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Effect of Vitamin D on basal and Luteinizing Hormone (LH) induced testosterone production and mitochondrial dehydrogenase activity in cultured Leydig cells from immature and mature rams

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ABSTRACT

The objectives of this study were to investigate the potential effects of $1\alpha,25\text{-}(OH)_2VD_3$ (biologically active form of Vitamin D) on basal and LH-induced testosterone production and mitochondrial dehydrogenase activity in Leydig cells from immature and mature rams cultured in vitro. Leydig cells were isolated from testes of immature and mature rams, treated without (control) or with increasing concentrations of LH (1, 10, 100 ng/ml) and/or $1\alpha,25\text{-}(OH)_2VD_3$ (1, 10, 100 nM). After 24 h, concentrations of testosterone in culture media were measured. After 96 h, mitochondrial dehydrogenase activity in Leydig cells were measured. In immature and mature ram Leydig cells, treatment with 10 and 100 ng/ml LH increased testosterone production and mitochondrial dehydrogenase activity. Treatment with $1\alpha,25\text{-}(OH)_2VD_3$ in the absence of LH did not increase testosterone production, but 10 and 100 nM $1\alpha,25\text{-}(OH)_2VD_3$ increased LH induced testosterone production for both immature and mature ram Leydig cells. Treatment with all doses of $1\alpha,25\text{-}(OH)_2VD_3$ in the absence of LH and 10 and 100 ng/ml LH in the absence of $1\alpha,25\text{-}(OH)_2VD_3$ increased mitochondrial dehydrogenase activity for cultured Leydig cells from immature and mature rams and 1 and 10 nM $1\alpha,25\text{-}(OH)_2VD_3$ treatment enhanced the LH induced increase in mitochondrial dehydrogenase activity. Result demonstrate Vitamin D_3 induced regulation of function of Leydig cells from immature and mature rams cultured in the presence or absence of LH and support a potential role for Vitamin D_3 in regulation of gonadal function in rams.

1. Introduction

Vitamin D is a steroid hormone long known for its key role in calcium and phosphorus metabolism and cell differentiation and proliferation (Walters, 1992; Studzinski et al., 1993; Bouillon et al., 1995). Actions of Vitamin D are

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mediated by the Vitamin D receptor (VDR) in target cells (Darwish and DeLuca, 1993; Mangelsdorf and Evans, 1995). In the body, the main source of Vitamin D is endogenous Vitamin D synthesized in the skin following ultraviolet B radiation mediated conversion of 7-dehydrocholesterol, via two hydroxylation steps in both the liver and kidney, to generate the active form of Vitamin D (1α,25-$(OH)_2VD_3$). The two hydroxylation steps are mediated by two hepatic cytochrome P450 enzymes and a renal cytochrome P450 enzyme (David and Prosser Jones, 2004; Blomberg Jensen, 2012). While evidence indicates the three cytochrome P450 enzymes are primarily expressed in the liver and kidney, recent studies demonstrated above enzymes and/or the VDR also exist in Leydig cells, Sertoli cells, germ cells, spermatozoa and the epithelial cells of the male reproductive tract (Merke et al., 1983; Schleicher et al., 1989; Johnson et al., 1996; Corbett et al., 2006; Aquila et al., 2008; Oliveira et al., 2008; Blomberg Jensen et al., 2010; Mahmoudi et al., 2013).

The most important function of Leydig cells is secreting testosterone. LH is an important regulator of testosterone production and Leydig cell proliferation. (Huhtaniemi and Toppari, 1995). In addition, mitochondrial dehydrogenase, such as succinodehydrogenase and malate dehydrogenase, are the key enzymes of glycometabolism. The activity of these enzymes reflect the cell viability and it can be measured by Cell Counting Kit-8 (CCK-8) assay. CCK-8 is widely used in cell proliferation and cytotoxicity tests (Umezu-Goto et al., 2002; Hamamoto et al., 2004; Qi et al., 2014), and CCK-8 substrate becomes yellow formazan by mitochondrial dehydrogenase. The production of the formazan is positively correlated with dehydrogenase activity. Moreover, the formazan has an absorption peak at 450 nm; therefore, the absorbance at 450 nm reflects the activity of mitochondrial dehydrogenase.

The presence of VDR and above cytochrome P450 enzymes in testis suggests that Vitamin D may affect Leydig cell function. Therefore, the objectives of this study were to investigate the effect of 1α,25-$(OH)_2VD_3$ on LH-induced testosterone production and mitochondrial dehydrogenase activity in cultured ram Leydig cells, and determine if effects of LH and 1α,25-$(OH)_2VD_3$ differ for cells collected from immature versus mature rams.

2. Materials and methods

2.1. Animals

Testes were collected from immature (1–2 month old) and mature (≥1 year old) healthy Small Tail Han sheep (perennial oestrus, particularly in the spring and autumn) at a local abattoir (112.32° east longitude, 37.18° north latitude, Jinzhong area, Shanxi, China) in autumn. Testes were transported to the laboratory on ice in sterile PBS.

2.2. Leydig cell isolation

The fat, epididymis and tunica albuginea were gently removed from the testis to expose the seminiferous tubule. Then the testis was placed into a sterile beaker containing PBS. The testis was nipped with tweezers and gently shaken until the PBS become turbid. The Leydig cells were thus gradually separated from the testis. After this, the PBS was centrifuged at 1,200 rpm for 5 min to pellet the cells. Cells were re-suspended in PBS and washed three times. Then Leydig cells were incubated with DMEM/F12 (Boster, Wuhan, China) supplemented with 10% FBS (Sijiqing, Hangzhou, China) for 12 h at 34 °C under 5% CO_2. After 12 h incubation, cells attached to plate were collected. Purity of the Leydig cells were assessed using 3β-Hydroxysteroid Dehydrogenase (3β-HSD) staining (Browning et al., 1983) and were 80–85% Leydig cells.

2.3. Effect of 1α,25-$(OH)_2VD_3$ and LH on testosterone production

The cell viability was estimated by trypan blue staining. Living Leydig cells were seeded in quadruplicate at 96-well plates for 1×10^5 cells/well. Cells were cultured with DMEM/F12 supplemented with 0.1% albumin from bovine serum (BSA; Boster, Wuhan, China), antibiotics (100 IU/ml penicillin and 0.1 mg/ml streptomycin; Boster, Wuhan, China), antimycotic (625 ng/ml amphotericin B; Boster, Wuhan, China) and different concentrations of sheep LH (0, 1, 10, 100 ng/ml; Sigma, Saint Louis, USA) and 1α,25-$(OH)_2VD_3$ (0, 1, 10, 100 nM; Selleck, Houston, USA). After culture for 24 h at 34 °C under 5% CO_2, the culture medium from each well was harvested, and concentrations of testosterone measured using a commercially available ELISA kit (Blue Gene, Shanghai, China). The sensitivity in this assay was 1.0 pg/ml. The intra-assay CVs for low, medium and high concentrations of testosterone were 7.2%, 5.8% and 7.6%, respectively. The inter-assay CVs for low, medium and high concentrations of testosterone were 8.2%, 9.4% and 9.7%, respectively.

2.4. Effect of 1α,25-$(OH)_2VD_3$ and LH on mitochondrial dehydrogenase activity

Living Leydig cells were seeded in quintuplicate at 96-well plates for 5×10^3 cells/well. Cells were cultured with DMEM/F12 supplemented with 10% FBS, antibiotics (100 IU/ml penicillin and 0.1 mg/ml streptomycin), antimycotic (625 ng/ml amphotericin B) and different concentrations of sheep LH (0, 1, 10, 100 ng/ml) and 1α,25-$(OH)_2VD_3$ (0, 1, 10, 100 nM). After culture for 96 h at 34 °C under 5% CO_2, mitochondrial dehydrogenase activity was measured using Cell Counting Kit-8 (CCK-8) assay (Boster, Wuhan, China).

For measurement of mitochondrial dehydrogenase activity, 10 μl CCK-8 substrate was added into each well and cells incubated at 34 °C for an additional 3 h. Absorbance at 450 nm was then recorded using a microplate reader (Thermo Scientific, Shanghai, China) and effects of treatments expressed as percentage change compared to untreated controls.

2.5. Statistical analysis

All data were analyzed in a two factor ANOVA including the main effects of LH and 1α,25-$(OH)_2VD_3$ as well as the interaction and experiment as a random effect using SAS 9.4. If the interaction was significant, differences between