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Regulation of CYP27B1 and CYP24A1 gene expression by recombinant pro-inflammatory cytokines in cultured human trophoblasts

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ABSTRACT

Placenta is an important source of endocrine and immunological factors. During pregnancy, calcitriol, the active metabolite of vitamin D, is also metabolized by decidua and placental tissue by means of CYP27B1 and CYP24A1 for synthesis and inactivation of calcitriol respectively. Calcitriol production is regulated by several factors in a tissue-specific manner. However, the association of pro-inflammatory cytokines on calcitriol metabolism has not been studied in human placenta. The aim of the present study was to investigate the effects of TNF-α, INF-γ, IL-6 and IL-1β upon CYP27B1 and CYP24A1 gene expression in primary cultures of human placental cells. Placentas were obtained immediately after delivery by cesarean section from normotensive women. Cytokine effects upon mRNA of CYPs in enriched trophoblastic cell preparations were evaluated by using qPCR. The results showed that incubation of trophoblasts in the presence of each cytokine resulted in a significant increase of both CYPs expression. Interestingly, TNF-α increased significantly the ratio of CYP24A1/CYP27B1 gene expression, while IFN-γ preferentially induced CYP27B1, whereas IL-1β and IL-6 stimulated gene expression of both CYPs in the same proportion. The results suggest that cytokines among other factors regulate calcitriol metabolism in human placenta; specifically, INF-γ may contribute to calcitriol production while TNF-α favors its catabolism.

1. Introduction

In addition to calcium homeostasis, the secosteroid 1α,25-dihydroxyvitamin D_3 (calcitriol) is involved in cell proliferation and differentiation as well as in the regulation of the endocrine and immune systems [1]. The kidney is the main source of calcitriol synthesis; however, there are extra-renal sites of calcitriol production such as the placenta among others [2–4]. In this organ, calcitriol acts in an autocrine/paracrine fashion. In cultured human trophoblast cells, we have shown that calcitriol affects gene and/or protein expression of human chorionic gonadotropin (hCG), calbindins, cytokines as well as the sex steroid hormones production [5–10]. Likewise, other groups have reported that calcitriol regulates human placental lactogen (hPL) and HOXA10 in trophoblast and endometrium, respectively [11,12]. These results highlight the important role of calcitriol on several physiological conditions including pregnancy.

Cytokines are small proteins secreted by immune cells and others including placental trophoblast cells [13,14]. During pregnancy, cytokines act in concert at the materno-placental interface balancing immunological tolerance. Indeed, both anti- and pro-inflammatory cytokines participate in several important events such as trophoblastic invasion, hormonal production and delivery process [15–19]. However, exacerbated production of pro-inflammatory cytokines as well as vitamin D deficiency are associated with miscarriage, preterm labor and preeclampsia [20,21].

Therefore, the aim of this study was to investigate the effects of several pro-inflammatory cytokines upon the gene expression of the enzymes involved in vitamin D metabolism: the 25-hydroxyvitamin D_3 [25-OHD_3] 1α-hydroxylase (CYP27B1) and the 25-OHD_3-24-hydroxylase (CYP24A1) involved in the synthesis and catabolic pathways of calcitriol, respectively.

Abbreviations: Calcitriol, 1α,25-dihydroxyvitamin D_3; CYP27B1, 25-hydroxyvitamin D_3 [25-OHD_3] 1α-hydroxylase; CYP24A1, 25-OHD_3-24-hydroxylase; ETC, enriched trophoblastic cell preparations; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; hCG, human chorionic gonadotropin; hPL, human placental lactogen; IL, interleukin; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha.

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Table 1
Oligonucleotides and probes used for qPCR analysis.

Gene bank/accession number	Upper primer	Lower primer	Amplicon (nt)	[a]Probe number
CYP27B1/NM_000785.2	CTT GCG GAC TGC TCA CTG	CGC AGA CTA CGT TGT TCA GG	123	63
CYP24A1/NM_000782.3	CAT CAT GGC CAT CAA AAC AA	GCA GCT CGA CTG GAG TGA C	65	88
TNF-α/M10988.1	CAG CCT CTT CTC CTT CCT GA	GCC AGA GGG CTG ATT AGA GA	123	29
IFNγ/NM_000619.2	GGC ATT TTG AAG AAT TGG AAA	TTG GAT GCT CTG GTC ATC TTT	111	21
hCGβ5/NM_000737.2	GCT CAC CCC AGC ATC CTA T	CAG CAG CAA CAG CAGCAG	131	79
GAPDH/AF261085.1	AGC CAC ATC GCT GAG ACA C	GCC CAA TAC GAC CAA ATC C	66	60

[a] From the universal probe library (Roche). nt = nucleotides.

2. Materials and methods

2.1. Reagents

Culture media, fetal bovine serum (FBS) and Trizol were from Invitrogen (CA, USA). TaqMan Master reaction, TaqMan probes, capillaries and the reverse transcription (RT) system were from Roche (Roche Applied Science, IN, USA), calcitriol (1α,25-dihydroxycholecalciferol) was kindly donated from Hoffmann-La Roche Ltd. (Basel, Switzerland). Recombinant TNF-α, IL-6 and IL-1β were from R&D Systems (MN, USA). Recombinant IFN-γ was from eBioscience (CA, USA). 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). Immunoassays for cytokines were from R&D systems Inc. (MN, USA).

2.2. Trophoblast cell culture

This protocol was approved by the Human Ethical Committee of 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 Perinatolgía "Isidro Espinosa de los Reyes" in Mexico City (No. 212250-21131). Written informed consent was obtained from all participants. All women were from the urban area of Mexico City, 23–34 years old, previously normotensive, with no history of diabetes mellitus, thyroid, liver, or chronic renal disease. Term placentae (37–41 weeks of gestation) were acquired following cesarean section. All women had uncomplicated pregnancies, with no evidence of active labor, cervical dilation or loss of the mucus plug. In addition, none had any clinical or microbiological signs of chorioamnionitis or of lower genital tract infection; multi-fetal pregnancies were excluded from this study.

Fetal cotyledons were dissected free of decidua and fetal membranes. Enriched trophoblastic cell preparations (ETC) were cultured as previously described [9,22]. 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 to 95% air at 37 °C. In order to remove all non-adherent cells, after allowing cells to attach to the surface of the flasks and before of treatments the cell culture media was aspirated and cells were washed twice with Hank's Balanced Salt Solution (HBSS). ETCs attached overnight were incubated in supplemented medium conditioned with charcoal-stripped 10% heat-inactivated-FBS in the presence of recombinant TNF-α, IFN-γ, IL-6 and IL-1β at different concentrations during 24 h. Total RNA was extracted using Trizol reagent [23]. The purity and yield of the RNA were determined spectrophotometrically and a constant amount of RNA (1 μg) was reverse transcribed using the transcriptor RT system. Extracted RNA was used for the study of hCG, TNF-α, IFN-γ and CYPs gene expression using qPCR. In addition, basal secretion of TNF-α and IFN-γ was determined by ELISA.

2.3. Cytokines effects on CYP27B1 and CYP24A1 gene expression

Real-time PCR was performed using the LightCycler® 2.0 (Roche), according to the following protocol: activation of Taq DNA polymerase and DNA denaturation at 95 °C for 10 min, proceeded by 45 amplification cycles consisting of 10 s at 95 °C, 30 s at 60 °C, and 1 s at 72 °C. In each case, identical PCR conditions were performed using 1 μl of cDNA, and results are presented as fold change of the gene of interest against the housekeeping gene encoding for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers and probes for PCR amplifications were designed by the *Universal Probe Library Assay Design Center* from Roche and respective sequences are listed in Table 1.

2.4. Quantification of TNF-α and IFN-γ secretion by ELISA

Quantification of cytokines was accomplished by ELISA following manufacturer's guidelines. The final cytokine concentration was calculated based on a standard curve constructed using recombinant cytokine standards. The detection limits of the assays were 15.62–1000 pg/ml (TNF-α and IFN-γ). According to the manufacturer, there is no significant cross-reactivity or interference by other cytokines in these assays.

2.5. Statistical analysis

Results were expressed as the mean ± standard deviation. One-way ANOVA was performed to assess statistical significance. Values of $P \leq 0.05$ were considered significant. Experiments were performed in triplicates from at least three separated cell cultures, each from different placentas.

3. Results

ETC were seeded (3×10^6 cells per flask) and after three days of culture the mononuclear trophoblasts aggregated and formed syncytia. This process was accompanied by a progressive increase in hCG gene expression, which was used as a biochemical marker for placental endocrine phenotype differentiation as described previously [10]. In addition, the well-known effects of calcitriol upon gene expression of CYP27B1 and CYP24A1 were used as controls of our cell culture system. As expected (data not shown) and as previously described [22,24], cAMP significantly increased hCG secretion in the culture media and calcitriol significantly stimulated and decreased CYP24A1 and CYP27B1 gene expression, respectively.

3.1. Cytokines effects upon CYP27B1 and CYP24A1 gene expression

In order to study the regulation of cytokines upon CYPs gene expression, we analyzed the effects of recombinant TNF-α, IFN-γ, IL-6 and IL-1β upon CYP27B1 and CYP24A1 gene expression in

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