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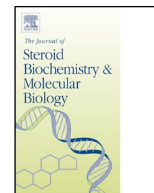

A time-course regulatory and kinetic expression study of steroid metabolizing enzymes by calcitriol in primary cultured human placental cells

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ABSTRACT

1,25-dihydroxivitamin D_3 (calcitriol), is a secoesteroid involved in several placental functions. In particular, we and others showed that calcitriol regulates peptides, proteins, cytokines and hormones production in human trophoblastic cells. On the other hand, calcitriol modifies the activity and expression of some steroidogenic enzymes, a process that is considered tissue-specific. However, the effects of calcitriol on the expression of enzymes involved in the synthesis of sex steroids in placental tissue have not yet been entirely studied. The aim of the present study was to investigate the effects of calcitriol upon gene expression of several steroid enzymes such as cytochrome P450scc (*CYP11A1*), type 1 3β-hydroxysteroid dehydrogenase(*3β-HSDI*), *17β-HSD3*, 17α-hydroxylase/17,20 lyase (*CYP17A1*) and aromatase (*CYP19A1*) in primary cultures of human placental cells. Cell cultures were performed using placentas obtained immediately after delivery by caesarean section from normotensive healthy women and calcitriol effects were evaluated, at level of transcription, by qPCR. The results showed that: 1) from basal expression values of the five genes studied, 3β-HSDI was the most expressed gene ($P < 0.05$); 2) basal expression of all enzymes was significantly higher in cultured syncytiotrophoblast than in cytotrophoblasts ($P < 0.05$); 3) the presence of calcitriol in cultured trophoblast cells generally resulted in a stimulatory effect of *CYP11A1*, *CYP19A1* and *17β-HSD3* gene expression at 3 h of treatment whereas *3β-HSDI* was induced at 6 h ($P < 0.05$). However, a time-dependent variable was also observed; 4) protein expression of CYP11A1 and 3β-HSDI were not modified significantly by calcitriol, however that of CYP19A1 was regulated in similar fashion as gene expression. In conclusion, calcitriol affected in a time-dependent manner the expression of steroids metabolizing enzymes in human placental cell cultures.
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1. Introduction

Calcitriol, is a secosteroid with both calcemic and non-calcemic biological activities [1]. Similar to steroids hormones, calcitriol is metabolized by human placenta, a tissue that is also considered a target for its hormonal actions. Indeed, this hormone regulates the expression or production of calbindins, cytokines, antimicrobial peptides as well as human chorionic gonadotropin (hCG), progesterone (P_4) and estradiol (E_2) in enriched trophoblastic cell preparations (ETC) [2–5]. In addition, it is well known that calcitriol differentially regulates CYP27B1 and CYP24A1, the two cytochromes involved in calcitriol synthesis and metabolism [6]. Likewise, its role in the regulation of other steroidogenic enzymes in a tissue-specific manner has been also reported [7].

Steroid hormones are synthetized from a common precursor called cholesterol and the steroidogenic enzymes involved are members of both the cytochrome (CYP) P450 superfamily and hydroxysteroid dehydrogenases (HSD) [8,9]. During pregnancy, the fluctuations of maternal steroids depend mainly of the placenta, and the major pathways involved in their synthesis have been well established [8]. In human placenta, after the conversion of

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cholesterol to pregnenolone by cytochrome P450 cholesterol side chain cleavage (P450scc; CYP11A1), pregnenolone is bioconverted mainly to P_4 by means of the type 1 3β-hydroxysteroid dehydrogenase (3β-HSDI). On the other hand, it well known that androgens from fetal tissues are necessary to produce the corresponding estrogens in the placenta means aromatase enzyme (CYP19A1). However, it has been showed *in vitro* the placental capability to express and synthetized androgens *de novo* by 17α-hydroxylase/17,20 lyase (CYP17A1) presence, a topic that is still discussed controversially [10,11].

Steroidogenic enzymes regulation is very complex [8,12], and the identification of factors regulating their expression has long been sought. At this respect, it has been demonstrated that some enzymes are target of the biological effects of calcitriol. In fact, it has been showed that calcitriol increased the 3β-HSDI activity in granulosa cells [13] and induces *3β-HSDI* and *CYP11A1* transcription in human glioma GI-1 cells [14]. In addition, it has been demonstrated that calcitriol up regulates *17β-HSD* type 2, type 4 and type 5 gene transcription in human prostate cancer lines cells, and both *CYP11A1* and *CYP17A1* in human adrenocortical NCI-H295R cells [15–17]. On the other hand, it has been shown that calcitriol stimulates aromatase activity in diverse cell types such as prostate, osteoblasts, human choriocarcinoma, purified immature rat Sertoli and rat granulosa cells [18–21]. Interestingly, Kinuta et al., [22] have showed that the activity and gene expression of *Cyp19A1* are decreased in the ovary, testis and epididymis of VDR null mutant mice, which indicates that calcitriol is an additional stimulator of aromatase. However, the effects of calcitriol on aromatase gene expression and enzyme activity are controversial, since is mainly inhibitory in breast cancer cells and human macrophages [7,23]. These findings indicate the differential tissue-specific effects of calcitriol.

We have previously shown that calcitriol induce P_4 and E_2 secretion in ETC [2]; however, its effects on the enzymes involved in this process have not been studied. Therefore, the aim of this study was to investigate the effects of calcitriol on expression of steroidogenic enzymes in primary cultures of human placental cells.

2. Materials and methods

2.1. Reagents

Culture media, fetal bovine serum (FBS), Trizol and all oligonucleotides for real time polymerase chain reaction (PCR) were by Invitrogen (CA, USA). Light Cycler® 480 probes master kit, TaqMan Master reaction, TaqMan probes, 96 well PCR microplates, the reverse transcription (RT) system and Proteases inhibitor cocktail were from Roche (Roche Applied Science and Roche Diagnostics, IN, USA). Calcitriol (1α,25-dihydroxycholecalciferol) was kindly donated from Hoffmann-La Roche Ltd (Basel, Switzerland). Deoxyribonuclease I and trypsin enzymes from bovine pancreas used for cell cultures were from Sigma-Aldrich (MO, USA). Percoll was from GE Healthcare (Uppsala, Sweden). CYP11A1 and CYP19A1 antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA) whereas CYP17A1 and 3β-HSDI antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and GAPDH antibody was from Millipore (Temecula, CA, USA).

2.2. Trophoblast cell culture

This protocol was approved by the Human Research Ethics Committee from the Instituto Nacional de Ciencias Médicas y Nutrición, Salvador Zubirán (No. BRE-535-12/15-1) and the Internal Review Board of the Instituto Nacional de Perinatología "Isidro Espinosa de los Reyes" in Mexico City (No. 212250-21131). Written informed consent was obtained from all participants. All pregnant women were from an urban area of Mexico City, 18–39 years old, previously normotensive, with no history of diabetes mellitus or thyroid, liver, renal disease. Term placentae (39–41 weeks of gestation) were acquired following caesarean section. All women had uncomplicated pregnancies, without evidence of active labor, cervical dilation or loss of the mucus plug. In addition, none had any clinical or microbiological signs of chorioamnionitis or lower genital tract infection; twin pregnancy was excluded from this study.

Placental cotyledons were dissected free of decidua and fetal membranes. The ETC were cultured as previously described [6,19]. Briefly: Villous tissue was enzymatically dispersed and cells were separated on density percoll gradients. Before plating, the viability of the percoll-enriched cells was estimated by dye exclusion (0.4% trypan-blue). Cells were plated at a density of 3×10^6 cells in flasks of 25 cm^2 with 4 mL each of supplemented medium [(DMEM HG) 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 mg/ml Fungizone], containing 10% heat-inactivated-FBS. Incubations were performed in humidified 5% CO_2-95% air at 37 °C. In order to remove all non-adherent cells, after 3 h to incubation, culture medium was aspirated and trophoblasts cells were washing with Hank's Balanced Salt Solution (HBSS) 1X. Treatments with calcitriol (1 or 100 nM) or its vehicle (ethanol 0.1%) were added in supplemented medium F-12 with penicillin and streptomycin. Afterward, RNA was extracted from cells for gene expression studies. Expression of human chorionic gonadotropin (hCGβ5) was used as control of the cell culture system and CYP24A1 gene as control of calcitriol function.

2.3. Calcitriol effects on CYP11A1, CYP17A1, CYP19A1, 3β-HSDI and 17β-HSD3 gene expression

Calcitriol effects upon gene expression were studied by extracting total RNA from treated cells using Trizol reagent [24]. In all cases, the amount and quality of RNA were estimated spectrophotometrically at 260/280 nm and a constant amount of RNA (2 μg) was reverse transcribed using a RT assay. Primers and probes for PCR amplifications are shown in Table 1. Identical PCR conditions were performed for all genes and in all cases normalized against glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) used as housekeeping gene.

Real time PCR amplifications were carried on a LightCycler® 480 II (Roche), as described below: The specific probe for each gene was added to a reaction mixture with 5.2 μL of PCR grade water, 0.1 μL of primer corresponding to the gene of interest [20 μM] and 3.5 μL of 2X enzyme. The mixture was homogenized and placed in plates of 96 wells to PCR. In each well 9 μL of the mixture and 1 μL cDNA are placed. The plate was then sealed and centrifuged at 1000 rpm for 30 s. Finally, the plate was placed in the thermocycler LightCycler480 II, where occurred activation of Taq DNA polymerase and DNA denaturation at 95 °C for 10 min, proceeded by 45 amplification cycles of 10 s at 95 °C, 30 s at 60 °C, and 1 s at 72 °C.

2.4. Calcitriol effects upon CYP11A1, CYP17A1, CYP19A1 and 3β-HSDI protein expression

In order to evaluate the calcitriol effects upon CYP11A1, CYP17A1, CYP19A1 *and* 3β-HSDI protein expression these were studied by Western blot. Briefly: Syncytiotrophoblast were incubated in the absence or presence of calcitriol 100 nM during 6 and 24 h. Afterwards cells were pelleted and lysed with RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mMNaCl, 1% Nonidet P-40, 0.1% SDS, pH 7.4) in presence of a proteases inhibitor cocktail. Protein content

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