human pregnancy and parturition (4, 10, 11). Although the precise biological functions of placental CRH during human pregnancy are not fully understood, it has been shown that CRH exerts a number of effects in a complex network within intrauterine tissues by autocrine/paracrine as well as endocrine fashions. For instance, CRH modulates prostaglandin production by the fetal membranes and placenta (12), induces vasodilation of the fetoplacental circulation by activating nitric oxide synthase (13, 14), and stimulates fetal adrenal DHEAS output directly (15) or indirectly via fetal pituitary ACTH (16). More recently we have demonstrated that placental CRH is able to regulate the expression of enzymes needed for estrogen and progesterone synthesis, thereby modulating estrogen and progesterone production by an autocrine/paracrine fashions (17, 18).

CRH belongs to a family of peptides that include urocortin I (UCNI), urocortin II (UCNII) and urocortin III (UCNIII) (19–21). During pregnancy, UCNI is synthesized and secreted by placenta and fetal membranes (22), which is similar to CRH. However, the secretion patterns of these two peptides are different, as maternal plasma CRH levels keep increasing until term (23), whereas UCNI levels remain relative constant during gestation and increase only after onset of parturition (24). UCNI has similar effects as CRH, augmenting matrix metalloproteinase, ACTH, and prostaglandin secretion from cultured human placental cells (25, 26). However, other studies showed that UCNI and CRH exhibit differential effects in gestational tissues. For example, UCNI seems to enhance myometrial contractility, whereas CRH has either relaxant or contractile effects on human pregnant myometrium (13, 26–28). Recently UCNII and UCNIII have also been reported to be expressed in placental cytotrophoblasts and syncytiotrophoblasts (14, 29), but treatment of cultured placental cells with UCNII and UCNIII fails to stimulate ACTH secretion (29).

All the CRH family members exert their effects by binding to specific cell surface G protein-coupled receptors. Two major CRH receptor subtypes have been identified, termed CRH-R1 and CRH-R2. These receptors share 70% homology at the amino acid level but have different binding properties for the members of CRH family. CRH and UCNI can bind to both CRH-R1 and CRH-R2 (19), whereas UCNII and UCNIII bind exclusively to CRH-R2 (20, 21). Human placenta has been shown to express both subtypes of CRH receptors (30, 31). Although our previous study had demonstrated CRH regulation of estrogen and progesterone production in placenta (17, 18), the effects of other CRH-related peptides and the specific subtype(s) of CRH receptors responsible for the actions of placenta-derived CRH and CRH-related peptides remains unknown. Therefore, in the present study, we conducted a series of experiments to determine the effects of endogenous CRH and UCNI produced locally on the production of estrogen and progesterone in placenta, determine the roles of CRH-R1 and CRH-R2, and define signaling pathway(s) of CRH-R in the regulation of estrogen and progesterone production.

Materials and Methods

Reagents

DMEM, deoxyribonuclease I, CRH, UCNIII, DHEAS, antalarmin, astressin-2b, SQ22536, H89, U73122, and chelerythrine chloride were obtained from Sigma Chemical Co. (St. Louis, MO). CRH and UCNI antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phospholipase C-β3 (PLC-β3)-phospho and PLC-β3 total antibodies were obtained from Epitomics, Inc. (Burlingame, CA). Trypsin, fetal calf serum, and Lipofectamine 2000 were purchased from Invitrogen Corp. (Carlsbad, CA). Percoll was obtained from Amersham (Uppsala, Sweden). The Gαs and Gαi activation assay kits were purchased from New East Biosciences (Malvern, PA). G protein (GP) antagonist-2A, a small inhibitory peptide of Gαq protein, and control peptide were purchased from Enzo Life Sciences (Waterloo, Australia).

Placental cell culture

Eighteen placentas were obtained from women with uncomplicated pregnancies who underwent elective cesarean section at term. Collections of placenta were performed with the approval of Changhai Hospital and Second Military Medical University Human Ethics Committees. Cytotrophoblasts were isolated and cultured according to modified Kliman's method as described previously (31, 32). Briefly, cotyledons were removed from the maternal side and dispersed with trypsin and deoxyribonuclease I. A purified fraction of cytotrophoblasts were obtained after Percoll gradient centrifugation. The cells were then plated into 12-well plates at a density of 1.2×10^6/well and grown in phenol red-free DMEM with 10% charcoal-stripped fetal calf serum at 37 C in 5% CO_2-95% air.

On the third day of culture, culture medium was changed to fetal calf serum-free DMEM in absence or presence of 1 μmol/liter DHEAS. CRH and UCNI antibodies, CRH, UCNIII, antalarmin, astressin-2b, SQ22536, H89, U73122, and chelerythrine were added at the indicated concentrations. Control cultures were maintained without additives. Each treatment was performed in triplicate for each preparation of cells. The cell media were collected after 24 h for determination of estradiol (E_2) and progesterone (P_4) production. A well of cells in each treatment was used to assess the cell viability by dimethylthiazoldiphenyltetra-zoliumbromide assay. It was found that all the treat-

ments did not affect the cell viability in this study (data not shown).

RNA interferences

The sequence-specific small interfering RNA (siRNA) targeting human CRH-R1 (sense, 5′-GGUUGGUGACAGCCGCCU ATT-3′; antisense, 5′-UAGGCGGCUGUCACCAACCTT-3′) and CRH-R2 (sense, 5′-GGAAUGUGAUUCACUGGAATT-3′; antisense, 5′-UUCCAGUGAAUCACAUUCCTT-3′) were designed and synthesized by Gene Pharma Corp. (Shanghai, China) for knockdown of the CRH-R1and CRH-R2 gene *in vitro*, respectively. The following siRNA (sense, 5′-UUCUCCGAACGU GUCACGUTT-3′; antisense, 5′-ACGUGACACGUUCGGAGA ATT-3′), without homology to any known human mRNA sequences in the National Center for Biotechnology Information RefSeq database (Bethesda, MD), was used as a negative control. The cultured placental cells were transfected with siRNA-CRH-R1, siRNA-CRH-R2, and negative control siRNA using Lipofectamine 2000 (Invitrogen) as described previously (33).

E_2 and P_4 assay

E_2 and P_4 were assayed using commercially available RIA kits (Shanghai Institute of Biological Product, Shanghai, China). The mean intra- and interassay coefficients of variation for E_2 were 5.78 and 6.96%, respectively. The mean intra- and interassay coefficients of variation for P_4 were 4.4 and 6.9%, respectively.

Measurement of activated Gαs and Gαi protein levels

The levels of activated, GTP-bound Gαs and Gαi proteins were measured using commercially available Gαs and Gαi activation assay kits. Trophoblast cells or CRH-R2 knockdown trophoblast cells were treated with increasing concentrations of CRH and UCNIII for 5 min in the absence and presence of antalarmin or astressin-2b, and cells were then scraped off the plate in the presence of lysis buffer. The cell lysate was centrifuged for 10 sec at 12,000 × *g*, and the supernatant was used to immunoprecipitate with antiactive Gαs or antiactive Gαi monoclonal antibody and the protein A/G beads. After incubating at 4 C for 1 h, the beads were washed three times (10 min each) in lysis buffer. Bound proteins were analyzed by Western blot with anti-Gαs or anti-Gαi monoclonal antibody. To control sampling errors and normalize results, the total Gαs or Gαi protein was also detected.

cAMP assay

Placental cells were treated with CRH and UCNIII at indicated doses for 5 min and then scraped off the plate in the presence of 50 mM sodium acetate (pH 4.75). Lysates were boiled at 95 C for 10 min and then quickly ultrasonicated in ice bath. The supernatants were collected by centrifuge, and the cAMP assays were performed according to the protocol of a commercial ^{125}I-

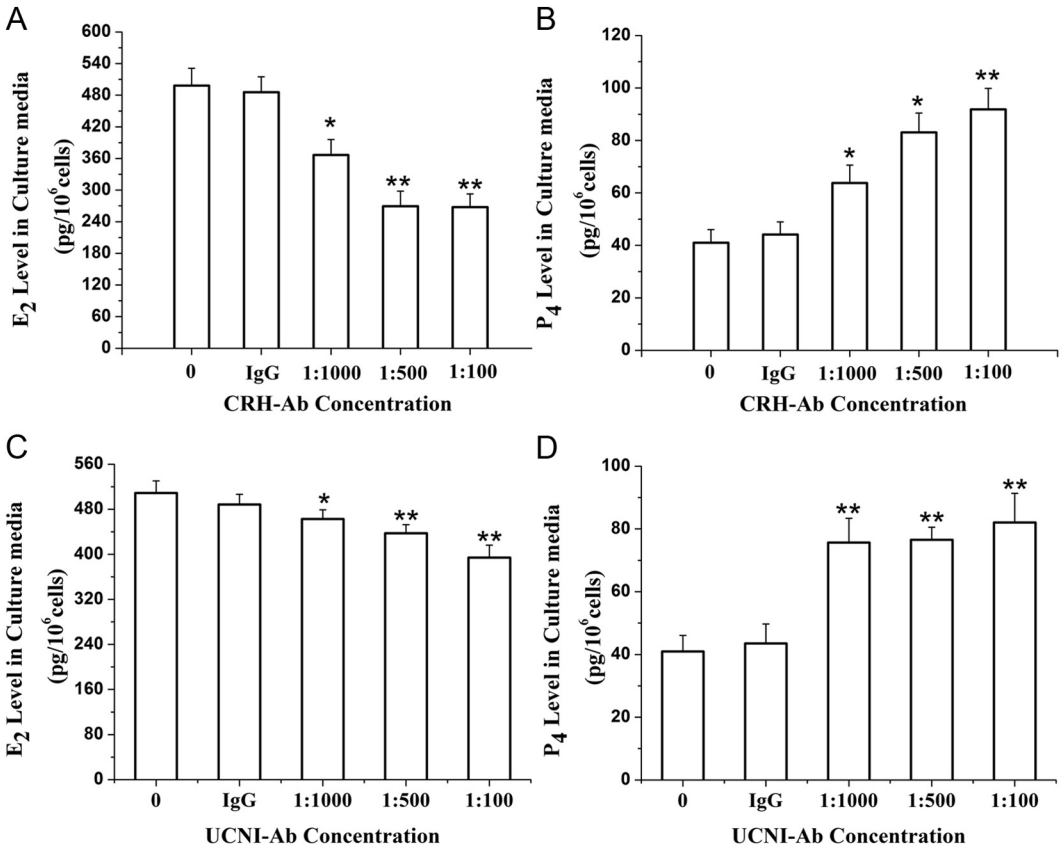

FIG. 1. Effects of CRH and UCNI antibodies on E_2 and P_4 production in cultured placental trophoblast cells. Cells were treated with indicated concentrations of CRH antibody (A and B) and UCNI antibody (C and D) for 24 h. Control cells were treated with normal IgG (2 μg/ml, 1:100). E_2 and P_4 levels in culture media were measured by RIA. Values are presented as mean ± SEM for a total of six placenta cultures obtained from six patients (n = 6) performed in triplicate. *, $P < 0.05$, **, $P < 0.01$ compared with vehicle control. CRH-Ab, CRH antibody; UCNI-Ab, UCNI antibody.

RIA kit (Huaying Biotechnology Research Institute, Peking, China).

Western blotting analysis

Cells were harvested in the presence of radioimmunoprecipitation assay lysis buffer containing phosphatase inhibitor cocktail (Roche, Indianapolis, IN). Seventy micrograms of proteins were denatured and separated by SDS-PAGE (8%) and subsequently transferred to nitrocellulose membranes by electroblotting. After the transfer, membranes were incubated in blocking buffer and then with PLC-β3phospho ([Ser 537, phosphorylated PLC-β3 (pPLC-β3)] antibody (1:1000 dilution in Tris-buffered saline and 0.1% Tween 20 with 5% nonfat milk), overnight at 4 C. Membranes were then washed and incubated with a secondary horseradish peroxidase-conjugated antirabbit antibody. The immunoreactive bands were visualized with enhanced chemiluminescence (Santa Cruz Biotechnology). The intensities of light-emitting bands were detected and quantified using Sygene Bio Image system (Synoptics Ltd., Cambridge, UK). Total PLC-β3 protein levels were also detected for sample loading correction and normalization.

Statistical analyses

The data were expressed as the mean ± SEM or, for illustrative purposes, presented as the mean percent control ± SEM in some cases. After the data were tested for normal distribution by using the Shapiro-Wilk test, they were then analyzed by one-way ANOVA, followed by Student-Newman-Keuls *post hoc* multiple comparison methods, with significance determined at the level of $P < 0.05$.

Results

Effects of CRH and UCNI antibody on E_2 and P_4 production

Our previous study has shown that the cultured placental trophoblasts secrete both CRH and UCNI, and CRH content was 186 ± 59 pg/ml, whereas UCNI content was 93 ± 29.7 pg/ml in the media up to 3 h of incubation (31). To investigate the effects of endogenous CRH and UCNI on steroid production, cells were treated with increasing concentrations of CRH and UCNI antibodies.

As shown in Fig 1, A and B, CRH antibody dose dependently reduced E_2 concentration in media in the presence of DHEAS (1 μmol/liter) as well as increased P_4 production in media, with the significant inhibition seen at an antibody concentration of 0.2 μg/ml (1:1000) after a 24-h treatment period. At a concentration of 2 μg/ml (1:100), CRH antibody resulted in a 46.4% inhibition in E_2 level

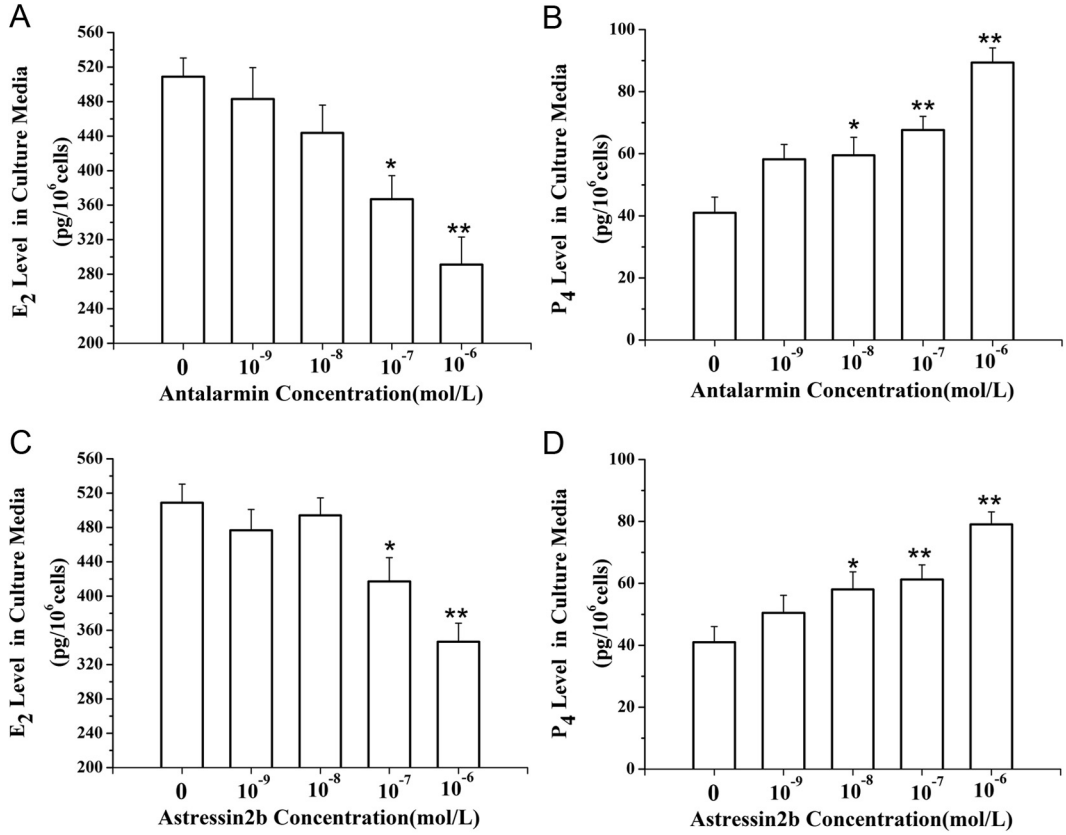

FIG. 2. Effects of CRH-R1 or CRH-R2 antagonist on E_2 and P_4 production in cultured placental syncytiotrophoblast cells. Cells were treated with increasing concentration of antalarmin, the specific CRH-R1 antagonist (A and B), or astressin-2b, the specific CRH-R2 antagonist (C and D) for 24 h. E_2 and P_4 contents in culture media were assayed by RIA. Values are presented as mean ± SEM for a total of six placenta cultures obtained from six patients (n = 6) performed in triplicate. *, $P < 0.05$, **, $P < 0.01$ compared with vehicle control.

and a 124.1% increase in P_4 level over basal production, respectively.

UCNI antibody exhibited the similar effect on E_2 and P_4 production. All doses tested caused significant decreases in E_2 level and increases in P_4 levels, At a concentration of 2 μg/ml (1:100), UCNI antibody resulted in 22.4% inhibition in E_2 level and up to 200.1% increase in P_4 level of control (Fig. 1, C and D).

Roles of CRH-R1 and CRH-R2 in the regulation of steroid production

To explore the roles of CRH-R1 and CRH-R2 in the modulation of steroid production, we examined the effects of specific CRH-R1 and CRH-R2 antagonist at first.

Treatment of cells with increasing concentrations of antalarmin, a CRH-R1 antagonist, decreased E_2 content and increased P_4 content in culture media in a dose-dependent manner. The significant effects of antalarmin on E_2 production and P_4 production were seen at the concentration of 0.1 μmol/liter and 0.01 μmol/liter, respectively (Fig. 2, A and B). The maximal effect was obtained by 1 μmol/liter antalarmin, which caused a 42% inhibition in E_2 production and a 123% increase in P_4 production, respectively.

Astressin-2b, a CRH-R2 antagonist, exhibited a similar effect as antalarmin. It dose dependently decreased E_2 production and increased P_4 production (Fig. 2, C and D).

To confirm the effects mediated by CRH-R1 and CRH-R2, sequence-specific siRNA targeting CRH-R1 and CRH-R2 were transfected into cells, respectively. As shown in Fig. 3A, transfection of siRNA-CRH-R1 resulted in about 88% decrease in CRH-R1 expression. Knockdown of CRH-R1 caused a significant decrease in basal E_2 production and an increase in basal P_4 production compared with the cells transfected with control siRNA (Fig. 3B). Treatment of CRH-R1 knockdown cells with

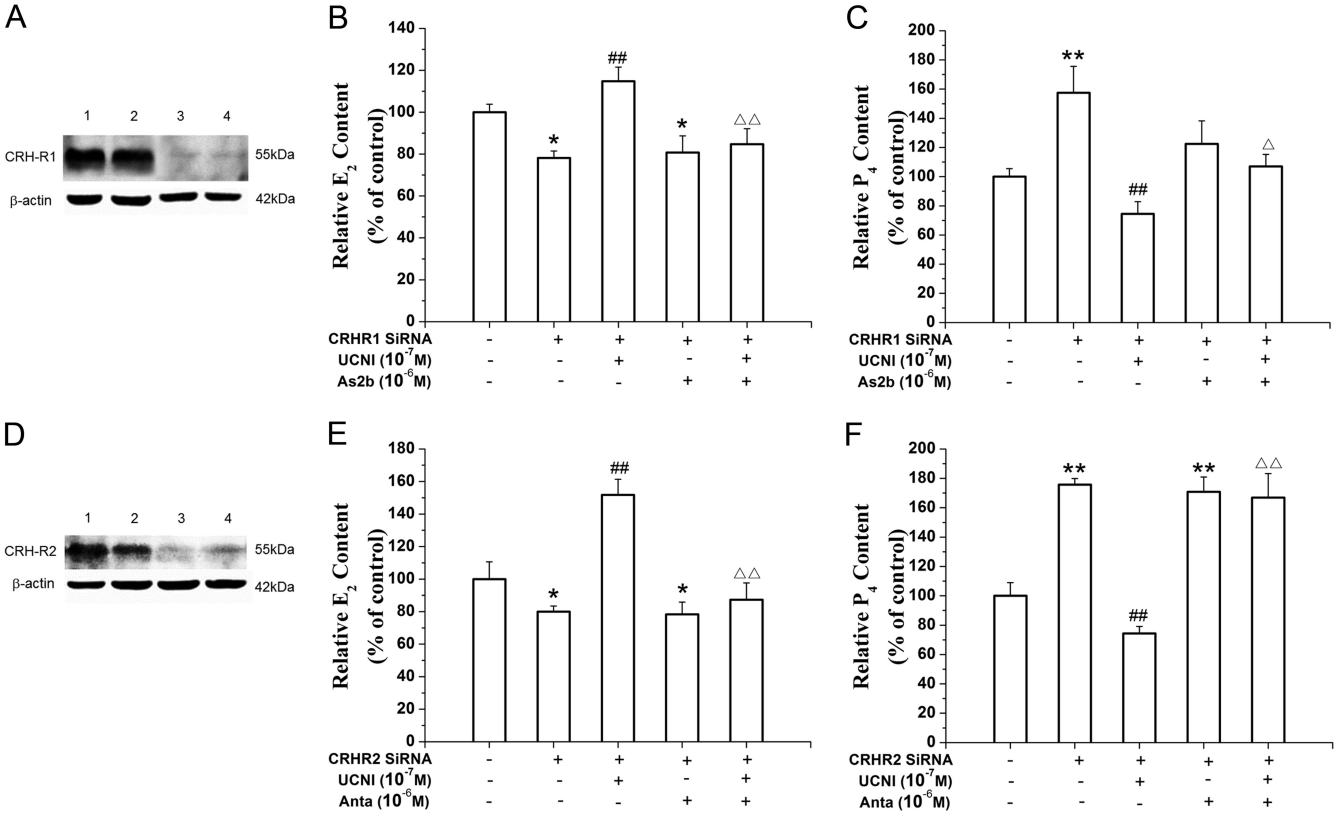

FIG. 3. E_2 and P_4 production in CRH-R1 or CRH-R2 knockdown placental cells. A–C, Changes in basal E_2 and P_4 production and the effect UCNI on E_2 and P_4 production in CRH-R1 knockdown cells. Cells were transfected with siRNA targeting CRH-R1 and then treated with UCNI (10^{-7} M) in the absence and presence of astressin-2b(10^{-6} M) for 24 h. E_2 and P_4 contents in culture media were assayed by RIA. A, Representative bands for protein expression of CRH-R1 in cells transfected with corresponding siRNA. Lane 1, Control cells; lane 2, cells transfected with negative control siRNA; lanes 3 and 4, cells transfected with siRNA targeting CRH-R1. B and C, Basal and UCNI-induced E_2 and P_4 production in CRH-R1 knockdown cells. D--F, Changes in basal E_2 and P_4 production and the effect UCNI on E_2 and P_4 production in CRH-R2 knockdown cells. Cells were transfected with siRNA targeting CRH-R2 and then treated with UCNI (10^{-7} M) in the absence and presence of antalarmin (10^{-6} M) for 24 h. E_2 and P_4 contents in culture media were assayed by RIA. D, Representative bands for protein expression of CRH-R2 in cells transfected with corresponding siRNA. Lane 1, Control cells; lane 2, cells transfected with negative control siRNA; lanes 3 and 4, cells transfected with siRNA targeting CRH-R2. E and F, Basal and UCNI-induced E_2 and P_4 production in CRH-R2 knockdown cells. Values are presented as mean percent control ± SEM (n = 4). *, $P < 0.05$, **, $P < 0.01$ compared with negative control siRNA; #, $P < 0.05$, ##, $P < 0.01$ compared with the basal level in the cells transfected with siRNA-CRH-R1 or siRNA-CRH-R2; △△, $P < 0.01$ compared with UCNI (10^{-7} M). Anta, Antalarmin; As2b, astressin-2b.

UCNI caused an increase in E_2 production and a decrease in P_4 production, which was blocked by astressin-2b (Fig. 3C).

Cells transfected with siRNA-CRH-R2 showed about 75% decrease in CRH-R2 expression compared with the cells transfected with control siRNA (Fig. 3D). Transfection of siRNA-CRH-R2 caused a decrease in basal E_2 production and an increase in basal P_4 production (Fig. 3E). UCNI treatment of these cells increased E_2 production and decreased P_4 production, which was blocked by antalarmin (Fig. 3F).

CRH-R1 activates Gαs/adenylyl cyclase (AC) and Gαq/PLC signaling pathways

CRH receptors have been reported to couple to multiple Gα proteins including Gαs, Gαi, Gαq/11, Gαz, and Gαo and then initiate multiple intracellular signaling pathways (34, 35). We specified the Gα proteins activated by CRH-R1 and CRH-R2.

Due to no CRH-R1-specific agonist available, CRH-R2 knockdown cells were used. The cells transfected with siRNA-CRH-R2 were treated with increasing concentration of CRH (10^{-9} to 10^{-7} M) for 5 min. A dose-dependent increase of GTP-bound Gαs protein level was found (Fig. 4A). CRH treatment increased cAMP production in a dose-dependent manner (Fig. 4B). These effects could be blocked by the CRH-R1 antagonist antalarmin.

Because there is no commercially available kit for Gαq detection, we instead determined the activation of PLC-β3, a downstream signaling molecule of Gαq protein. As shown in Fig. 4, C and D, CRH treatment was shown to stimulate

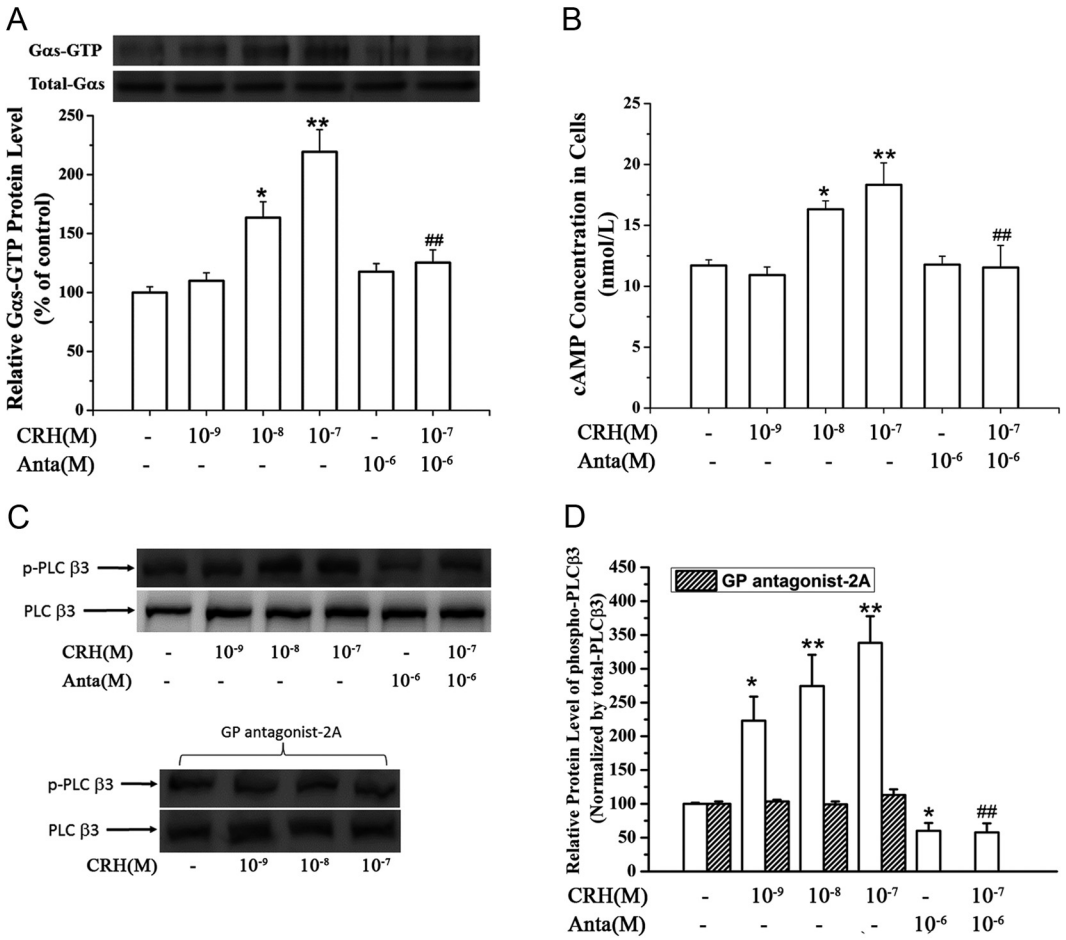

FIG. 4. CRH-R1 activates Gs and Gq signaling pathways. Cultured placental cells were transfected with siRNA-CRH-R2 and then treated with CRH at the indicated concentrations in the absence or presence of antalarmin (10^{-6} M) for 5 min. Levels of GTP-bound Gαs protein, cAMP content, and pPLC-β3protein were analyzed as described in *Materials and Methods*. A, Effect of CRH on the expression of GTP-bound Gαs protein. Representative protein bands of GTP-bound Gαs protein and total Gαs protein were presented on the top of corresponding histogram. B, Effect of CRH on cAMP production in placental cells. C and D, The effect of CRH on pPLC-β3 protein and the effect of GP antagonist-2A on CRH-induced pPLC-β3 in CRH-R2 knockdown cells. Cells were transfected with siRNA-CRH-R2 and then treated with CRH at the indicated doses in absence or presence of antalarmin for 5 min. Or after transfection of cells with siRNA-CRH-R2, the cells were transfected with a small inhibitory peptide of Gq protein GP antagonist-2A for 2 h. Cells were then treated with CRH at the indicated doses for 5 min. C, Representative protein bands of pPLC-β3 and total PLC-β3 in response to CRH treatment. D, Summary histogram of CRH effect on pPLC-β3. Values are presented as mean ± SEM (B) or mean percent control ± SEM (A and D) for a total of four placenta cultures obtained from four patients (n = 4). *, $P < 0.05$, **, $P < 0.01$ *vs.* vehicle control; ##, $P < 0.01$ compared with CRH (10^{-7} M). Anta, Antalarmin.

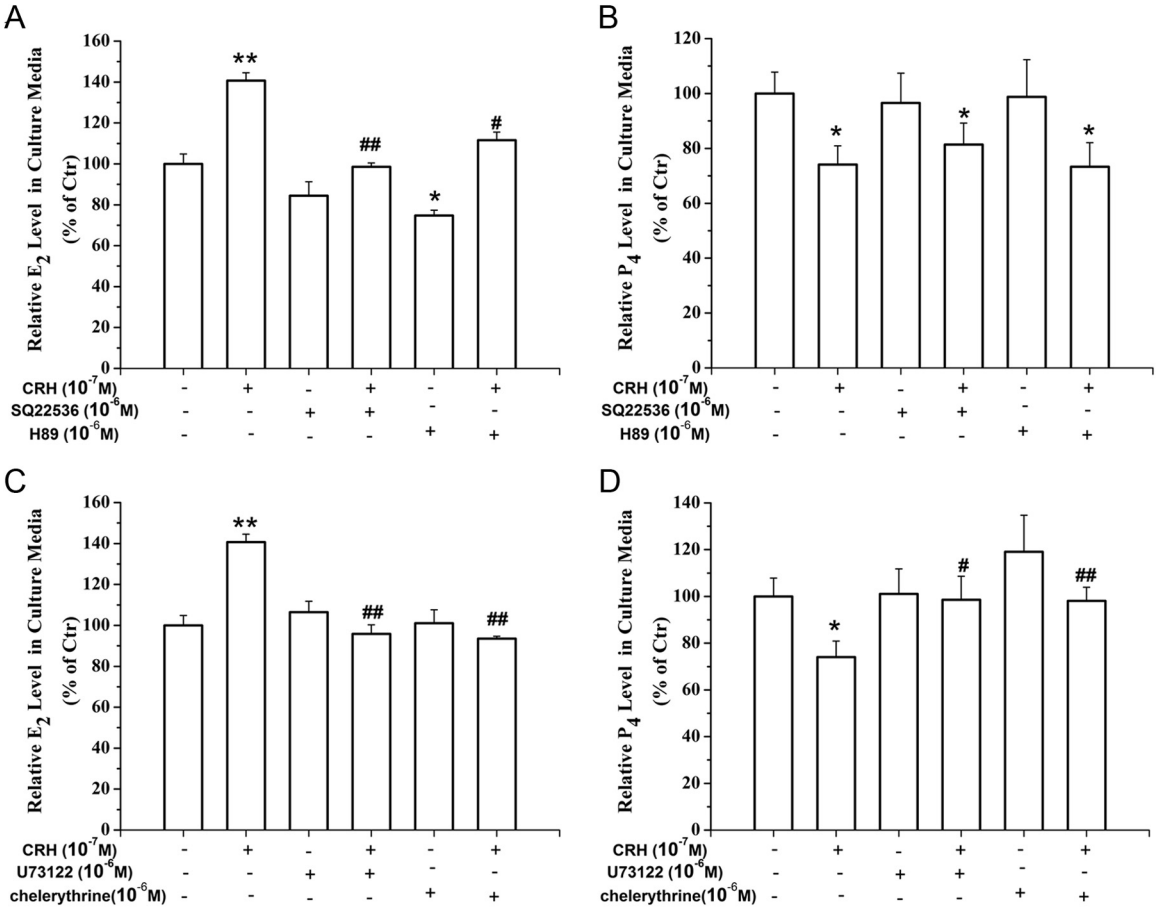

FIG. 5. The roles of the AC-cAMP-PKA and PLC-PKC signaling pathway in the regulation of E_2 and P_4 production caused by CRH in CRH-R2 knockdown placental cells. Cells were transfected with siRNA-CRH-R2 and then treated with the indicated concentration of CRH in the presence or absence of the AC inhibitor SQ22536 and the PKA inhibitor H89 (A and B) or the PLC inhibitor U73122 and the PKC inhibitor chelerythrine (C and D). E_2 and P_4 production were measured by RIA. Data were expressed as mean percent control ± SEM (n = 4). *, $P < 0.05$, **, $P < 0.01$ compared with vehicle control; #, $P < 0.05$, ##, $P < 0.01$compared with CRH (10^{-7} M).

pPLC-β3, the activated form of PLC-β3, in a dose-dependent manner, which was also blocked by antalarmin.

To confirm that CRH-induced pPLC-β3 is dependent on Gαq activation, a small inhibitory peptide of Gαq protein GP antagonist-2A was transfected into the cells. It was found that blockage of Gαq activation by GP antagonist-2A totally abolished CRH-induced pPLC-β3 in CRH-R2 knockdown cells (Fig. 4, C and D).

The roles of AC/protein kinase A (PKA) and PLC/protein kinase C (PKC) signaling in CRH-R1 regulation of steroid production

To further determine the signaling pathway involved in CRH-R1regulation of E_2 and P_4 production, the inhibitors of AC, PKA, PLC, and PKC were used.

Application of either AC inhibitor SQ22536 or PKA inhibitor H89 blocked CRH-induced E_2 production in CRH-R2 knockdown cells. Neither SQ22536 nor H89 blocked CRH-induced inhibition of P_4 production in CRH-R2 knockdown cells (Fig. 5, A and B).

Application of either PLC inhibitor U73122 or PKC inhibitor chelerythrine blocked CRH-induced E_2 production as well as CRH-induced inhibition of P_4 production in CRH-R2 knockdown cells (Fig. 5, C and D).

CRH-R2 activates Gαi and Gαq signaling pathways

As shown in Fig. 6, A–C, UCNIII treatment did not affect the level of GTP-bound Gαs protein but caused an increase in GTP-bound Gαi protein and a decrease in cAMP production in dose-dependent manner. These effects were blocked by CRH-R2 antagonist, astressin-2b.

UCNIII treatment induced pPLC-β3 expression in a dose-dependent manner (Fig. 6, D and E), which was blocked by astressin-2b. Blockage of Gαq protein with GP antagonist-2A abolished UCNIII-induced pPLC-β3 expression (Fig. 6, D and E).

The role of PLC/PKC signaling in CRH-R2 regulation of steroid production

Because CRH-R2 stimulates PLC but inhibits AC/cAMP signaling, we examined the role of PLC signaling in

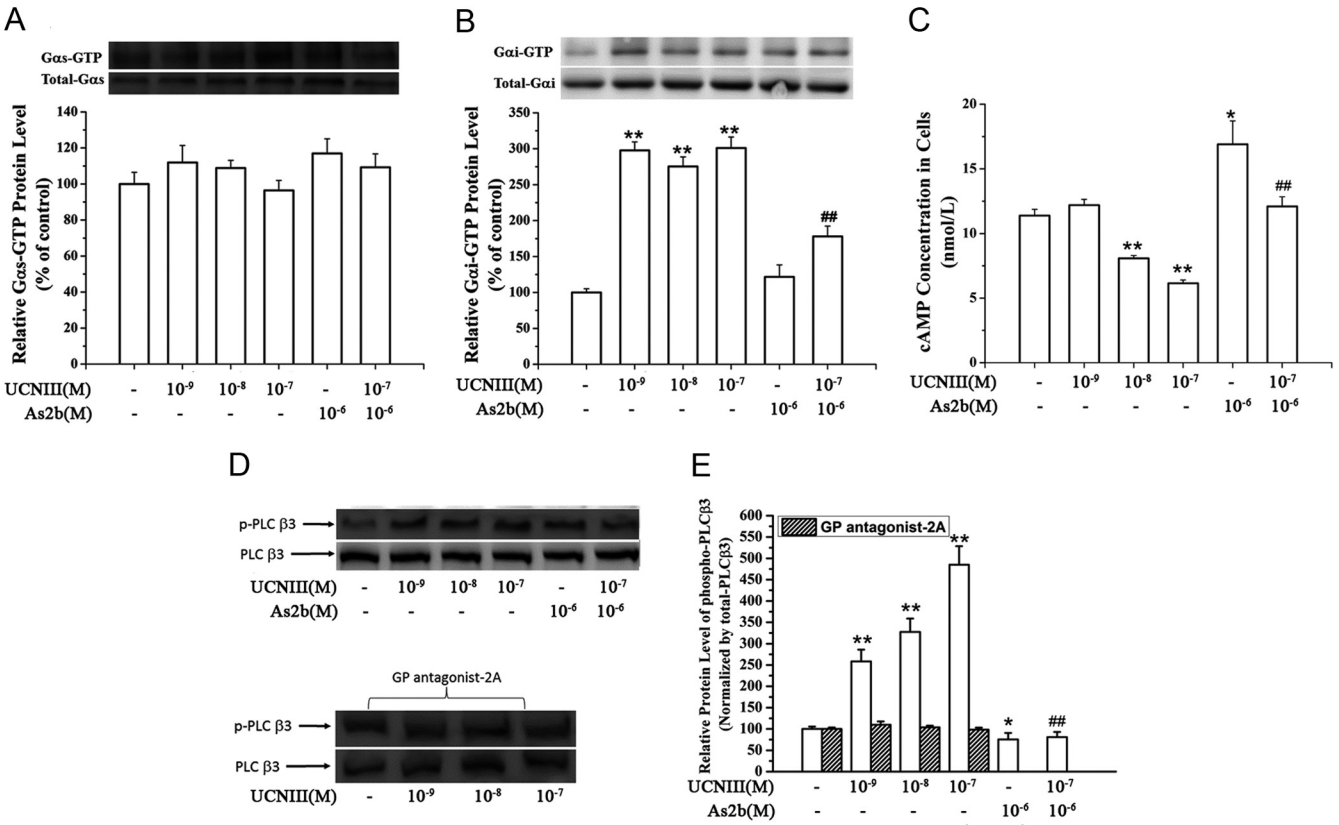

FIG. 6. CRH-R2 activates Gi and Gq signaling pathways. Placental cells were treated with specific CRH-R2 agonist UCNIII at the indicated concentrations in the absence or presence of astressin-2b (10^{-6} M) for 5 min. Levels of GTP-bound Gαs protein, GTP-bound Gαi protein, cAMP content, and pPLC-β3 protein were determined as described in *Materials and Methods*. A and B, Effect of UCNIII on the expression of GTP-bound Gαs protein and GTP-bound Gαi protein. Representative protein bands of GTP-bound Gαs/Gαi protein and total Gαs/Gαi protein were presented on the top of corresponding histogram. C, Effect of UCNIII on cAMP production in placental cells. D and E, Effects of UCNIII on the pPLC-β3 and the effect of GP antagonist-2A on UCNIII-induced pPLC-β3. Cells were treated with increasing concentration of UCNIII in the absence or presence of astressin-2b for 5 min, or cells were transfected with a small inhibitory peptide of Gq protein GP antagonist-2A for 2 h and then treated with CRH at the indicated doses for 5 min. The levels of pPLC-β3 were analyzed by Western blotting. D, Representative protein bands of pPLC-β3 and total PLC-β3 in response to UCNIII treatment. E, Summary histogram of UCNIII effect on pPLC-β3. Values are presented as mean ± SEM (C) or the mean percent control ± SEM (A, B, D, and E) for a total of four placenta cultures obtained from four patients. *, $P < 0.05$, **, $P < 0.01$ *vs.* vehicle control; ##, $P < 0.01$ compared with UCNIII (10^{-7} M). As2b, Astressin-2b.

the CRH-R2 regulation of steroid production. As shown in Fig. 7, the PLC inhibitor U73122 and the PKC inhibitor chelerythrine blocked increased E_2 production and decreased P_4 production caused by UCNIII.

Discussion

In this study, we demonstrated that, when endogenous CRH and UCNI secreted by placental trophoblasts were bound by their antibodies, there was an increase in P_4 production and a decrease in E_2 production in placental cells. Their effects were mediated by CRH-R1 and CRH-R2. CRH-R1 stimulated Gs and Gq signaling pathways, whereas CRH-R2 activated Gi and Gq signaling pathways. CRH-R1 increased E_2 production via AC-PKA and PLC-PKC signaling and decreased P_4 production via PLC-PKC signaling. CRH-R2 increased E_2 production and inhibited P_4 production via PLC-PKC signaling.

It has long been assumed that human placenta, unlike the gonads and the adrenal gland, is an incomplete endocrine organ lacking CYP17 enzymatic activity and thus depended on the fetal and maternal compartments for C-19 steroid precursors to generate estrogens in gestation (6). Therefore, in the present study, DHEAS (10^{-6} M) were added to supply the precursors for generation of estrogens in the cultured placental cells. Because E_2 production is supposed not to be synthesized from progesterone, increased P_4 production is not due to decreased E_2 production and vice versa. However, most recently, Escobar *et al.* (7) demonstrated that human placentas express CYP17 and generate androgen *de novo*. Thus, we could not exclude the possibility that the change in P_4 is due to a change in estrogen production in cultured placental cells. Nota-

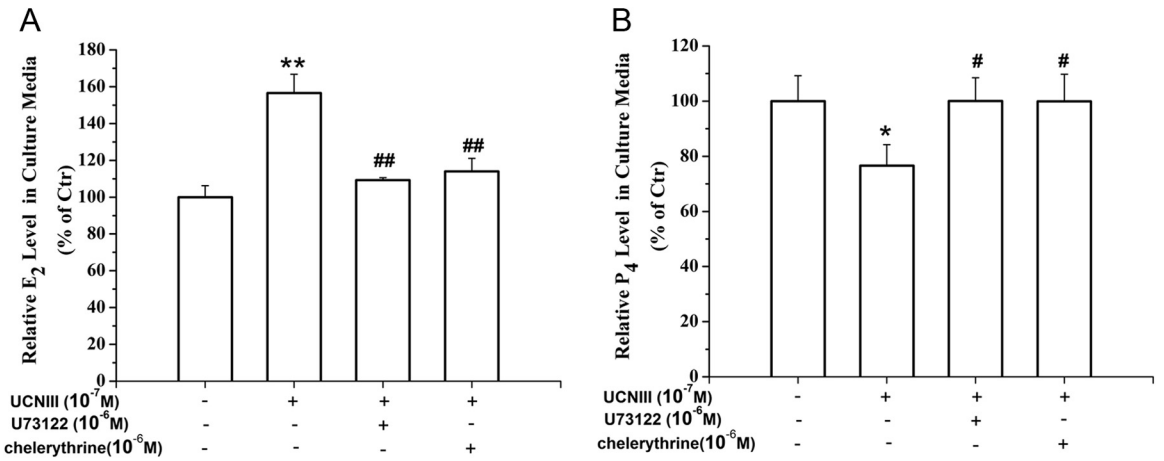

FIG. 7. The role of the PLC-PKC signaling pathway in the regulation of E_2 and P_4 production induced by UCNIII in placental cells. Cells were treated with the indicated concentration of UCNIII in the presence or absence of the PLC inhibitor U73122 and the PKC inhibitor chelerythrine. E_2 (A) and P_4 (B) contents in culture media were measured by RIA. Data were expressed as mean percent control ± SEM (n = 4). *, $P < 0.05$, **, $P < 0.01$ compared with vehicle control. #, $P < 0.05$, ##, $P < 0.01$ compared with UCNIII (10^{-7} M).

bly, our previous studies have shown that CRH stimulates the expression of all the enzymes required for estrogen synthesis but inhibits all the enzymes required for progesterone synthesis (17, 18).

It is known that functions of CRH and its related peptides are mediated by two subtypes of CRH receptors, CRH-R1 and CRH-R2 (35). Both CRH-R1 and CRH-R2 have been localized to placental syncytiotrophoblasts (31). CRH-R1 and CRH-R2 have been found to trigger similar effects but sometimes opposite actions in various tissues. For instance, CRH-R1 and CRH-R2 exert opposite effect on prostaglandin production (31) and glucose transporter-1 but similar effect on glucose transporter-3 in placental trophoblasts (33). The present study demonstrated that CRH-R1 and CRH-R2 exhibited similar effects on E_2 and P_4 production in placental trophoblasts. In addition, our findings that CRH-R2 activation stimulated E_2 production in placenta are consistent with the study of Imperatore *et al.* (36) in which they have demonstrated that UCNII simulates E_2 production via CRHR2 in cultured placental cells.

CRH receptors have been demonstrated to be coupled with multiple G proteins and subsequently activate divergent signaling pathways in various tissues (35). Karteris *et al.* (34) reported that CRH stimulated Gq, Go, and Gz but not Gi and Gs in placental tissues, and CRH could increase inositol 1,4,5-triphosphate but not cAMP production. However, their study did not define the signaling activated by individual CRH receptors. The present study demonstrated that both CRH-R1 and CRH-R2 were coupled to multiple G proteins but activated differentially signaling pathways. Gq-PLC signaling was activated by both CRH-R1 and CRH-R2, whereas Gs and Gi were activated by CRH-R1 and CRH-R2, respectively. Our findings that CRH-R1 was coupled to Gs, whereas CRH-R2 was coupled to Gi might partly explain the observation by Karteris *et al.* (34) that CRH did not stimulate Gi and Gs as well as cAMP production in placenta.

It has been shown that cAMP/PKA activation induces aromatase, leading to an increase in estrogen production in placental cells (37). Recently the study by Klempan *et al.* (38) indicated that PKC activation could stimulate estrogen production in human choriocarcinoma cell line JEG-3

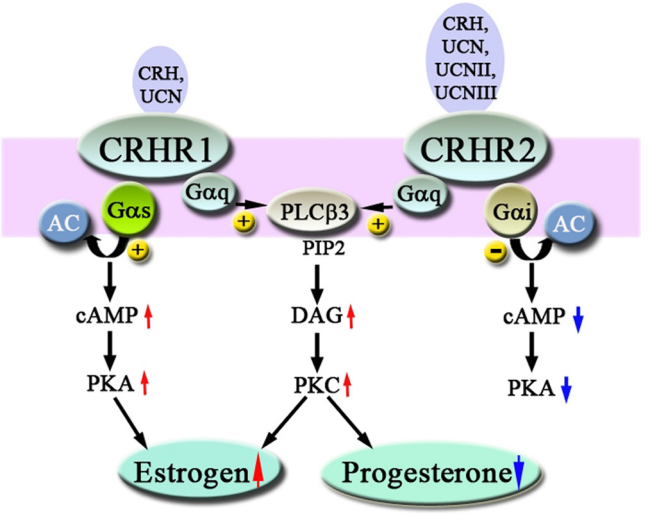

FIG. 8. Scheme illustrating the proposed regulation of estrogen and progesterone production by CRH and its related peptides in human placenta. CRH-R1 primarily couples to Gs and Gq protein. When CRH or UCN binds CRH-R1, it stimulates the AC-cAMP-PKA and PLC-PKC signaling pathways, which stimulates estrogen production. CRH-R1 activation can inhibit progesterone production via the PLC-PKC signaling pathway but not by the AC-cAMP-PKA signaling pathway. When CRH-R2 activation by UCNII and UCNIII as well as UCN and CRH, it induces Gαi and Gαq activation and then stimulates the PLC-PKC signaling pathway, which stimulates estrogen production and inhibits progesterone production.

and BeWo. Our study also demonstrated that CRH-R1 and CRH-R2 simulated estrogen production via AC/PKA and PLC/PKC signaling pathways in primary placental cells. Barrera *et al.* (39) reported that PKA is not involved in P_4 production in primary human placental cells. Our previous study had shown that CRH inhibits P_4 production through a PKC-dependent pathway (17). The present study confirms that PLC/PKC is involved in inhibition of P_4 production by CRH and its related peptides and indicates that AC/PKA is not involved in CRH-R1 and CRH-R2 regulation of P_4 production in placental cells.

Various factors within fetal-placental unit, such as human chorionic gonadotropin, glucocorticoids, and prostaglandins (8, 9), are involved in the control of estrogen and progesterone biosynthesis in placenta. Previously we found that CRH is a local factor involved in placental steroid production (17, 18). Our present data demonstrated that CRH-related peptides such as UCNI and UCNIII can act on CRH receptors to modulate steroid production during pregnancy. Thus, it suggests that there is a network of endogenous factors controlling steroid production in placenta, which may be important for maintenance of pregnancy and initiation of parturition.

In most mammals, estrogen production is further increased whereas progesterone production is dramatically decreased at the end of pregnancy, which is a pivotal event for the onset of parturition in these animals (40). Although human parturition is not preceded by these systemic changes in estrogen and progesterone, a decrease in the progesterone to estrogen ratio occurs toward term (41, 42). In humans, a major form of estrogen produced by placenta is estriol. More recently Smith *et al.* (43) have demonstrated that, in late pregnancy, progesterone/estriol decreases and CRH and estriol concentrations are significantly positively associated in maternal circulation. Given that placental CRH and UCNI production is increased toward the onset of labor (23, 24), our findings that CRH and UCNI stimulate E_2 production but inhibit P_4 production indicate that CRH and its related peptides might be involved in the changes in progesterone to estrogen ratio toward the onset of labor.

In conclusion, CRH and its related peptides act on CRH-R1 and CRH-R2 to stimulate E_2 and inhibit P_4 production in placental cells. CRH-R1 and CRH-R2 stimulated divergent signaling pathways (Fig. 8). Gs-AC-cAMP and Gq-PLC signaling pathways were activated by CRH-R1, whereas the Gi-AC and Gq-PLC signaling pathways were activated by CRH-R2. CRH-R1 increased E_2 production via AC-PKA and PLC-PKC signaling and decreased P_4 production via PLC-PKC signaling. CRH-R2 increased E_2 production and inhibited P_4 production via PLC-PKC signaling. CRH and its related peptides might be involved in change in the progesterone to estrogen ratio during human pregnancy.

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