Vitamin D Regulates Steroidogenesis and Insulin-like Growth Factor Binding Protein-1 (IGFBP-1) Production in Human Ovarian Cells

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Abstract

Vitamin D Receptor (VDR) is expressed in both animal and human ovarian tissue, however, the role of vitamin D in human ovarian steroidogenesis is unknown. Cultured human ovarian cells were incubated in tissue culture medium supplemented with appropriate substrates, with or without 50 pM–150 pM or 50 nM – 150 nM of 1,25-$(OH)_2D_3$, and in the presence or absence of insulin. Progesterone, testosterone, estrone, estradiol, and IGFBP-1 concentrations in conditioned tissue culture medium were measured. Vitamin D receptor was present in human ovarian cells. 1,25-$(OH)_2D_3$ stimulated progesterone production by 13% ($p<0.001$), estradiol production by 9% ($p<0.02$), and estrone production by 21% ($p<0.002$). Insulin and 1,25-$(OH)_2D_3$ acted synergistically to increase estradiol production by 60% ($p<0.005$). 1,25-$(OH)_2D_3$ alone stimulated IGFBP-1 production by 24% ($p<0.001$), however, in the presence of insulin, 1,25-$(OH)_2D_3$ enhanced insulin-induced inhibition of IGFBP-1 production by 13% ($p<0.009$). Vitamin D stimulates ovarian steroidogenesis and IGFBP-1 production in human ovarian cells likely acting via vitamin D receptor. Insulin and vitamin D synergistically stimulate estradiol production. Vitamin D also enhances inhibitory effect of insulin on IGFBP-1 production.

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Introduction

1,25-$(OH)_2$ vitamin D is the active form of vitamin D. Although its primary role is to regulate calcium and phosphorus metabolism, recently many other actions of vitamin D have been identified. Effects of vitamin D are mediated through vitamin D receptor, a member of nuclear receptor superfamily of ligand-activated transcription factors. Presence of vitamin D receptor in animal ovaries and its role in female reproduction in mice, rats, and Chinese hamster are documented in several studies [1–5].

Although presence of vitamin D receptor in human ovary has been reported in one study [6], this finding has not been confirmed and the role of vitamin D, if any, in human ovarian function is not known. Previous studies have demonstrated that insulin upregulates steroidogenesis and inhibits IGFBP-1 production in granulosa cells. However, the interactions between vitamin D and insulin in the ovary have not been explored.

The primary goals of this study were to confirm the presence of vitamin D receptor in human ovarian cells and to examine the effects of vitamin D on regulation of steroid hormone and IGFBP-1 production in human ovarian cell systems. We also examined whether Vitamin D and insulin interact in regulation of ovarian steroidogenesis and IGFBP-1 production. The institutional review boards at Beth Israel Medical Center and at the Weill Medical College of Cornell University approved all studies described in this report.

Methods

Cell culture systems

Granulosa cells were obtained during in vitro fertilization over the course of 24 months and were pooled from several patients at a time to ensure adequate cell number for the experiments. The diagnoses included male factor, tubal factor and uterine factor infertility, endometriosis, and anovulation. For mixed ovarian cell culture containing granulosa, stromal and thecal cells, ovarian tissue samples were collected from 26 women at the time of indicated oophorectomy. The patients'

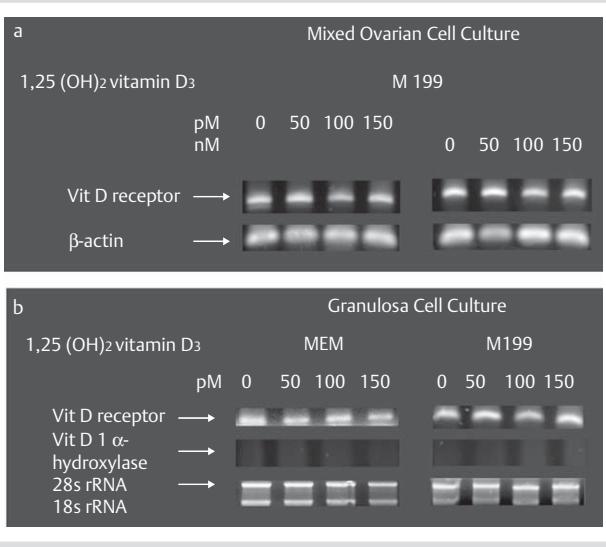

Fig. 1 Effects of 1,25-$(OH)_2D_3$ on vitamin D receptor mRNA expression in mixed ovarian cell culture and in granulosa cell culture. **a**: Representative RT-PCR analysis of vitamin D receptor expression and β-actin expression in mixed ovarian cell culture in conditioned M199 tissue culture medium and in the presence of different concentrations of 1,25-$(OH)_2D_3$. **b**: Representative RT-PCR analysis of vitamin D receptor mRNA, 28 s rRNA, 18 s rRNA, and vitamin D 1-α-hydroxylase mRNA expression in MEM Eagle medium or M199 medium in the presence of different concentrations of 1,25-$(OH)_2D_3$.

age ranged from 27–50 years. Both culture systems are described in details in our previous publications [7,8].

Both mixed ovarian cells and granulosa cells were incubated in M199 or MEM Eagle tissue culture medium supplemented with Fetal Bovine Serum (FBS) and steroid hormone substrates (30 µM pregnenolone, or 30 µM dehydroepiandrosterone, or 3 µM testosterone, or 3 µM androstenedione) with or without 1,25-$(OH)_2D_3$ (50 pM–150 pM or 50 nM–150 nM) and with or without insulin (10, 10^2, 10^3 ng/ml) [7,8]. We used the active form of vitamin D, 1,25-$(OH)_2D_3$, to examine its effects on the steroid hormone and IGFBP-1 production in M199 tissue culture medium that contains vitamin D_2, since we confirmed that in our ovarian cell cultures, 1-α-hydroxylase expression was absent (**Fig. 1**).

Steroid hormone and IGFBP-1 production

During in vitro fertilization granulosa cells are exposed to high concentrations of gonadotropins and become highly luteinized. As a result, progesterone production in these cells is maximized and becomes supraphysiological. Therefore, progesterone experiments were carried out in mixed ovarian culture, which contains nonluteinized granulosa cells. Testosterone is produced in thecal and stromal cells, but not in granulosa cells. Hence, testosterone studies were carried out in mixed ovarian cell cultures. Estrogen is primarily synthesized in granulosa cells, which abundantly expresses aromatase enzyme that converts androgens to estrogen. Thus, estrogen studies were carried out in granulosa cells.

Progesterone, testosterone and estradiol concentrations in the tissue culture medium were measured using radioimmunoassay (RIA). Estrone was measured using Enzyme Linked Immuno-Sorbent Assay (ELISA).

IGFBP-1 concentration in tissue culture medium was measured using ELISA. Detectable amounts of IGFBP-1 were produced by the granulosa cells obtained during IVF. In our preliminary experiments, IGFBP-1 production in mixed ovarian cell culture was close to the lower limit of detection sensitivity in the available assay and the results, therefore, were highly variable. For this reason the IGFBP-1 studies were conducted in the granulosa cell cultures. Under our experimental conditions, IGF-I concentration in the conditioned tissue culture medium was negligible as all the experiments were carried out in IGF-I free media, with 0.1 % FBS being the only source of IGF-I.

Total RNA isolation and RT-PCR

Mixed ovarian cells or granulosa cells were incubated either in medium M199 or in vitamin D free MEM Eagle medium for total RNA isolation experiments. The total RNA was isolated using RNAqueous-4PCR kit according to the manufacturer's recommendations (Ambion Inc., Austin, Texas, USA). The concentration of RNA was determined by reading the absorbance in spectrophotometer at 260 and 280 nm.

GeneAmp EZ rTth RNA PCR kit was used for RT-PCR reaction. Thermostable recombinant DNA polymerase was used as both a reverse transcriptase and as a DNA polymerase in a single PCR reaction. The vitamin D receptor forward primer was 5′-GCC CAC CAT AAG ACC TAC GA-3′ and the reverse primer was 5′-AGA TTG GAG AAG CTG GAC GA-3′. β-Actin, house protein was used to demonstrate the relative abundance of mRNA under each set of conditions in mixed ovarian cell culture. β-Actin forward primer was 5′-AAA CTG GAA CGG TGA AGG TG-3′ and reverse primer was 5′-AGA GAA GTG GGG TGG TTT T-3′. Because the granulosa cells are specialized cells, the expression of β-actin in these cells is not as abundant as in other types of ovarian cells. Therefore, in granulosa cell culture equal amount of total RNA was used to analyze relative abundance of 28 s rRNA and 18 s rRNA under each set of conditions suggested by the manufacturer (Ambion Inc., Austin, Texas, USA). RNA was quantified by measuring optical density at 260 nm. An amount of 2–3 µg purified total RNA, primer, deoxyribose nucleotides and rTth DNA polymerase were used in the concentrations suggested by the manufacturer for RT-PCR (Applied Biosystems, Branchburg, NJ, USA). The reverse transcription reaction was carried out at 65 °C for 40 min and the PCR reaction was carried out in a 2-temperature PCR (94 °C for 15 s followed by 62 °C for 40 s) for 30 cycles (GeneAmp PCR system 9 700). To rule out genomic DNA contamination in the RNA preparation used for RT-PCR, the reverse transcription step was omitted in a separate sample (data not shown). The cDNA was separated on 2.5 % agarose gel in TBE buffer and was stained with 2 µg/ml ethidium bromide to visualize DNA bands on a UV transilluminator.

Materials

Androstenedione, dehydroepiandrosterone, pregnenolone, and testosterone were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Insulin was obtained from Roche Applied Science (Indianapolis, IN, USA). 1,25-$(OH)_2D_3$ was obtained from EMD Biosciences (La Jolla, CA, USA). RIA kit was obtained from Diagnostic Systems Laboratories, Inc. (Webster, TX, USA) and ELISA kit was obtained from American Laboratory Products Company (ALPCO) Diagnostics (Salem, NH, USA).

Statistical analysis

All experiments were carried out in quadruplicate. For hormone production experiments, Student *t*-test was used to compare mean values of each set of data. Pairwise *t*-test was used to compare mean values according to insulin concentrations in the presence or absence of 1,25-$(OH)_2D_3$. Because the effects of vitamin D on the mRNA expression and steroid hormone production were not dose-dependent, data obtained from experiments with different concentrations of vitamin D were pooled and then compared to control cell population. Since absolute concentrations of steroid hormones and IGFBP-1 in tissue culture medium at baseline differed among samples from different patients, results were normalized to each sample's baseline level, which was considered 100%.

Results

Vitamin D receptor in the human ovary

Vitamin D receptor mRNA was expressed in both the mixed ovarian and purified granulosa cell cultures (**Fig. 1a, b**). 1,25-$(OH)_2D_3$ at concentrations of 0–150 pM or 0–150 nM had no effect on expression of vitamin D receptor mRNA whether the cells were incubated in the vitamin D-free MEM Eagle medium or M199 medium, which contains 250 nM vitamin D_2.

mRNA expression of 1-α-hydroxylase, the enzyme that converts 25-$(OH)D_3$ to its active form 1,25-$(OH)_2D_3$, was absent in the granulosa cell cultures (**Fig. 1b**). The amount of mRNA used for each set of conditions was similar when examined by mRNA expression of house protein β-actin or 28 s rRNA or 18 s rRNA (**Fig. 1**).

1,25-$(OH)_2D_3$ effect on steroidogenesis

1,25-$(OH)_2D_3$ increased progesterone production in mixed ovarian cell culture by 13% compared to control ($p<0.001$), but had no significant effect on testosterone production (**Fig. 2a**). 1,25-$(OH)_2D_3$ stimulated estradiol or estrone production in purified granulosa cell culture by up to 6% ($p<0.02$) and up to 21% ($p<0.005$) compared to control, respectively (**Fig. 2b**).

Interaction of 1,25-$(OH)_2D_3$ with insulin

In the granulosa cell culture, insulin stimulated estradiol production by 13% ($p<0.04$) (**Fig. 2c**). 1,25-$(OH)_2D_3$ acted synergistically with insulin to increase estradiol production by up to 50% ($p<0.001$) (**Fig. 2c**). In contrast, insulin and 1,25-$(OH)_2D_3$ had no significant synergistic effect on progesterone, testosterone, or estrone production (data not shown).

IGFBP-1 production

1,25-$(OH)_2D_3$, used alone, increased IGFBP-1 production by up to 24% compared to control ($p<0.001$) in purified granulosa cells (**Fig. 2d**). In the presence of insulin, 1,25-$(OH)_2D_3$, enhanced insulin-induced inhibition of IGFBP-1 production by 13% ($p<0.009$) (**Fig. 2e**).

Discussion

The physiological role of vitamin D in human reproduction and ovarian steroidogenesis is not well understood. There are several animal studies suggesting the importance of vitamin D in reproduction. For example, in the study of vitamin D receptor null

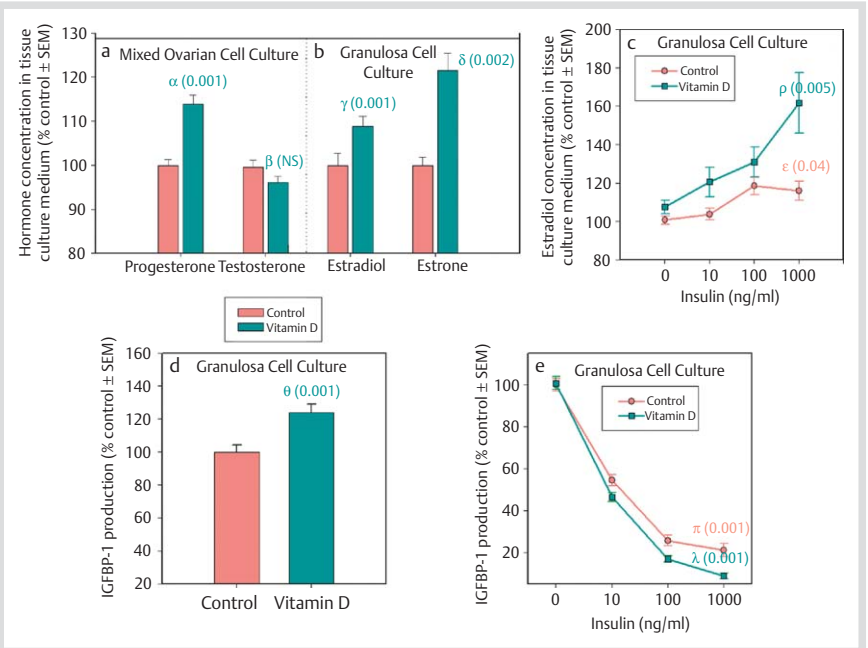

Fig 2 **a**: 1,25-$(OH)_2D_3$ effect on steroid hormone production in mixed ovarian and granulosa cell cultures. Steroid hormone production was assessed in the absence or presence of 1,25-$(OH)_2D_3$. Progesterone production in the mixed ovarian cell culture, α, $p<0.001$ ($n=13$); testosterone production in the mixed ovarian cell culture, β, p <NS ($n=12$). **b**: Estradiol production in granulosa cells, γ, $p<0.05$ ($n=5$); estrone production in granulosa cells, δ, $p<0.002$ ($n=8$); **c**: Synergistic effect of 1,25-$(OH)_2D_3$ and insulin on estradiol production in purified granulosa cell cultures; ε, $p<0.04$ ($n=6$) compared to control [in the absence of 1,25-$(OH)_2D_3$ and in the absence of insulin]; ρ, $p<0.005$ ($n=6$) compared to control [in the absence of 1,25-$(OH)_2D_3$ and in the presence of insulin]. **d**: 1,25-$(OH)_2D_3$ effect on IGFBP-1 production in human granulosa cells, θ, $p<0.001$ ($n=7$) compared to control [in the absence of 1,25-$(OH)_2D_3$]; **e**: Synergistic effect of 1,25-$(OH)_2D_3$ with insulin on IGFBP-1 production in purified granulosa cell culture, π, $p<0.001$ ($n=10$), compared to control [in the absence of 1,25-$(OH)_2D_3$ and in the absence of insulin], λ, $p<0.009$ ($n=10$), compared to control [in the absence of 1,25-$(OH)_2$ D_3 and in the presence of insulin]. Data are normalized to the baseline IGFBP-1 production.

mutant mice, serum level of estrogens in these animals was significantly lower than in heterozygous mice [3], suggesting a role of vitamin D in estrogen synthesis.

Presence of vitamin D receptor in human ovarian tissue was demonstrated in one study but has not been confirmed [8]. In this report, we have confirmed that vitamin D receptor is present in the human ovary and, for the first time, described the direct effect of 1,25-$(OH)_2D_3$ on steroid hormone production. We have demonstrated that, under conditions of our experiments, 1,25-$(OH)_2D_3$ stimulated estrogen and progesterone production in human ovarian cells, but had no effect on the testosterone production.

Stimulation of aromatase activity by 1,25-$(OH)_2D_3$ in the human skin fibroblasts has been previously demonstrated [9]. Our finding of increased estrogen production in the presence of 1,25-$(OH)_2D_3$ may be explained by augmentation of aromatase activity by 1,25-$(OH)_2D_3$ in the human ovary.

We examined the effect of 1,25-$(OH)_2D_3$ on the components of steroidogenic signaling cascade, but 1,25-$(OH)_2D_3$ had no significant effect on steroidogenic acute regulatory protein (StAR), 3-β-hydroxysteroid dehydrogenase (3-βHSD), or aromatase mRNA expression (data not shown). This lack of findings can be possibly explained by the small magnitude of the 1,25-$(OH)_2D_3$ effects on the steroid hormone production. These effects range between 10–20% and the corresponding effects on the steroidogenic enzyme mRNA expression, if any, may be too small to be detected in our experiments.

Our results also demonstrate that 1,25-$(OH)_2D_3$ acted synergistically with insulin to stimulate estradiol production. Although the mechanism of this synergism remains unknown, increased insulin sensitivity in the presence of vitamin D has been described in the studies of glycemic control [10]. Therefore in the ovary, vitamin D may be acting as an insulin sensitizer.

IGFBP-1 is one member of IGFBP family whose production is regulated by insulin. In vivo IGFBP-1 is produced in the liver, but also in other organs, including the ovary. IGFBP-1 production is regulated by insulin and IGF-I. We had previously demonstrated that in granulosa cells system, insulin and IGF-1 inhibit IGFBP-1 production, similar to their effects in the liver [7]. We examined IGFBP-1 production in the presence of vitamin D and insulin to determine whether, similar to its effect on steroidogenesis, vitamin D exerts insulin-sensitizing effect on IGFBP-1 production in human ovarian cells. Our studies demonstrated that vitamin D and insulin synergistically enhanced inhibition of IGFBP-1 production, although vitamin D, when used alone, stimulated IGFBP-1 production. In summary, vitamin D has both an independent and an insulin-sensitizing effect on IGFBP-1 production in human ovarian cells and these effects are opposite in direction. Mechanisms of this interesting phenomenon need to be investigated further.

We conclude that vitamin D stimulates production of progesterone, estrone and estradiol in cultured human ovarian cells both independently and, in the case of estradiol, synergistically with insulin. Vitamin D also stimulates IGFBP-1 production when used alone. When used with insulin, however, vitamin D enhances insulin-induced inhibition of IGFBP-1 production in cultured human ovarian cells. Further studies are needed to determine the full range of vitamin D effects in the human ovary, their mechanisms and clinical significance.

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