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ORIGINAL ARTICLE

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Regulation of progesterone production in human term trophoblasts in vitro by CRH, ACTH and cortisol (prednisolone)

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Abstract *Background*: In most mammals, onset of labor is accompanied with progesterone withdrawal. In humans, cortisol blockade of progesterone is a possible mechanism involved in the initiation of labor. Therefore, aim of the study was to clarify the effect of CRH, ACTH and cortisol (prednisolone) on the release of progesterone by term trophoblast cells in vitro. *Methods*: Cytotrophoblast cells were prepared from human term placentas by standard dispersion of villous tissue followed by a percoll gradient centrifugation step. Trophoblasts were incubated with CRH, ACTH as well as with prednisolone *Results*: The release of progesterone is decreased in CRH- and ACTH-treated trophoblast cell cultures compared to untreated trophoblast cells. Addition of prednisolone in varying concentrations leads to an increase of trophoblast progesterone production. *Conclusions*: The results suggest that CRH and ACTH directly modulate the endocrine function of trophoblasts in culture by downregulating progesterone production. Prednisolone on the other hand showed a stimulating effect on progesterone production in term trophoblast cells in vitro. Because blockade of progesterone is a possible mechanism involved in initiation of labor, we may speculate that CRH and ACTH are directly involved in the auto- or paracrine regulation of this procedure.

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Introduction

The human placenta produces large amounts of steroids during pregnancy (Pepe and Albrecht 1995). Cortisol, a δ4-C21 steroid, belongs to the group of glucocorticoid hormones (Anderson et al. 1978). Lacking enzyme P 450c17, the placenta is not capable of synthesizing cortisol from cholesterol, a δ5-C27 steroid and pregnenolone, an inactive δ5-C21 steroid. However, the placenta expresses P450scc (side chain cleaving enzyme), 3β hydroxysteroid dehydrogenase and 11β hydroxysteroid dehydrogenase (11βHSD) activity (McMullen et al. 2004) and therefore, the placenta is able to metabolize C19 steroids of maternal and fetal origin for estrogen and cortisol biosynthesis (Sun et al. 1998). Especially 11βHSD (McMillen et al. 2000) allows the conversion of cortisone to biologically active cortisol within trophoblasts cells (McMullen et al. 2004). Normally, cortisol homeostasis is regulated by a negative feedback system. Cortisol inhibits the release of ACTH from the anterior pituitary gland and the secretion of CRH by parvicellular neurons of the hypothalamus.

In a prospective, longitudinal cohort study of 485 pregnant women (McLean et al. 1995), CRH was introduced as a marker of a placental clock that determines the length of gestation and the timing of labor and delivery. Maternal plasma concentrations of CRH are dramatically increased during the third trimester of pregnancy and at term (Jeske et al. 1990). In maternal circulation the secreted CRH is bound by a circulating CRH-binding protein (CRHBP) (Karteris et al. 1998). During later stages of pregnancy, maternal plasma levels of CRHBP are reduced and therefore leading to an increase of biologically active CRH. Placental CRH, which is identical in immunoreactivity and bioactivity to hypothalamic CRH (Sasaki et al. 1988) has been

localized in the cytotrophoblast and syncytiotrophoblast (Riley et al. 1991). CRH-mRNA was detected by Northern blot analysis in cultured human cytotrophoblasts and syncytiotrophoblasts (Frim et al. 1988; Robinson et al. 1988). The progressive increase in maternal plasma CRH concentrations in human pregnancy is accompanied by a corresponding rise in the concentration of maternal ACTH. Because CRH stimulates ACTH secretion in a dose-dependent manner in trophoblast cells in vitro (Petraglia et al. 1987), it has been suggested that placental CRH exerts local paracrine actions on the production and secretion of placental ACTH. These results are in accordance with those of Rees et al. (1975) showing elevated ACTH levels during pregnancy. Plasma ACTH levels showed a progressive rise throughout the second and third trimester, which were autonomous and not subject of feedback control. Short-term stimulation experiments with ACTH (Nolten and Rueckert 1981), measured in seven women, in each trimester of pregnancy and 3 months post partum, showed an increase in the production of cortisol. Responsiveness of the cortisol production to stimulation with ACTH increased as pregnancy advanced. Concentration of plasma cortisol and corticosteroid binding globulin is elevated in pregnancy (Nolten et al. 1980, 1981). Cortisol concentrations rise in umbilical cord serum with gestational age in late pregnancy (Murphy 1982). The rise in the level of cortisol is steepest immediately before normal time of onset of labor and cannot be attributed to the stress associated with labor. Investigations of Herzc et al. (Hercz et al. 1987) indicated that between the twenty-eighth and fortieth week of pregnancy the cortisol concentration increased only in serum of the umbilical artery, but even at term the cortisol level of maternal vein blood was higher than of umbilical cord blood (Kohno et al. 1984a, Kohno et al. 1984b).

In nonprimate mammals, end of pregnancy is associated with a fall in maternal progesterone concentration, which contributes to the initiation of labor (Karalis et al. 1996). In the human placenta, cortisol is able to compete with the action of progesterone in CRH regulation.

Cortisol blockade of progesterone is a possible molecular mechanism involved in initiation of labor. Because there exist no studies about the influence of CRH, ACTH and prednisolone on progesterone production in term trophoblasts, aim of the present study was to investigate the progesterone production in isolated term trophoblast cells after stimulation with CRH, ACTH or prednisolone and in unstimulated controls.

Materials and methods

Culture of trophoblasts

Cytotrophoblast cells were isolated from three different term human placentas following planned cesarean section and were processed according to Kliman et al. (1986) with slight modifications (Jeschke et al. 2003, 2004). Briefly, villous tissue was minced and transferred to a trypsin/DNAse I mixture (142.4 mg trypsin and 5 mg DNAse I, Sigma-Aldrich, Taufkirchen, Germany). Remaining tissue fragments were digested twice in trypsin/DNAse I solution (71,2 mg trypsin and 5 mg DNAse I, Sigma-Aldrich). Cell suspensions obtained by the three digestion steps were pooled and loaded on a preformed Percoll (Amersham Biosciences) gradient. Density zone containing trophoblast cells was removed.

Treatment of trophoblast cultures with CRH, ACTH and prednisolone

Percoll-gradient purified trophoblasts were adjusted to a cell concentration of 1×10^6 cells/ml in DEMEM culture medium supplemented with 10% fetal calf serum and gentamicin (200μg/ml). An amount of 5 ml of the throphoblast cell suspension were incubated in cell culture flasks in humidified 5% CO_2–95% air at 37°C. Stimulation experiments were performed in the presence of CRH (Bachem, Weil am Rhein, Germany, 500 ng/ml), ACTH (Bachem, 500 ng/ml) or prednisolone (Solu-Decortin H 50, Merck, Darmstadt, Germany, 1 μg/ml, 10 μg/ml and 100 μg/ml). Untreated cell cultures were used as controls. The effect of CRH, ACTH and prednisolone on the secretion of progesterone was studied by comparing the kinetics of progesterone release of untreated (controls) and CRH, ACTH and prednisolone stimulated trophoblast cell cultures. At designated times fresh medium and stimulating substances were added. At 30 min after stimulation aliquots of the culture media were removed and frozen at −20°C. Cells were cultured for up to 120 hours.

Identification of secreted hormones

The analyzer SR1 from BioChemImmunoSystems GmbH determined the secretion of progesterone. Samples were diluted 1:3 with hormone-free diluents because of the intensively colored culture medium. Sensitivity and the intra-assay coefficient of variation of progesterone EIA (<6.4 nmol/l and 6.8% at a medium value of 11.5 nmol/l; 5.4% at 65.6 nmol/l, respectively) demonstrate the accuracy of the applied methods.

Statistical analysis

The Wilcoxon′s signed rank tests for paired samples was used for comparison of the means. Statistical computerization of the analysis used the SPSS software package (SPSS, Chigaco, IL, USA). $P<0.05$ was considered statistically significant.

Results

Characterization of isolated trophoblast cells

Trophoblast identity of the isolated cells was confirmed by their ability to produce specific hormones (hCG, hPL and progesterone) and by immunocytochemistry and incubation with 5 μg/ml of an anti-cytokeratin antibody (anti-cytokeratin AE1/AE3, Roche, Mannheim, Germany) followed by HRP-conjugated secondary antibody (Rabbit anti Mouse IgG HRP-conjugated, and staining with chromagen 3,3'-diaminobenzidine (DAB; Dako, Glostrup, Denmark) (Fig. 1). Viability of trophoblast cells was tested by using trypan blue exclusion assay (Sigma-Aldrich, Munich, Germany).

Inhibition of progesterone production by CRH and ACTH

Analysis of progesterone in stimulated and unstimulated cell cultures revealed that CRH and ACTH stimulated trophoblast cells show a lower secretion of progesterone compared to unstimulated cells over a cultivation period of 120 h (Fig. 2). There was a significant difference between the progesterone production of stimulated and unstimulated cell cultures ($P < 0.02$ for CRH and $P < 0.007$ for ACTH). Although the inhibitory effect of ACTH is larger compared to CRH (reduction to 77.7% progesterone production by ACTH and to 81.8% for CRH compared to unstimulated controls) there are no significant differences ($P > 0.23$) between both substances.

Stimulation of progesterone production by prednisolone

To test the effect of prednisolone on progesterone secretion of trophoblast cells in vitro, we stimulated trophoblast cells with increasing prednisolone concentrations (1–100 μg/ml) (Fig. 3). There was a significant difference between the progesterone production of stimulated and unstimulated cell cultures ($P < 0.003$ for 1 μg prednisolone, $P < 0.004$ for 10 μg prednisolone and $P < 0.002$ for 100 μg prednisolone). The stimulation effect of prednisolone is not dose dependent.

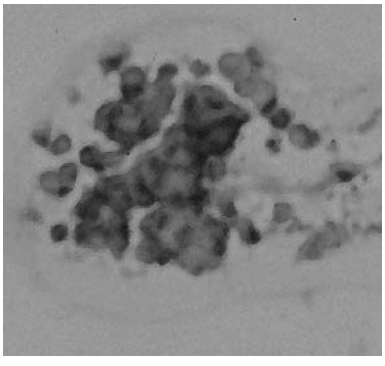


Fig. 1 Isolated trophoblast cell express cytokeratin (AE1/AE3, Roche, Mannheim, Germany), visualized with 3,3'-diaminobenzidine (DAB) (brown-staining, 10x lens)

Discussion

In this study, we demonstrated that CRH and ACTH inhibited secretion of progesterone in human trophoblast cells. In a recent study, we showed that both CRH and ACTH also stimulated cortisol production in term trophoblast cells in vitro (Hocker et al. 2003). In addition, we showed that cortisol (prednisolone) is able to stimulate progesterone production in trophoblast cells. Although the human placenta lacks the enzyme P459 17–20, the cortisol output is increased markedly toward term. The placenta expresses P450 side chain cleavage and 3β hydroxysteroid dehydrogenase activity (Arcuri et al. 1999). Particularly within trophoblast cells, 11βHSD (Driver et al. 2001) allows the conversion of biologically inactive cortisone to biologically active cortisol. On the other hand, 11βHSD also regulates the amount of maternal cortisol that crosses the placenta to reach the fetal compartment. The increase in 11β-HSD causes a change in transplacental corticosteroid metabolism, which results in activation of the HPAA in the fetus. As a result of this cascade of events, there is an increase in the expression of pituitary POMC/ACTH and key enzymes, e.g. 3β-HSD and P-450 17alpha-hydroxylase, important for de novo cortisol formation by, and consequently maturation of, the fetal adrenal gland. In turn, cortisol has well-defined actions on surfactant biosynthesis and consequently fetal lung maturation, as well as effects on placental CRH/POMC release, which may be important to the initiation of labor (Pepe and Albrecht 1995). CRH plays a major role in reproductive physiology and is found in several intrauterine sites, including the syncytiotrophoblast. It has been suggested that there is a placental clock that determines the length of gestation (Challis 1995; Inder et al. 2001), and the maternal plasma level is an indicator in this event. Altered cortisol dynamics have been reported during gestation as well. Goland et al. (1992) showed the relationship between elevated maternal plasma CRH-concentration and elevated cortisol concentration during pergnancy. The hypothalamic neuropeptide corticotropin-releasing hormone (CRH) is produced in several organs of the female reproductive system including the endometrial glands, decidualized stroma, trophoblast, syncytiotrophoblast and placental decidua (Makrigiannakis et al. 1995a, 1995b, 1997, 2001). The biological role of intrauterine CRH is not fully understood. However, it has been suggested that CRH might participate in local immune phenomena associated with embryo implantation (Makrigiannakis et al. 2003a, b, 2004a, b).

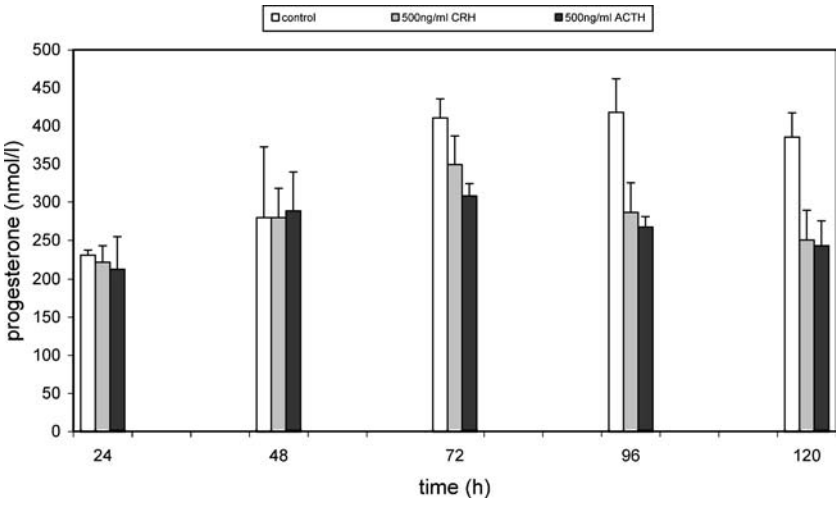
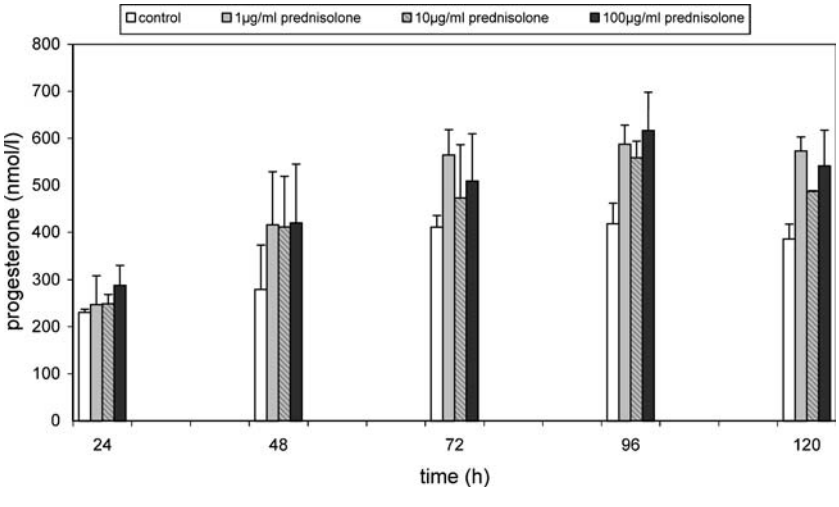


Fig. 2 Effect of CRH and ACTH on progesterone production of trophoblast cells in vitro. Data (mean ± SD) represent progesterone concentration in aliquots of the culture media at designated times, $n = 3$ for every group. Differences between the controls and stimulated cultures are significant for cultures stimulated with CRH and ACTH ($P < 0.02$ and 0.005)

Steroid hormones of placental and fetal adrenal origin have important roles in regulating key physiological events essential to the maintenance of pregnancy and development of the fetus for extrauterine life. Progesterone has suppressive actions on lymphocyte proliferation and activity and on the immune system to prevent rejection of the developing fetus and placenta (Stites and Siiteri 1983). Progesterone also suppresses the calcium–calmodulin–MLCK system and thus activity of uterine smooth muscle, thereby promoting myometrial quiescence to ensure the maintenance of pregnancy (Pepe and Albrecht 1995).

In summary, we have shown that ACTH and CRH inhibited the progesterone production of trophoblast cells and in addition prednisolone upregulates progesterone production. Stimulation of progesterone by prednisolone may be due to a different mechanism than the positive CRH–ACTH–cortisol feedback in placenta. Prednisolone upregulates CRH and ACTH which should result in downregulation of progesterone (Hocker et al. 2003). Our results show the opposite. Prednisolone stimulated progesterone. There may be exist additional stimulation mechanism than CRH–ACTH–cortisol axes. In a recent study we found that prednisolone stimulated hCG synthesis in term trophoblasts in vitro (Hocker et al. 2004). Because hCG also stimulates progesterone, this effect could reverse the CRH and ACTH stimulating effect of prednisolone. It is already known that placental CRH determines the length of gestation and the timing of labor and delivery by a ''placental clock'', which is active from an early stage in human pregnancy. The present study shows that CRH and ACTH are directly involved in progesterone regulation in trophoblast cells. Because blockade of proges-

Fig. 3 Effect of prednisolone on progesterone production of trophoblast cells in vitro. Data (mean ± SD) represent progesterone concentration in aliquots of the culture media at designated times, $n = 3$ for every group. Differences between the controls and stimulated cultures are significant for cultures stimulated with prednisolone ($P < 0.003$, 0.004 and 0.002)



terone is a possible mechanism involved in initiation of labor, we may speculate that CRH and ACTH are directly involved in the auto- or paracrine regulation of this procedure. But further investigations are necessary on the precise mechanism of progesterone stimulation/inhibition by different glucocorticoids.

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