SHORT COMMUNICATION

The Influence of Testosterone on the Expression and Function of Vitamin D_3 Receptor (VDR) Protein in the Porcine Ovarian Follicle

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Short title: Androgens and VDR in porcine ovarian follicle.

Summary

Recently it has been shown that vitamin D_3 acting *via* its cognate receptor (VDR) regulates the growth, differentiation and function of female reproductive tissues including ovary. The aim of the study was to examine the effect of testosterone (T) and its antagonist 2-hydroxyflutamide (HF) on VDR protein expression and function in porcine ovarian follicles. Medium size antral follicles expressing great amount of androgen receptors and represent high steroidogenic activity were used in this research. After 6 h incubation of whole follicles with T, HF or T+HF, immunohistochemical analysis of VDR revealed its nuclear localization in granulosa and theca interna cells in control and experimental groups. The expression of VDR protein was shown as a band of 48 kDa. There were no significant differences between either experimental group and the control. T influenced the function of VDR through decreased formation of VDR/RXR (retinoid X receptor) complexes ($P<0.05$) in both granulosa and theca interna cells, but HF abolished this effect only in granulosa cells ($P<0.05$). These results suggest that androgens regulate the response of follicular cells to vitamin D_3 in pigs ovary *via* regulation of VDR transcriptional activity.

Key words

Androgens • Vitamin D_3 receptor • Ovarian follicle • Pig

It is well established that androgens are crucial steroid hormones involved in the regulation of ovarian function (Walters 2015). Our previous *in vivo* and *in vitro* studies on porcine ovary using the anti-androgen flutamide or its metabolite 2-hyroxyflutamide (HF), revealed that androgens affect early folliculogenesis (Knapczyk-Stwora *et al*. 2013) and further antral follicle functions (Duda *et al.* 2014). Besides contributions to normal ovarian physiology, clinical data support a role for androgens in ovarian pathologies such as polycystic ovary syndrome (PCOS) (Walters 2015).

There is increasing evidence that vitamin D_3 (VD) regulates female reproduction through cognate VD receptor (VDR) expressed in the ovary, uterus and placenta (Shahrokhi *et al.* 2016). In the ovary, VD was shown to influence follicular development, steroidogenesis and expression of ovarian reserve markers, including anti-Müllerian hormone. Furthermore, there is a proposed relationship between VD deficiency and PCOS-associated ovulatory dysfunction, insulin resistance and hyperandrogenism (Irani and Merhi 2014). Importantly, recent data revealed that VD supplementation in women with PCOS significantly reduced total testosterone level (Azadi-Yazdi *et al.* 2017). Thus, mutual interactions between androgens and VD within ovary seem to be very interesting and important for normal ovarian function.

VDR belongs to the superfamily of steroid hormone receptors and acts as a transcriptional factor. The response to VD involves its binding to VDR, which further undergoes heterodimerization with retinoid X receptor (RXR) to initiate either activation or repression of transcription (Christakos *et al.* 2016). Taking into account the highlighted role of androgens and VD in ovarian physiology and pathology, as well as data suggesting cross-talk between VD and androgens and their cognate receptors (Ahonen *et al.* 2000), our aim was to examine for the first time the effect of testosterone and its antagonist HF on VDR protein expression and function in porcine ovarian follicles.

All chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Porcine ovaries were obtained from sexually mature pigs at a local abattoir and placed immediately after slaughter in cold phosphate-buffered saline (PBS; pH 7.4) supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml, amphotericin B 2.5 μg/ml). Tissues were transported to the laboratory within 2 h of collection and rinsed with PBS. Healthy, follicular phase, medium size antral follicles (5 - 6 mm) were excised from different ovaries (in each experiment ten ovaries from five animals were used) and cultured in 4-well plates (37˚C; 95% air:5% CO_2) in DMEM-F12 medium, supplemented with antibiotics and 10% fetal bovine serum, for 6 h using the following treatments: (i) medium alone (control), (ii) testosterone (T; 10^{-7} M), (iii) 2-hydroxyflutamide (HF; 1.7×10^{-4} M) and (iv) HF+T. After incubation, whole follicles were fixed for 24 h in 10% neutral buffered formalin for immunohistochemical studies (n = 6/group) or separated granulosa and theca interna cells were frozen in liquid nitrogen for protein isolation (n = 6/group). The experiment was carried out on three separate occasions.

To reveal the cellular distribution of VDR within porcine ovarian follicles the immunohistochemistry was conducted as previously described (Grzesiak *et al.* 2015). In brief, after antigen retrieval in 0.01 M citrate buffer (pH 6.0) and blockade of endogenous peroxidase in 0.3% H_2O_2, the nonspecific binding was prevented by incubation in 5% normal goat serum prior to incubation with rabbit anti-VDR antibody (1:50, cat. no. 12550, Cell Signaling Technology, Beverly, MA, USA; overnight incubation at 4°C in a humidified chamber), biotinylated goat anti-rabbit IgG (1:300, cat. no. BA-1000, Vector Laboratories, Burlingame CA, USA) and avidin-biotin-peroxidase complex (Vectastain Elite ABC Reagent, Vector Laboratories). For the negative control reaction, slides were incubated with non-immune rabbit IgG instead of primary antibody and processed as above. Sections were

photographed using a Nikon Eclipse Ni-U microscope and a Nikon Digital DS-Fi1-U3 camera (Nikon, Tokyo, Japan) with corresponding software.

Western blot analysis was performed to examine VDR protein expression. Prior protein isolation follicles were subjected to mechanical separation of granulosa and theca interna layers. The purity of granulosa and theca interna samples were confirmed by the examination of their specific markers expression (cytochorme P450 aromatase and cytochrome P450 17α-hydroxylase/c17,20 lyase, respectively). Protein extraction and Western blot analysis were performed as described (Grzesiak *et al.* 2015). A primary anti-VDR antibody (1:500 at room temperature for 1.5 h) and secondary horseradish peroxidase-conjugated anti-rabbit IgG (1:3000, cat. no. PI-1000, Vector Laboratories, 1 h) were used. To control for variable amounts of protein, the membranes were stripped and reprobed with mouse anti-β-actin antibody (1:3000, cat. no. A5316) and horseradish peroxidase-conjugated anti-mouse IgG (1:3000, cat. no. PI-2000, Vector Laboratories). Signals were detected using luminol reagent and visualized with ChemiDoc Imaging System (UVP). Analysis of images was performed using the public domain ImageJ program (National Institutes of Health, Bethesda, MD, USA). The bands were densitometrically quantified and normalized to their corresponding β-actin bands.

Transcriptional activity of VDR was determined using VDR/RXR co-immunoprecipitation procedure with Immunoprecipitation Kit (Protein G) (Roche, Mannheim, Germany, Grzesiak *et al.* 2015) according to the manufacturer's protocol. In brief, 100 μg of total protein extracted from granulosa and theca interna cells of control and experimental groups were pre-cleaned with Protein G and incubated with 2 μg of anti-VDR antibody overnight at 4°C on a rotator. Next, the samples were mixed with Protein G and incubated for 3 h at 4°C on a rotator to precipitate the immunocomplexes. The precipitates were washed and agarose-bound complexes were eluted by denaturating samples in the

presence of reductant. The samples were immunobloted with rabbit anti-RXR antibody (1:100, cat. no. sc-774, Santa Cruz Biotechnology Inc., CA, USA). RXR bands in precipitated material from the control, T, HF and T+HF groups were densitometrically quantified and normalized to their corresponding RXR bands in the whole homogenates (expressed as arbitrary units).

Statistical analysis was conducted using Statistica v.13 program (StatSoft, Inc., Tulsa, OK, USA). The nonparametric Kruskal-Wallis test was used since data were not normally distributed according to the Shapiro-Wilk test. Differences were considered statistically significant at the 95% confidence level (P <0.05).

The results of the present study reveal for the first time the expression of VDR protein in porcine ovarian follicles (Figure 1B) immunolocalized in the nuclei of granulosa and theca interna cells (Figure 1A). Thus, porcine follicular cells are a target for direct VD action. By this time, the expression of VDR has been shown in human, rat (Ahonen *et al.* 2000) and chicken (Wojtusik and Johnson 2012) ovary but predominantly in granulosa cells. In pigs, both granulosa and theca interna compartments demonstrate high steroidogenic activity (Conley *et al.* 1994). Therefore, it is likely that VD influences steroid biosynthesis in porcine follicles. Indeed, recent *in vitro* studies on porcine granulosa cells showed enhanced insulin- and follicle-stimulating hormone- induced progesterone secretion (Smolikova *et al.* 2013) and augmented estradiol production (Hong *et al.* 2017) following VD treatment. However, further studies related to androgens and VD interactions are required.

Herein we have examined the influence of testosterone (T) and its antagonist 2-hydroxyflutamide (HF) on VDR expression in porcine granulosa and theca interna layers. Western blot analysis did not show any changes in VDR protein level in either experimental group (Figure 1B). However, similar patterns of VDR and AR protein expression (Slomczynska and Tabarowski 2001) in porcine follicles might indicate potential cross-talk

between both receptors. VDR transcriptional activity depends on its dimerization with RXR. Therefore, the next step of our research was to analyze the effect of T and/or HF on formation of VDR/RXR complexes. Co-immunoprecipitation revealed that T decreased VDR/RXR dimerization in both follicular compartments. Additionally, HF abolished this effect only in granulosa cells (Figure 2). Ting *et al.* (2005) demonstrated the involvement of AR coregulator ARA70 in VDR signal transduction. They found that AR activation mediates suppression of VDR transactivation *via* competition for ARA70. These results are in agreement with our present findings and explain the negative influence of T on the ability of VDR to heterodimerization. Removing this effect by HF in granulosa but not theca interna cells might be the first step in elucidating the role of androgen signaling in response of different follicular cells to VD. These outcomes appear to be crucial especially in the light of theca interna cells androgens overproduction and subsequent hyperandrogenism in women with PCOS (Irani and Merhi 2014).

Concluding, our research provides novel information about VDR protein expression within porcine follicles, identifying granulosa and theca interna cells as targets for VD action. Furthermore, T-impaired VDR activation in both follicular compartments suggests a role of androgen signaling *via* AR in the regulation of VDR transcriptional activity. This may influence follicular responses to VD and contribute to female reproductive pathologies.

Conflict of Interest

There is no conflict of interest to declare.

Acknowledgements

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Legend to figures

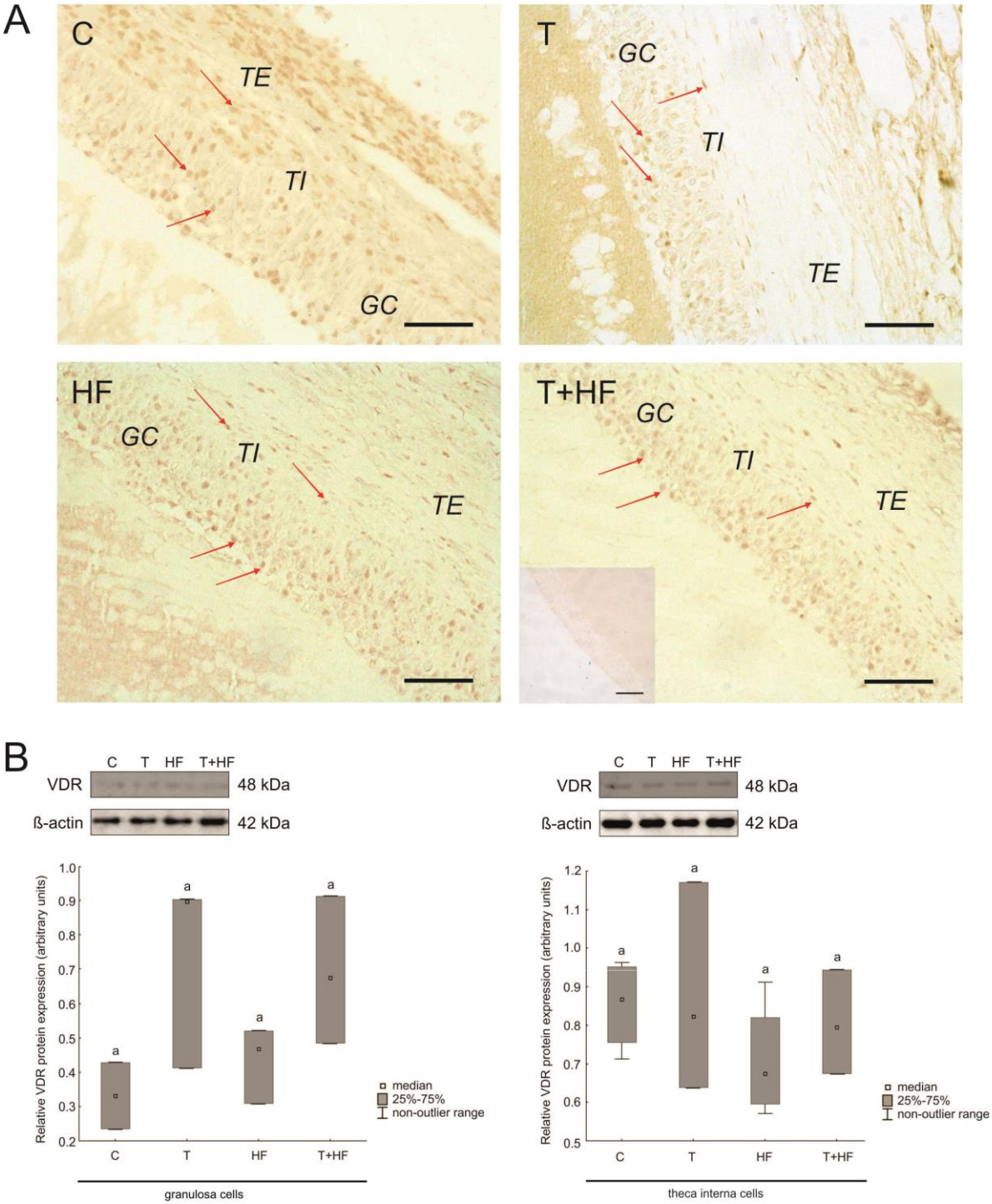
Fig. 1. Immunohistochemical localization of vitamin D_3 receptor (VDR) (A) and Western blot analysis of VDR protein expression (B) in granulosa and theca interna cells from control (C), testosterone (T; 10^{-7} M), hydroxyflutamide (HF; 1.7×10^{-4} M) and testosterone +

hydroxyflutamide (T+HF) -treated porcine ovarian follicles. (A) Red arrows indicate positive nuclear reaction. Control sections in which the primary antibody was replaced by rabbit IgG did not exhibit any positive staining (inset). (B) Representative Western blots are shown. VDR expression was expressed as the ratio to β-actin. The box plot shows medians (dots within boxes) and 25/75 percentiles (box sizes). The same letter superscripts indicate lack of differences between groups (Kruskal-Wallis test; $P<0.05$)

GC – granulosa cells, TI – theca interna cells, TE – theca externa cells, bar = 50 μm.

Fig. 2. Co-immunoprecipitation of retinoid X receptor (RXR) in homogenates of granulosa and theca interna cells from control (C), testosterone (T; 10^{-7}M), hydroxyflutamide (HF; 1.7×10^{-4}M) and testosterone + hydroxyflutamide (T+HF) -treated groups. Representative blots show immunoprecipitation with anti-VDR antibody. Immunocomplexes were subjected to immunoblotting and stained with anti-RXR antibody. RXR bands were densitometrically quantified and normalized to their corresponding RXR bands in the whole homogenates (total RXR). The box plot shows medians (dots within boxes) and 25/75 percentiles (box sizes). Different letter superscripts indicate differences between groups (Kruskal-Wallis test; $P<0.05$).

Fig. 1


A
C
TE
TI
GC
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GC
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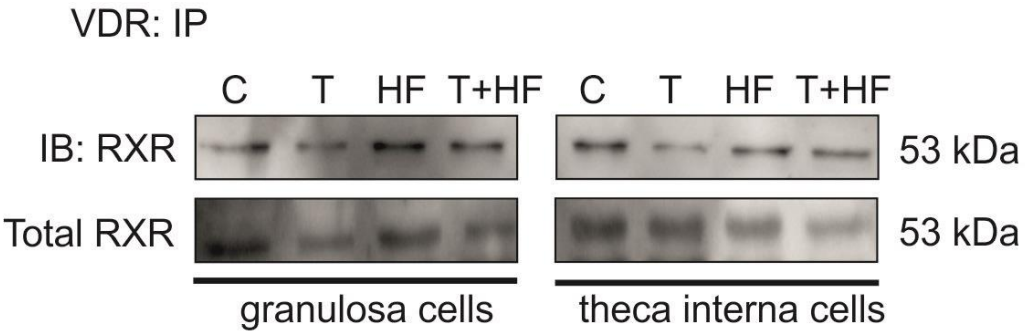
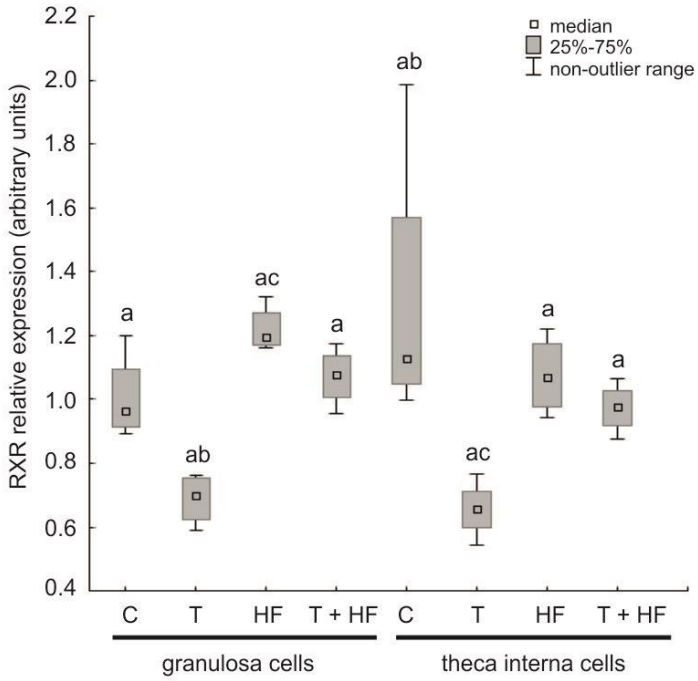
HF
GC
TI
TE
T+HF
GC
TI
TE
B
C T HF T+HF
VDR
48 kDa
ß-actin
42 kDa
Relative VDR protein expression (arbitrary units)
median
25%-75%
non-outlier range
granulosa cells
theca interna cells

Fig. 2


VDR: IP
C T HF T+HF C T HF T+HF
IB: RXR
53 kDa
Total RXR
53 kDa
granulosa cells
theca interna cells


median
25%-75%
non-outlier range
RXR relative expression (arbitrary units)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
a
ab
ac
a
ab
ac
a
a
C T HF T + HF C T HF T + HF
granulosa cells
theca interna cells