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REGULATION OF PROGESTERONE SECRETION IN HUMAN SYNCYTIOTROPHOBLAST IN CULTURE BY HUMAN CHORIONIC GONADOTROPIN

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Summary—In the present investigation we sought to define the specific sites in the pathway of placental progesterone biosynthesis that underlie the action of human chorionic gonadotropin (hCG). When the cells were challenged with dibutryl cAMP (dbcAMP), forskolin or isobutylmethylxanthine, they produced significantly higher amounts of progesterone which in the presence of the hCG antibody was reduced to the level of the control set of cells. Trophoblast cells cultured in serum free medium with 25-hydroxycholesterol (25-OHC) produced increased amounts of progesterone. In the presence of hCG antibody at a concentration which neutralized the secreted hCG, the steroid production was completely blocked, even when the 25-OHC was added to the medium. Also, direct quantitation of the cytochrome *P*450 SCC enzyme in the absence of hCG indicated a significant decrease. The exogenous addition of low density lipoproteins (LDL) increased the progesterone secretion by the trophoblast cells in culture. Neutralization of hCG by the antibodies, however, drastically reduced the LDL induced progesterone secretion, which was restored by the addition of dbcAMP to the medium. Based on these findings, we suggest a stimulatory effect of hCG on normal trophoblast cells at the level of LDL utilization and cytochrome *P*450 SCC enzyme. Since dbcAMP could mimic these actions of hCG, the data suggest a possible autocrine/paracrine role of hCG on the trophoblast cells. An additive effect of hCG and cAMP on progesterone secretion observed in our studies, indicate that apart from hCG, adenylate cyclase activity may also be regulated by other factors.

INTRODUCTION

Human chorionic gonadotropin (hCG) is detected as early as 6–7 days following fertilization [1, 2], and is responsible, at least transiently, for stimulating progesterone secretion from the corpus luteum [3, 4], thereby maintaining pregnancy during the early stages. The time of transition from corpus luteum to placental production of progesterone has been reported to be around the 7th week of gestation [3, 4]. Detailed studies in rhesus monkeys have however, shown that this luteoplacental shift may occur even earlier—as early as 14 to 16 days after conception as reflected by the sustained rise in maternal serum progesterone levels and maintenance of pregnancy despite bilateral ovariectomy [5, 6].

The mechanism by which hCG regulates the luteal progesterone synthesis is well established [7–9]. However, the regulatory mechanisms controlling the synthesis of progesterone by the human placenta and the involvement of hCG in this process is largely unknown. Progesterone formation by placental tissue under *in vitro* conditions has been shown to be modulated by cAMP [10], prolactin [11], estradiol [12] and insulin growth factor-1 (IGF-I) [13]. All these factors are of local origin and suggest that the regulation of placental steroidogenesis may involve autocrine and/or paracrine mechanisms.

With the exception of stimulating adenylate cyclase activity *in vitro* [14], hCG has been shown to have no effect on the specific steps of placental progesterone formation, i.e. low density lipoprotein (LDL) uptake or an increase in cytochrome *P*450 SCC, leading to increased conversion of cholesterol to pregnenolone [15–18]. These steps in the steroidogenic pathway are however, activated by hCG in the corpus luteum [9].

Recent advances into the isolation and culture of relatively pure preparations of cytotrophoblasts have now made it possible to study the regulation of placental progesterone synthesis under *in vitro* conditions. Using such a

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system we have earlier reported that syncytial cells formed under culture conditions by fusion of cytotrophoblast cells in culture secrete both hCG and progesterone [19]. Complete neutralization of hCG by monoclonal antibodies abrogated the steroid production by these cells. Based on this observation we had proposed that placental progesterone production is under the regulation of hCG. In the present investigation we have sought to elucidate the specific sites in the pathway of placental progesterone biosynthesis which may be regulated by hCG.

EXPERIMENTAL

Materials

Chemicals. Trypsin (Type III), DNase (Type I), dibutryl cAMP, forskolin, isobutylmethylxanthine (MIX) and 25-hydroxycholesterol (25-OHC) were obtained from Sigma Chemical Co. (U.S.A.). The source of DMEM and fetal bovine serum was GIBCO (U.S.A.). Antibiotics were purchased from Flow Laboratories (U.S.A.) and Percoll was obtained from Pharmacia, (Sweden). [1,2,6,7-^{3}H]Progesterone (80–110 Ci/mmol) and ^{125}I (100 mCi/ml, for iodination of hCG) were purchased from Amersham Int. (England). All other chemicals were obtained from local sources and were of analytical grade.

Antibodies. The monoclonal antibody against hCG was a generous gift from Dr G. P. Talwar, National Institute of Immunology (New Delhi, India). The antibody was of IgG_1 isotype and its association constant (K_a) for binding with hCG was 3.0×10^{10} l/mol. This antibody recognized a conformation common to the native hormone and the β subunit of hCG. It bound nearly as well with hCG and β-hCG, but had very low reactivity (<1%) with the α subunit and hLH (LER-960). It was devoid of reaction with FSH, TSH and other pituitary hormones [20]. The same antibody was also used in quantitating hCG by RIA. The antibody for progesterone used for RIA was obtained under the Matched Assay Reagents Programme of the Indian Council of Medical Research (New Delhi, India). This antibody was of very high specificity and had no significant cross reactivity with the related steroids [21].

Lipoproteins. Serum LDL (hydrated density 1.019–1.063 g/ml) and lipoprotein poor serum (LPPS, hydrated density >1.2 g/ml) were prepared from 100 ml fetal bovine serum following the method of Havel *et al.* [22].

Method

Cell preparation and culture. Term placentae were obtained immediately following spontaneous vaginal delivery from uncomplicated pregnancies; collected in normal saline and were processed aseptically within an hour of delivery. Portions of villous tissue (20–30 gm) were separated from the surrounding connective tissue and blood vessels and processed essentially as published earlier [23]. Essentially, the villous tissue was minced to very fine pieces and subjected to differential trypsin digestion (0.125% for 3×20 min) with DNase in Ca^{++}, Mg^{++} free Hanks Balanced Salt Solution (HBSS) containing penicillin (100 IU/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml) and gentamycin (50 μg/ml). The heterogenous cell population thus obtained was further purified on discontinuous Percoll gradient (0–70%). The homogenous population of cytotrophoblasts were plated in 16 mm, 24-well tissue culture clusters (Costar) at a concentration of 0.3×10^6 cells/well/ml in DMEM containing 10% fetal bovine serum and the antibiotics. The plates were maintained at 37°C in sterile humid atmosphere for a period of 6–7 days with daily changes of the media. The media was analysed for progesterone and hCG by RIA. Total cell protein was determined by the method of Lowry *et al.* [24].

Neutralization of secreted hCG by hCG antibody. The amount of antibody needed to neutralize the hCG in the medium was predetermined by checking hCG neutralization capacity as described earlier [19]. The antibody dilution (10^4) at which no free hCG could be detected after PEG precipitation was then used in the culture system. This confirmed the total neutralization of secreted hCG by the antibodies.

Incubation of cytotrophoblasts with various ligands. In a separate set of experiments, the cells were challenged after 72 h of plating with dbcAMP, MIX or forskolin at the concentrations indicated in the figure legends. The effect of LDL on progesterone secretion was investigated in cells maintained in media supplemented with LPPS.

Cytochrome P450 SCC activity. Cytochrome P450 SCC activity was assessed by measuring the amount of progesterone secreted by the trophoblasts maintained in serum free media containing 0.1% BSA and supplemented with 25-OHC in presence or absence of hCG. The

25-OHC served as the substrate for conversion to progesterone [25].

Spectral analysis of mitochondrial P450 SCC content. The cells were harvested after 96 h of culture (in presence or absence of hCG antibody), washed and resuspended in 10% w/v of homogenizing buffer [PBS containing 0.1% BSA (RIA grade), 0.15 M sucrose and 1 mM EDTA], and homogenized in a glass–Teflon homogenizer. The homogenate was filtered through cheese cloth into a 15 ml centrifuge tube. The residue was rehomogenized and filtered. The combined filtrate was first centrifuged at 600 *g* for 10 min at 4°C. The supernatant was decanted and centrifuged again at 5000 *g* for 1 min at 4°C. The pellet containing the mitochondria was resuspended in homogenizing buffer (2 ml), and subjected to quantitation of cytochrome *P*450 SCC according to the method of Omura and Sato [26]. The excitation coefficient of 91 cm^{-1} mM^{-1} was used to calculate the cytochrome *P*450 content from the absorbence difference between 450 and 490 nm.

Radioimmunoassay. The spent culture media were assayed for progesterone following WHO protocol and hCG was quantitated according to the method of Salahuddin *et al.* [27, 28]. The intra- and interassay coefficient of variation for progesterone was 1.4 ± 0.05 and 3.9 ± 0.16 and for hCG it was 2.8 ± 0.091 and 6.6 ± 0.21, respectively.

Statistical analysis

The data is expressed in terms of mean ± SEM. The level of significance was determined by one way ANOVA.

RESULTS

The cytotrophoblastic cells cultured from the human term placentae differentiated into syncytiotrophoblasts by 72 h of plating and by 96 h the cells secreted peak levels of progesterone (16.2 ± 1.1 ng/mg protein) and hCG (84.3 ± 3.2 ng/mg protein).

Effect of cAMP, forskolin and MIX on progesterone secretion

In the presence of either dbcAMP (1.5 mM) or forskolin (10 μM) or phosphodiesterase inhibitor MIX (1.5 mM), the trophoblast cells secreted 25.8 ± 1.9, 24.6 ± 1.3 and 22.4 ± 1.5 ng of progesterone/mg protein, respectively, which were significantly higher ($P < 0.001$) as compared to the control levels (Fig. 1). Progesterone secretion was completely blocked in the presence of hCG antibody (ab) at a dilution of 10^4. The amount of progesterone secreted by the cells cultured in the presence of hCG ab and challenged either with dbcAMP, forskolin or MIX was similar to the levels of untreated control set of cells. Figure 1 inset shows the dose–response curve of dbcAMP with or without hCG antibody. At a saturating concen-

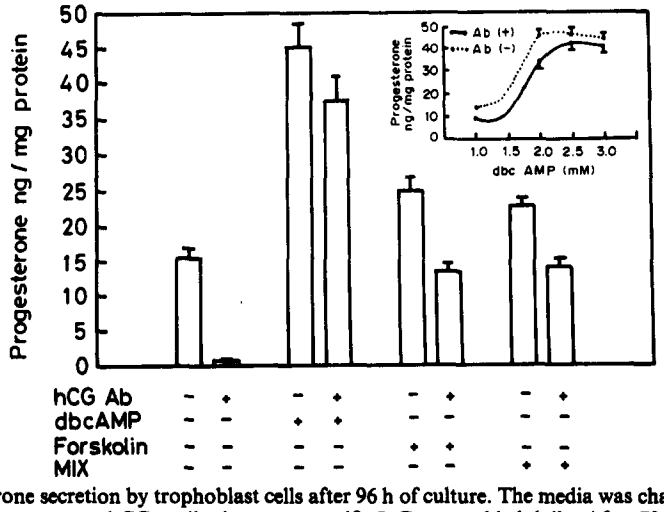

Fig. 1. Progesterone secretion by trophoblast cells after 96 h of culture. The media was changed daily after plating; wherever necessary hCG antibody or nonspecific IgG were added daily. After 72 h of plating, the fresh media, in addition to antibodies or IgG also contained either dbcAMP (2.5 mM), forskolin (10 μM) or MIX (1.5 mM). This medium was removed after 24 h (96 h after plating), stored at −20°C and was subsequently assayed for progesterone. The inset shows the dose–response curve of dbcAMP (1.0–3.0 mM). The data are the mean values (±SEM) obtained using cells from six wells and are representative of 3 different experiments.

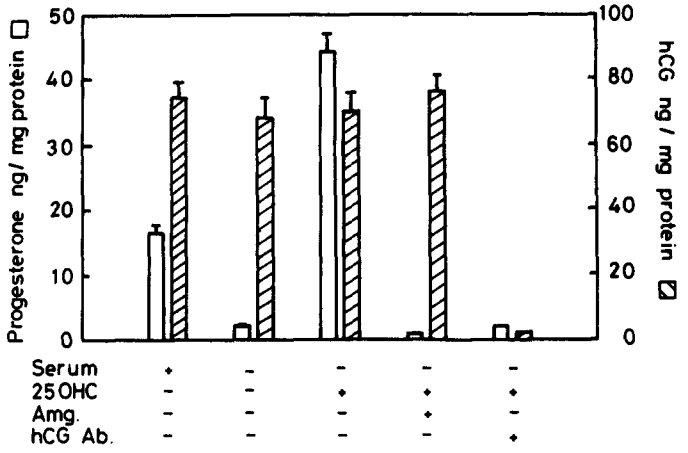

Fig. 2. The effect of 25-OHC and aminoglutethemide on progesterone and hCG secretion by syncytiotrophoblasts in culture in presence or absence of hCG antibody. 25-OHC (20 μM) was added to the cells in serum free media containing 1% BSA after 72 h of culture which resulted in significantly higher amounts of progesterone secretion ($P < 0.001$). This increase in progesterone secretion was completely blocked when either aminoglutethemide (50 μm) or hCG ab were included in the culture media. Presence of aminoglutethemide had no effect on secretion of hCG. The data are the mean values (±SEM) obtained using cells from six wells and are representative of 3 different experiments.

tration of 2.5 mM of dbcAMP the amount of progesterone secretion in the presence or absence of hCG ab remained statistically similar (Fig. 1).

Effect of hCG antibody on P450 SCC activity

Cytochrome *P*450 side chain cleavage enzyme activity is the rate limiting step in the conversion of cholesterol to progesterone. Since 25-OHC is more soluble in aqueous medium than cholesterol and enters the cells easily, it serves as a substrate for the conversion to progesterone under the condition of substrate excess [25]. Hence, the activity of cytochrome *P*450 SCC was measured in the cells maintained in serum free medium to which 25-OHC was added. In serum free medium, though the hCG secretion remained unaffected, the cells were unable to produce progesterone due to the nonavailabity of the precursor present in serum. In the presence of 25-OHC as substrate, progesterone secretion increased to a maximum of 44.2 ± 3.1 ng/mg protein with no change in hCG secretion (Fig. 2). In the presence of hCG antibody at a concentration which neutralized the secreted hCG, progesterone secretion was completely blocked. The secretion of the steroid was also inhibited by the addition of 50 μm aminoglutathemide to the incubation medium (Fig. 2).

Table 1. Effect of hCG antibody on cytochrome *P*450 SCC content of trophoblast cells in culture

Treatment	Cytochrome *P*450 nmol mg^{-1} protein
Control	4.88 ± 0.45
hCG antibody[a]	0.29 ± 0.09***

***$P < 0.001$.
[a]hCG antibody was added after 24 h of plating with daily changes of the media. Cytochrome *P*450 was measured at 96 h of culture.

Spectral analysis of mitochondrial P450 SCC content

The absolute value of cytochrome *P*450 SCC content in the trophoblasts as determined by spectral analysis was 4.88 nmol/mg protein (Table 1). Neutralization of hCG by addition of the antibody reduced the *P*450 content by 93% ($P < 0.001$).

Effect of LDL on progesterone secretion by trophoblasts

In order to examine the role of hCG on LDL induced progesterone secretion, LDL was added in graded concentrations from 0–300 μg LDL ml^{-1} protein to the cells cultured in LPPS. No progesterone secretion was observed in the absence of LDL, but the secretion of the steroid increased with increasing concentrations of LDL (Fig. 3). At an optimum concentration of 200 μg LDL, the cytotrophoblasts secreted 43.1 ± 2.6 ng progesterone/mg protein. This exogenous addition of LDL (0–300 μg) had no effect on hCG secretion. Neutralization of hCG by the antibodies, however, drastically reduced the LDL induced progesterone secretion

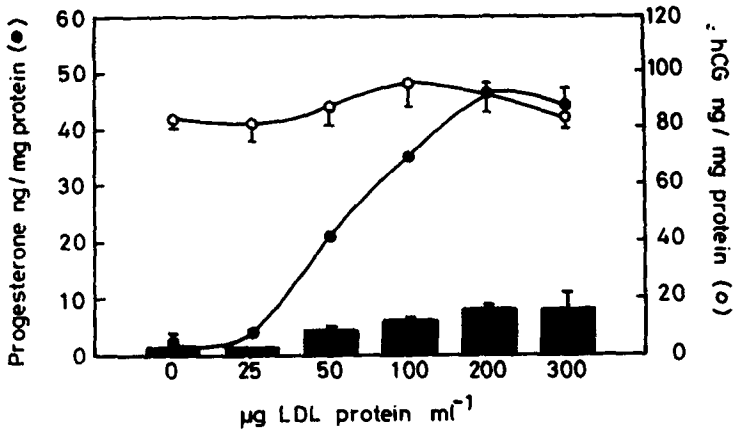

Fig. 3. The effect of LDL on progesterone (●—●) and hCG (○—○) secretion by human trophoblasts. The cells were cultured in LPPS. LDL (0–300 μg protein ml^{-1}) was added after 72 h of plating and the media assayed for progesterone and hCG. With increasing concentrations of LDL progesterone secretion increased significantly (ANOVA, $P < 0.001$) whereas hCG secretion remained unaltered (ANOVA, nonsignificant). In presence of hCG ab, the increase in progesterone secretion (■) was marginal and less significant (ANOVA, $P < 0.5$). The data are the mean values (±SEM) obtained using cells from six wells and are representative of 3 different experiments.

(Fig. 4). Addition of dbcAMP (2.5 mM) restored the LDL induced progesterone secretion to the high levels (43.1 ± 1.8 ng/mg protein) in spite of the presence of hCG antibody in the medium (Fig. 4).

DISCUSSION

In vitro culture of villous trophoblast cells provides a suitable model for the study of the dynamics of production and secretion of placental hormones. Cytotrophoblasts isolated from term placental tissue and maintained in culture aggregate fuse to form large multinucleated syncytia by 72 h of plating. Of note is the fact that cytotrophoblasts fuse only with trophoblastic elements revealing a specificity to this process. The endocrine activity of these cells follows their morphological changes as previously observed [29, 30]. Along with the formation of syncytia the cells begin to elaborate hCG and progesterone. The production of both these hormones is very low during the initial 72 h of culture and increases significantly by 96 h, which are maintained for several days. It is interesting to note that these syncytial cells secrete both progesterone and hCG, whereas in other systems e.g. in corpus luteum, secretion of progesterone (local) is a consequence of gonadotropin (pituitary/placental) action. Whether any such regulatory mechanisms controlling the synthesis of progesterone occurs in the trophoblasts is not known [15–17, 31]. The biggest difficulty in studying such an inter-

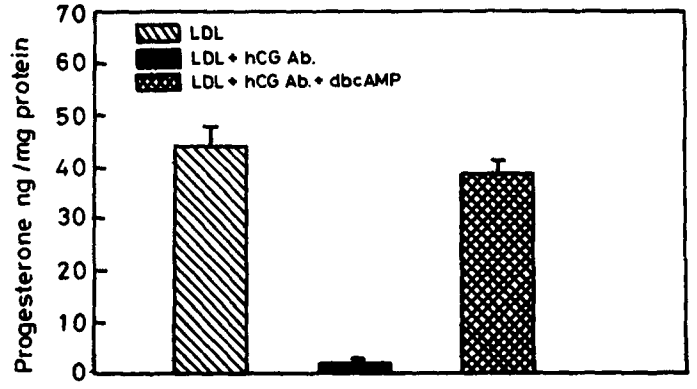

Fig. 4. Reversal of the inhibition caused by hCG ab on LDL induced progesterone secretion by dbcAMP. LDL (200 μg) was added to all the culture wells. Addition of hCG ab alone (■) resulted in almost nondetectable amounts of progesterone secretion. Further addition of dbcAMP (2.5 mM ■) restored the secretion of progesterone comparable to that obtained after stimulation of cells with LDL alone. The data are the mean values (±SEM) obtained using cells from six wells and are representative of 3 different experiments.

relationship is the spontaneous secretion of these two hormones by the trophoblast cells in culture. We have recently been able to overcome the problem by neutralizing the hCG secreted by the cells in culture using specific monoclonal antibodies. The trophoblast cells were unable to produce progesterone in the absence of hCG suggesting a possible autocrine regulation of the steroid synthesis by the gonadotropin [19]. In the present investigation efforts have been made to elucidate the mechanism of such an activation of steroid synthesis by hCG.

The findings of the present study indicate that the ligands which increase intracellular cAMP concentration, also stimulate the progesterone secretion. In the absence of hCG the cAMP analogue dbcAMP, the phosphodiesterase inhibitor MIX and adenylate cyclase activator forskolin, stimulated progesterone production by the trophoblast cells in culture. Since hCG has been shown to stimulate placental adenylate cyclase activity [14], its presence in the culture medium along with the ligands may further enhance progesterone secretion as evident from the dose–response curve of dbcAMP. Taken together these results are indicative of the positive effect of hCG on progesterone secretion. The stimulatory effect of cAMP on progesterone secretion by human trophoblast cells has been reported previously [10, 32–34]. Ringler *et al.* [35] have demonstrated an increase in hCG and progesterone secretion by 8-Br-cAMP in a dose dependent manner by human trophoblast cells in culture. The results from these studies and the observations of Moore *et al.* [36] that cAMP induces phosphorylation of several specific placental proteins suggest that placental progesterone secretion is coupled to the activation of the cAMP dependent protein kinase (PK) system. It therefore appears that the level of expression of adenylate cyclase activity which can be stimulated by hCG [14] may be a prerequisite for the progesterone synthesis. An additive effect of hCG and cAMP on progesterone secretion observed in our studies, suggests that apart from hCG, adenylate cyclase activity may be regulated by other factors also, which may include prolactin [11].

Stimulation of trophoblast cells by 8-Br-cAMP has been shown to result in an accumulation of the mRNA encoding adrenodoxin and cytochrome *P*450 SCC as well as subunits for α- and β-hCG [33–35, 37]. Our studies with 25-OHC as the precursor show a stimulatory effect of hCG on placental cytochrome *P*450 SCC. Also direct quantitation of *P*450 SCC in the absence of hCG indicated a significant decrease (Fig.3). Both these observations suggest a positive regulation of cytochrome *P*450 SCC by hCG in the trophoblast cells.

The principal source of cholesterol for placental progesterone synthesis appears to be the LDL-cholesterol [38,39]. Utilization of LDL by trophoblast cells involves binding of LDL to specific receptors on the cell surface [40]. Our studies indicate that the utilization of LDL for progesterone synthesis is an hCG dependent process, mediated probably through activation of cAMP. This follows from our observations that in the presence of hCG the progesterone secretion increased with increasing concentration of LDL but in the absence of hCG, the steroid secretion was completely blocked even though LDL was provided in the medium (Fig. 3). Addition of dbcAMP in the culture medium reversed the inhibitory effect of hCG antibodies on progesterone production to the levels obtained in the presence of hCG.

The inhibitory effect of progesterone on acyl coenzyme-A-cholesterol acyl transferase (ACAT) activity ensures against sequestration of cholesterol in an esterified form, thereby providing a continuous supply of cholesterol for progesterone biosynthesis [41]. Since hCG markedly stimulates the metabolism of cholesterol to pregnenolene through activation of cytochrome *P*450 SCC, the depletion of cellular cholesterol, therefore, could also be one of the major factors responsible for increased LDL receptor expression, update and metabolism.

In summary the data presented demonstrate a stimulatory effect of hCG on progesterone secretion in normal trophoblast cells involving multiple regulatory processes which may be at the level of adenylate cyclase activation, LDL uptake and regulation of the cyt*P*450 SCC enzyme. Since, the syncytiotrophoblasts secrete both hCG and progesterone, our data suggest a possible autocrine/paracrine interaction between these two hormones.

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