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5 α -dihydrotestosterone reduces renal Cyp24a1 expression *via* suppression of progesterone receptor

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Abstract

Androgens act in concert with vitamin D to influence reabsorption of calcium. However, it is unclear whether androgens directly regulate vitamin D homeostasis or control other cellular events that are related to vitamin D metabolism. To examine whether the expression of vitamin D-related genes in mouse kidney is driven by androgens or androgen-dependent effects, the androgen receptor and other sex steroid receptors were monitored in orchidectomized mice treated with 5 α -dihydrotestosterone (DHT). Our results revealed that exposing orchidectomized mice to DHT inhibited the expression of progesterone receptor (Pgr) with or without estrogen receptor α expression, the latter was confirmed by ER-positive (MCF7 and T47D) or -negative (PCT) cells analysis. The loss of Pgr in turn decreased the expression of renal 24-hydroxylase *via* transcriptional regulation because Cyp24a1 gene has a progesterone receptor-binding site on promoter. When male kidneys preferentially hydroxylate 25-hydroxyvitamin D₃ using 24-hydroxylase rather than 25-hydroxyvitamin D₃-1- α hydroxylase, DHT suppressed the Pgr-mediated 24-hydroxylase expression, and it is important to note that DHT increased the blood 25-hydroxyvitamin D₃ levels. These findings uncover an important link between androgens and vitamin D homeostasis and suggest that therapeutic modulation of Pgr may be used to treat vitamin D deficiency and related disorders.

Key Words

- ▶ 5 α -dihydrotestosterone
- ▶ kidney
- ▶ androgen withdrawal
- ▶ Cyp24a1
- ▶ progesterone receptor
- ▶ renal tubule cells

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Introduction

Androgens perform physiologically essential functions in the development and differentiation of the male reproductive tract, but their anabolic effects are also seen in non-reproductive tissues (Bardin & Catterall 1981). For instance, androgens increase the life span of bone cells through anti-apoptotic activity (Kasperk *et al.*

1989), and their effects are linked with transcriptional activity of the androgen receptor (Kousteni *et al.* 2001). Androgens induce both cell proliferation and hypertrophy in the prostate, whereas they are only involved in cell hypertrophy in the kidney (Catterall *et al.* 1986). Although kidneys do not require androgens

for normal functions (Catterall *et al.* 1986), it is possible that androgens have a significant role in the homeostasis of the body. While estrogen deficiency has been shown to be involved in the osteoporosis related to calcium and vitamin D (Morris *et al.* 2010), studies have also indicated that androgens can act in concert with vitamin D to influence important physiological events including mobilization of bone calcium (Greenspan *et al.* 2005) and UV light-induced vitamin D production in the skin (Xue *et al.* 2015). In patients afflicted with coronary artery disease, levels of 25-hydroxyvitamin D (calcidiol) in the blood were found to be much greater in men than that in women (Verdoia *et al.* 2015). Interestingly, obese men were more prone to vitamin D deficiency when compared to women (Johnson *et al.* 2012). Increased aromatase activity in obese individuals evidently reduces levels of androstenedione by converting it to estrone in adipose tissues (Zumoff 1982). Another study suggests a link between the impairment of testicular function and low levels of 25-hydroxyvitamin D₃; vitamin D-deficient male rat has an insufficient fertility, such as sperm concentration, sperm morphology and motile sperm count (Kwiecinski *et al.* 1989). As a mediator for local vitamin D production, 25-hydroxylase *CYP2R1* has been detected in testes and Leydig cells producing androgen (Bieche *et al.* 2007, Foresta *et al.* 2011). These findings highlight the need for further studies on the cellular functions of androgen in relation to vitamin D metabolism. There is also considerable evidence that the imbalance in calcium homeostasis may originate from sex steroid hormone deprivation or vitamin D disorder and from declining calcium reabsorption efficiency in the kidney due to aging. For instance, renal vitamin D receptor (VDR) expression correlates inversely with serum sex steroid levels (Iida *et al.* 1995), while estrogen influences the expression of enzymes involved in 1 α ,25-dihydroxyvitamin D₃ (calcitriol) production and metabolism (Lechner *et al.* 2006).

As the major circulating vitamin D metabolite in the body, 25-hydroxyvitamin D₃ is synthesized in the liver by 25-hydroxylase enzymes such as 25-hydroxylase *CYP27A1* or *CYP2R1* (Zhu *et al.* 2013). The converted 25-hydroxyvitamin D₃ is endogenously activated by 25-hydroxyvitamin D₃-1 α hydroxylase (*CYP27B1*) at the 1 α position in the renal cortex, which gives rise to the active form of vitamin D, 1 α ,25-dihydroxyvitamin D₃ (Jones *et al.* 1998). Both 25-hydroxyvitamin D₃ and 1 α ,25-dihydroxyvitamin D₃ can be further hydroxylated by the vitamin D-inactivating enzyme, 24-hydroxylase (*CYP24A1*). 1 α ,25-dihydroxyvitamin D₃ plays a major role

in regulating calcium homeostasis. Low levels of 1 α ,25-dihydroxyvitamin D₃ increase the release of parathyroid hormone that stimulates *CYP27B1* expression, while high levels of 1 α ,25-dihydroxyvitamin D₃ induce *CYP24A1* expression (Jones *et al.* 1998). In addition to its role in vitamin D homeostasis, *CYP24A1* may be implicated in cancer, as overexpression of *CYP24A1* has been noted in several tumors including lung, breast colon cervical and ovarian cancer (Deeb *et al.* 2007). When vitamin D₃ used as an antitumor agent in patients with prostate cancer, *CYP24A1* is also used as a predictive marker of vitamin D₃-based therapies (Tannour-Louet *et al.* 2014). In early studies, 5 α -dihydrotestosterone (DHT) has been shown to significantly inhibit the expression of *Cyp24a1* mRNA in a prostate cancer cell line (Lou *et al.* 2005). However, little is known about the mechanism underlying the regulation of *CYP24A1* expression by androgens.

The kidney is well recognized as an androgen target organ, where various overexpressed genes resulting from androgen exposure have been identified (Berger & Watson 1989). Interestingly, it is also established that *CYP27A1* and *CYP24A1* are mainly localized in the proximal convoluted tubule of the kidney (Kurokawa *et al.* 1982). To examine whether the expression of vitamin D-related genes is affected by androgens, levels of transcripts corresponding to the androgen- and vitamin D-related genes were monitored in the mouse kidney and in the immortalized PKSV-PCT mouse proximal convoluted tubule (PCT) epithelial cell line, which has characteristics of epithelial cells in the S1–S2 region of the proximal convoluted tubule (Cartier *et al.* 1993) and expresses the *Kap* gene in response to androgen (Soler *et al.* 2002). Interestingly, we observed that androgen suppresses the expression of *Cyp24a1* mRNA without affecting the expression of vitamin D receptor (*Vdr*) and that the reduced *Cyp24a1* level is correlated with reduced expression of progesterone receptor. Our work highlights the complexity of androgen actions in vitamin D catabolism.

Materials and methods

Animals and treatment

Male mice in a C57BL/6N background obtained from Orient Bio (Seongnam, Korea) and were housed in a pathogen-free facility at Chungnam National University under a standard 12-h light:12-h darkness cycle and fed standard chow with water provided *ad libitum*. All mouse experiments were performed in accordance with the Chungnam Facility Animal Care Committee

(CNU-00606). To remove endogenous androgen, 6-week-old mice were subjected to surgical orchiectomy. They are separated for treatment group (each group, $n=5$ or 6). After 2 weeks post surgery, mice were treated for 5 days with either DHT (50 µg/day; A8380; Sigma-Aldrich) or bicalutamide (1 mg/day; B9061; Sigma-Aldrich) in a corn oil solution by subcutaneous injection, and then kidneys were isolated at 24 h following final injection. For analysis, all mice were killed with CO₂ asphyxiation.

Cell culture

All cell culture reagents were from Life Technologies. The T47D and MCF7 breast cancer cells grown at 37°C in a 5% CO₂ atmosphere in DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 U/mol) and streptomycin (100 µg/mL). The PKSV-PCT (PCT) cell line was kindly provided by Dr A. Vandewalle (INSERM U478, Paris, France) and grown at 37°C in a 5% CO₂ atmosphere in DMEM/F12 medium supplemented with 2% fetal bovine serum, insulin (5 µg/mL), dexamethasone (5×10^{-8} M), selenium (60 nM), transferrin (5 µg/mL), triiodothyronine (5×10^{-8} M), EGF (10 ng/mL), D-glucose (20 mM), penicillin (100 U/mol) and streptomycin (100 µg/mL).

Gene knockdown and gene overexpression assay

Briefly, 4×10^5 PCT cells were seeded into six-well tissue culture plates 1 day before transfection in 2 mL phenol red-free DMEM/F12 medium (Life Technologies) containing 2% dextran charcoal-treated fetal bovine serum (Thermo Fisher Scientific). Transient transfection of *PRa* or *PRb* expression vectors (5 µg), and a *PGR* siRNA (Qiagen Flexitube gene Solution; Qiagen Korea) were performed using lipofectamine reagent (Life Technologies) according to the protocol recommended by Life Technologies. At 24 h after transfection, the cells were washed twice with PBS and harvested by scraping. After centrifugation, cell pellets were re-suspended in 100 µL 0.25 M Tris-Cl, pH 7.8, and cells were lysed by 3 freeze-thaw cycles.

Western blotting

Cell and kidney samples were homogenized in lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, 0.5% Nonidet P-40 containing protease inhibitor phenylmethylsulfonyl fluoride (P7626; Sigma-Aldrich) at 4°C for 30 min. Protein was quantified using the Bradford assay PRO-MEASURE (Intron Biotechnology,

Sungnam, Korea) and proteins were resolved on 7.5–10% SDS-PAGE gels. The membranes were blocked for 1 h in PBS containing 0.1% Tween 20 (PBS-T) and 5% skim milk and then incubated overnight at 4°C with a primary antibody (see below) in the same buffer. The blots were then washed three times in PBS-T for 15 min to remove excess antibody and then the membranes were incubated for 1 h with secondary antibodies in PBS-T+5% skim: anti-rabbit (bs-0295G-HRP; MA, USA or 211-032-171; Jackson Laboratories) or anti-mouse (115-035-174; Jackson Laboratories). Following 3 washes in PBS-T, proteins were detected using Lumi-Light Western blotting substrate (Roche Korea). Primary antibodies used were progesterone receptor (sc-7208; Santa Cruz Biotechnology), CYP24A1 (ab175976; Abcam), VDR (sc-9164; Santa Cruz Biotechnology), CYP27B1 (sc-67260; Santa Cruz Biotechnology) and actin (sc-1616; Santa Cruz Biotechnology).

ChIP assay using progesterone receptor

ChIP was used to measure the association of progesterone receptor with bound genes. As previously described (Hong *et al.* 2013), ChIP samples from murine PCT cells were prepared with cell culture medium containing formaldehyde (1% final), and DNA–protein crosslinking was allowed to occur at room temperature for 10 min with rotation. The crosslinked cells were washed twice with cold PBS and re-suspended in nuclei lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS) supplemented with protease inhibitors (Roche Korea). Samples were sonicated on ice at power 10 for 30 s pulses using a VirSonic 100 (Virtis) sonicator at 30 s intervals to prevent the samples from heating. Sonicated material was centrifuged at 16,200 *g* for 15 min at 4°C to remove cellular debris, and chromatin (100 µg) was diluted in 2.5X ChIP dilution buffer (0.5% Triton X-100, 2 mM EDTA, 100 mM NaCl, 20 mM Tris-HCl, pH 8.1) and incubated overnight with anti-PR polyclonal antibody (sc-7208; Santa Cruz Biotechnology) or a rabbit IgG antibody as a control (sc-2027; Santa Cruz Biotechnology) and 60 µL of Dynabeads Protein A (10008D; Life Technologies). Beads were washed 6 times with LiCl buffer (1% NP-40, 500 mM LiCl, 1% Na-deoxycholate, pH 8.0, 100 mM Tris-HCl, pH 8.1). The beads were next washed briefly with TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0) and de-crosslinked (1% SDS, 0.1 M NaHCO₃) at 65°C overnight. De-crosslinked samples were purified using the QIAquick Spin Kit (Qiagen Korea). *PGR* standard ChIP enrichments were quantified by qPCR analysis using specific primers and normalized to

the average enrichments obtained using 2 control primer sets amplifying non-PGR bound genomic regions.

Total RNA extraction and quantitative real-time PCR

Total RNA extracts from mouse kidney or PCT cells were prepared using the TRIzol Reagent (Thermo Fisher Scientific). Reverse transcription was performed at 37°C for 60min using 1µg of total RNA and 200 units of M-MLV together (Intron Biotechnology) with random primers and reagents provided by Thermo Fisher Scientific. Quantitative RT-PCR was carried out using specific primers (Table 1), a SYBR Premix Ex Taq (Takara-Clontech Laboratories) and a CFX Connect Real-Time PCR Detection System (Bio-Rad) equipped with a 96-well optical reaction plate. Negative controls, containing water instead of sample cDNA, were used in each plate. All experiments were run in triplicate, and mRNA values were calculated based on the cycle threshold and monitored for an amplification curve.

ELISA

Circulating 25-hydroxyvitamin D levels were detected in serum from naïve or orchidectomized mice acutely treated with DHT for the indicated time points. Enzyme-linked immunosorbent assay (ELISA) kits were obtained from IBL international GMBH (IBL International, Hamburg, Germany), the serum levels of 25-hydroxyvitamin D3 levels were measured according to the manufacturer's protocols, and absorbance was determined at 450nm using a microplate ELISA reader (Bio-Rad).

Data analysis

Data were analyzed using one-way and two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test and presented as the mean±s.e.m. using Prism Graph Pad (v4.0; GraphPad Software). *P* values <0.05 were considered statistically significant.

Results

Identification of androgen response genes whose expression is either enhanced or suppressed by the presence of DHT

To assess whether endogenous androgens influence sex steroid hormone-mediated actions, we first performed surgical castration (orchietomy) on male mice. After removal of the principal source for endogenous androgen production, mice were treated with DHT (50µg/day) or DHT-bicalutamide-treated (1mg/day) for 5 days. We observed clear decreases in *Kap* mRNA levels in the kidneys of all orchietomy groups when compared to the corresponding sham-operated control groups (Fig. 1A). We noted that the synthesis rate of *Kap* mRNA could be maintained by small amounts of residual testosterone as shown in a previous study (Watson & Paigen 1988), which in turn confirmed that the orchidectomized mice were still responsive to androgens. In the absence of endogenous androgens, DHT treatment induced the expression of *Kap* mRNA, while DHT-induced *Kap* mRNA levels were significantly inhibited by androgen receptor (Ar) blocker, bicalutamide (Fig. 1A). To further investigate, we then monitored the levels of mRNA corresponding to the androgen, estrogen, and progesterone receptors.

Table 1 Primers used for real-time or conventional PCR, related to Figs 1, 2, 3, 4 and 5.

Gene name	Upper primer (5'–3')	Lower primer (5'–3')	Species
<i>Kap</i>	TTCTGTGGTCTGACTGTGGC	CGTGAAGTCCATGATCTGCT	Mouse
<i>Ar</i>	CTGGGAAGGGTCTACCCAC	GGTGCTATGTTAGCGGCCTC	Mouse
<i>ESr1</i>	TGTGTCCAGCTACAAACCAATG	CATCATGCCCACTTCGTAACA	Mouse
<i>Pgr</i>	CTCCGGGACCGAACAGAGT	ACAACAACCCTTTGGTAGCAG	Mouse
<i>VDR</i>	ACCCTGGTGACTTTGACCG	GGCAATCTCCATTGAAGGGG	Mouse
<i>Cyp24a1</i>	CTGCCCCATTGACAAAAGGC	CTCACCGTCGGTCATCAGC	Mouse
<i>Cyp27b1</i>	GCACAGTTTACGTTGCCGAC	CGTTAGCAATCCGCAAGCA	Mouse
<i>Gapdh</i>	GCTGAGTATGTCGTGGAGTC	TTGGTGGTGCAGGATGCATT	Mouse
<i>AR</i>	GACGACCAGATGGCTGTCTT	GGGCGAAGTAGAGCATCCT	Human
<i>ESR1</i>	CCCACTCAACAGCGTGTCTC	CGTCGATTATCTGAATTTGGCCT	Human
<i>PGR</i>	ACCCGCCCTATCTCAACTACC	AGGACACCATAATGACAGCCT	Human
<i>GAPDH</i>	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG	Human
<i>Cyp24a1</i>	GCACAGCCTCTGATGTTTCA	AGCTCTGGACTTTTCCACCA	ChIP
<i>Pgr</i>	GAGCCTGGTTACTGCAAAGG	CGACATTCTGGGACTCATCC	ChIP
<i>Ppargc1a</i>	CCGGCAACCTAGAACACAGT	CAGCATAGCCCTCTCCAGTC	ChIP
<i>Sox17</i>	TCTACCTGGCTTCTCTGCT	TCCCATGCCTTTATTCTGG	ChIP

Figure 1

Influence of 5 α -dihydrotestosterone (DHT) on sex steroid receptors in kidney. After orchidectomy and recovery of mice for 2 weeks, male mice ($n=5$ or 6 per each group) were treated with vehicle alone (vehicle), with DHT (50 μ g/day) or DHT plus bicalutamide (1 mg/day) for 5 days, and *Kap* (A), *Ar* (B), *Esr1* (C) and *Pgr* (D) mRNA levels were determined by quantitative RT-PCR. *Gapdh* mRNA was used as internal control. Values represent means $\pm$ S.E.M. * $P<0.05$ vs orchidectomy vehicle-alone (control) treated group. # $P<0.05$ vs DHT-bicalutamide-treated group.

The *Ar* mRNA levels in the kidneys of sham-operated mice were similar to those of orchidectomized mice, even after androgen treatment and subsequent bicalutamide treatment (Fig. 1B). Interestingly, the estrogen receptor α (*Esr1*) and progesterone receptor (*Pgr*) were clearly overexpressed in orchidectomized mice when compared to in sham-operated mice. In orchidectomized mice, DHT treatment significantly decreased *Esr1* (Fig. 1C) or *Pgr* (Fig. 1D) mRNA levels in the kidney, when compared to in vehicle-treated or DHT-bicalutamide co-treated mice. This suggests that the DHT treatment induced androgen response transcripts and reduced estrogen response transcripts. It is generally accepted that estrogen induces *PGR* mRNA and protein levels, and there is evidence that *ESR1* binds the estrogen receptor response element (ERE) of the *PGR* gene (Petz *et al.* 2004). Likewise, *Pgr* level is controlled by *Ar* through its competition with activated estrogen receptor on ERE site in the *Pgr* promoter (Peters *et al.* 2009).

To determine whether DHT alters the expression of sex steroid hormone receptors, we conducted a separate set of experiments in which *ESR1*- and *PGR*-positive cell lines, T47D (Yu *et al.* 2017) and MCF7 cells (Levenson & Jordan 1997), were incubated in medium depleted of steroids (i.e., containing 2% dextran charcoal-treated

Figure 2

Influence of 5 α -dihydrotestosterone (DHT) on expression of sex steroid receptors in human hormone-dependent breast cancer cell. T47D or MCF-7 cells were treated with vehicle alone or with DHT, and *AR* (A and D), *ESR1* (B and E), *PGR* (C and F) mRNA levels were determined by quantitative RT-PCR. Cells were grown in phenol red-free DMEM/F12 medium supplemented with 2% charcoal-treated FBS for 5 days before daily treatments with DHT at 10 nM. *GAPDH* mRNA was used as internal control. Values represent means $\pm$ S.E.M. of three separate experiments, $P<0.05$ vs vehicle-alone (control)-treated group.

FBS) and supplemented with 10 nM DHT. After 24 h, *AR* mRNA levels in DHT-treated cells were similar to those observed in vehicle-treated cells (Fig. 2A and D). More importantly, both *ESR1* and *PGR* mRNA levels in DHT-treated cells decreased significantly when compared to those seen in vehicle-treated control cells (Fig. 2B, C and F). This observation raises the possibility that DHT suppresses *PGR* levels with and without the activity of estrogen receptor.

To validate the role of androgen receptor or estrogen receptor in *PGR* expression, we examined the expression levels of *PGR* mRNA in ER α -negative HEK 293 cells that had been transfected with cDNA constructs encoding the human androgen receptor or estrogen receptor α . As expected, ER α -containing HEK 293 cells induced expression of the *PGR* mRNA, while *PGR* mRNA levels in AR-containing HEK 293 were not different from those in control-HEK 293 cells (Supplementary Fig. 1A, see section on supplementary data given at the end of this article). Although AR can inhibit activation of target genes that mediate the stimulatory effects of 17 β -estradiol on T47D cells (Peters *et al.* 2009), our result suggests that *PGR* inhibition is due to androgens and not AR-mediated signaling (Fig. 2C and F and Supplementary Fig. 1A). While the *PGR* mRNA expression is decreased in HEK

Figure 3

Impact of siRNA-mediated knockdown or *PGR* overexpression on the *Cyp24a1* and *Vdr* mRNA expression. PCT cells were transfected with either 100 nM control siRNA (con si) or progesterone receptor siRNA (Pgr si), and then cultured in medium containing 2% FBS for a further 24 h (A). *Cyp24a1* (B), and *Vdr* (C) mRNA levels were determined by quantitative RT-PCR, using *Gapdh* mRNA as an internal control. The values represent means $\pm$ S.E.M. of four experiments. $*P < 0.05$ when *PGR* siRNA treated PCT cells are compared to control siRNA treated PCT cells. PCT cells were transfected with *PGRa* or *PGRb* expression vectors, and then cultured in medium containing 2% FBS for a further 24 h (D, m=mouse, h=human). *Cyp24a1* (E), and *Vdr* (F) mRNA levels were determined by quantitative RT-PCR, using *Gapdh* mRNA as an internal control. The values represent means $\pm$ S.E.M. of four experiments. $*P < 0.05$ when *PGRa* or *PGRb* expression vectors treated PCT cells are compared to control vector treated PCT cells.

293 cells following treatment with DHT (Supplementary Fig. 1B), AR mRNA levels were unchanged compared to those in vehicle-treated HEK 293 cells (Supplementary Fig. 1C). These results indicate that androgen-mediated *Pgr* suppression might be concerned with suppression of estrogen receptor.

Progesterone receptor modulates *Cyp24a1* expression in mouse proximal convoluted tubule (PCT) epithelial cells

Modulation of sex steroid hormone receptors may occur in cells where sex steroids act in concert with vitamin D to influence important physiological events, such as calcium homeostasis (Van Cromphaut *et al.* 2003). To examine whether the loss of progesterone receptor in

PCT cells enhances or blocks the expression of vitamin D-related genes, *Cyp24a1* and *Vdr*, we depleted *Pgr* in PCT cells by treating them with a siRNA specific for *Pgr* mRNA (Fig. 3A). The siRNA treatment not only reduced *Pgr* mRNA levels, but also reduced *Cyp24a1* mRNA levels in these cells (Fig. 3B). On the other hand, renal *Vdr* mRNA levels remained similar in PCT cells treated with *PGR* siRNA when compared to cells treated with control siRNA (Fig. 3C). When we increased *PGR* production in PCT cells by transfecting them with a vector expressing human *PGR* (Fig. 3D), we observed an increased expression of *Cyp24a1* (Fig. 3E). However, *Vdr* mRNA levels were not significantly different in cells expressing human *PGR* compared with cells that had been transfected with a control vector (Fig. 3F). These observations suggest that stimulation of *PGR* activity

Figure 4

Standard ChIP qPCR analysis of *PGR* occupancy on *Cyp24a1* in the mouse PCT cell line. After PCT cells were cultured in medium containing 2% FBS for a further 24 h, they were treated with 10 nM progesterone for 1 h, and then crosslinked by formaldehyde. The region of *PGR* occupancy on *Cyp24a1* gene is located 7775bp upstream the transcription initiation site (170,322,016 in chromosome 2). The full palindromic PRE motif was indicated with red box (A). *Pgr*, *Ppargc1a*, and *Sox17* gene promoter were introduced with positive control (B and C). A full color version of this figure is available at <http://dx.doi.org/10.1530/JME-17-0187>.

may result in increased Cyp24a1 expression in PCT cells. A recent ChIP sequencing (ChIP-seq) study using the mouse uterus identified binding events in the *Pgr* regulatory regions (Rubel *et al.* 2012). Therefore, we examined whether 10 nM progesterone-treated PCT cells exhibit increased recruitment of *PGR* to the progesterone response element (PRE) found in gene *Cyp24a1* of mouse chromosome 2 (Fig. 4A). As shown in a previous study (Rubel *et al.* 2012), an enrichment in *PGR* binding was observed in the promoter regions of genes *Pgr*, *Ppargc1a* and *Sox17*. More importantly, the ChIP-seq experiment confirmed that *PGR* was indeed recruited to a regulatory region in gene *Cyp24a1* that contained the consensus progesterone response element, AGAATA/TGTTCT (Fig. 4B). As shown in Fig. 4B and C, binding of *PGR* to this site was significantly augmented in progesterone-treated PCT cells. To validate this result, promoter regions of genes *Pgr*, *Ppargc1a* and *Sox17* were introduced as positive controls in the *PGR* ChIP experiment (Fig. 4B and C). Our finding suggests that *PGR* regulates

Figure 5

Influence of 5α-dihydrotestosterone (DHT) on vitamin D-related genes. After orchietomy and recovery of mice for 2 weeks, male mice were treated with vehicle alone (vehicle), with DHT (50 μg/day) or DHT plus Bicalutamide (1 mg/day) for 5 days, and renal *Cyp24a1* (A), *Vdr* (B), and *Cyp27b1* (C) mRNA levels were determined by quantitative RT-PCR. *Gapdh* mRNA was used as internal control. Values represent means ± s.e.m. §*P* < 0.05 vs sham control group. *P < 0.05 vs orchietomy vehicle-alone (control) treated group. #*P* < 0.05 vs DHT-bicalutamide treated group. In panel (D), the levels of renal CYP24A1, and VDR compared to PRA expression were analyzed by Western blotting. For these experiments, 40 μg samples of protein extracts were analyzed, and β-ACTIN were also examined as internal controls. Relative amounts of protein expression in orchietomy vs sham control was evaluated based on Western blot analysis. PCT cells were treated with vehicle alone (control) or with DHT, and *Pgr* mRNA levels were determined by RT-PCR (E), and *Cyp24a1* (F), and *Vdr* (G) mRNA levels were determined by quantitative RT-PCR. Cells were grown in phenol red-free DMEM/F12 medium supplemented with 2% charcoal-treated FBS for 5 days before daily treatments with DHT at 10 nM. *Gapdh* mRNA was used as internal control. In panel (H), the levels of CYP24A1, and VDR in the corresponding PRA expression were assessed by Western blotting. For these experiments, 40 μg samples of protein extracts were analyzed, and β-ACTIN were also examined as internal controls. The values represent means ± s.e.m. of four experiments. *P < 0.05 when DHT-treated PCT cells are compared to vehicle-treated PCT cells.

Cyp24a1 expression, and in turn, controls vitamin D₃ 24-hydroxylase activity.

DHT modulates the level of plasma 25-hydroxyvitamin D3 by progesterone-dependent Cyp24a1 expression

Orchidectomized mice exhibited lower renal *Cyp24a1* mRNA levels (Fig. 5A) when compared to sham-operated animal. More importantly, renal *Cyp24a1* mRNA levels in orchidectomized mice were significantly decreased by DHT when compared to those in vehicle-treated mice or DHT-bicalutamide co-treated mice (Fig. 5A). On the other hand, renal *Vdr* mRNA levels were similar between DHT-treated mice and vehicle-treated mice (Fig. 5B). We further observed from Western blots that DHT treatment markedly decreased the abundance of renal 24-hydroxylase protein (Cyp24a1) relative to that of the vehicle-treated group (Fig. 5C). These observations suggest that the decreased renal PRA expression observed in DHT-

Figure 6

Influence of DHT to vitamin D and vitamin D-related genes expression. Level of 25-hydroxyvitamin D in mouse serum is determined by enzyme-linked immunosorbent assay (A). Values represent means $\pm$ S.E.M. * P < 0.05 vs orchidectomy vehicle-alone (control) treated group. # P < 0.05 vs DHT-bicalutamide treated group. Schematic representation highlighting the molecular link between androgen (DHT), progesterone receptor, CYP24A1, and the vitamin D metabolites (B). *Cabp9k* and *Cabp28k* mRNA levels were determined by quantitative RT-PCR (C and D). *Gapdh* mRNA was used as internal control. Values represent means $\pm$ S.E.M. * P < 0.05 vs sham control group.

treated group may suppress 24-hydroxylase expression in orchidectomized mice (Fig. 5C). We cultured PCT cells in a medium depleted of steroids (i.e., containing 2% dextran charcoal-treated FBS) and then treated them in the same medium with and without 10 nM DHT for 24 h. Using mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as an internal standard, it was evident that *Pgr* mRNA levels were reduced in these cells after DHT treatment, as shown in Fig. 5D. Further comparison of DHT-treated PCT cells with vehicle-treated cells revealed a significant decrease in *Cyp24a1* mRNA levels (Fig. 5E) and slight differences in *Vdr* mRNA levels (Fig. 5F). Parallel Western blot analyses showed that both progesterone receptor alpha (PRA) and 24-hydroxylase protein levels were suppressed in DHT-treated PCT cells when compared to those seen in vehicle-treated controls (Fig. 5G). These observations are consistent with the notion that an androgenic environment inhibits PRA expression, and this in turn suppresses 24-hydroxylase expression. To determine whether reduced 24-hydroxylase expression by DHT influences the levels of vitamin D₃, we monitored plasma 25-hydroxyvitamin D₃ in orchidectomized mice following treatment with DHT with and without bicalutamide for 5 days. We observed a significant increase in 25-hydroxyvitamin D₃ in DHT-treated orchidectomized mice, as compared to that in corresponding vehicle-treated mice (Fig. 6A). In addition, co-treatment with bicalutamide almost completely blocked the DHT-stimulated increase in serum 25-hydroxyvitamin D₃ (Fig. 6A), indicating that this stimulation must also be mediated by the androgen receptor, and this is linked with the previous results that show DHT regulating the expression of *Pgr* (Fig. 5E and H) regardless presence of estrogen receptor. To summarize,

our results demonstrate that androgen (DHT) signaling controls 24-hydroxylase degradation by regulating *Pgr* expression at the transcriptional and translational levels (Fig. 6B). It is also supported by induction of vitamin D-related genes, including *Calbindin-9k* and *Calbindin-28k* mRNA levels (Fig. 6C and D), suggesting testosterone significantly increased in active vitamin D-response gene. We also observed significant increase in *Cyp27b1* mRNA levels in all orchidectomized mice when compared to sham-operated animal (Fig. 6E). This observation is consistent with the notion that an administration of estradiol to males increased *Cyp27b1* activity (Pike *et al.* 1978, Lechner *et al.* 2006).

Discussion

It is also established that androgen deficiency increases the risks of the common bone fractures associated with osteoporosis (Ross & Small 2002, Alibhai *et al.* 2010). Androgen deprivation is a hallmark of prostate cancer therapy, but it is associated with side effects such as low serum 25-hydroxyvitamin D₃ and osteoporosis (Diamond *et al.* 2004). In addition, there is a suggested evidence between radical orchidectomy and lower concentrations of 25-hydroxyvitamin D₃ (Foresta *et al.* 2010). In light of the importance of androgen in male reproduction and its link with vitamin D, which is a global regulator of calcium uptake and is implicated in osteoporosis and various types of cancer, we sought to investigate the role of androgen in the biosynthesis and metabolism of vitamin D.

Kidney androgen-regulated protein (Kap) gene is one of the most well-studied targets of androgen action in the mouse kidney (Toole *et al.* 1979, Meseguer & Catterall 1990).

Our studies revealed that DHT treatment of orchidectomized mice increased mRNA levels of androgen-responsive *Kap* when compared to those of vehicle-treated mice. Renal *Kap* gene expression is restricted to the PCT cells where it is more sensitive to androgen induction than other androgen-regulated genes, evidently because testosterone fails to induce *Kap* expression in *Tfm/y* Ar-deficient mice (Meseguer & Catterall 1990). While DHT-stimulated *Kap* expression at the transcriptional level, treatment of DHT had the opposite effect on *Esr1* and *Pgr* expression in the mouse kidney (Petz *et al.* 2004, Peters *et al.* 2009). *In vitro* analysis showed that DHT exposure significantly decreased the expression of *ESR1* and *PGR* transcripts in breast cancer cells (T47D and MCF7) expressing estrogen, androgen and progesterone receptors. These findings are particularly interesting in light of reports that DHT treatment induced the activity of androgen receptor and reduced the expression of progesterone receptors in T47D cells (Liberato *et al.* 1993). Furthermore, there is evidence that testosterone suppressed *ESR1* expression in mammary glands of non-human primates (Poulin *et al.* 1989, Zhou *et al.* 2000, Dimitrakakis *et al.* 2003). Moreover, early studies reported that the *PGR* is regulated by estrogen in breast cancer cells (Nardulli *et al.* 1988, Read *et al.* 1988). It is now known that the *PGR* gene contains estrogen response element site in its promoter region (Petz *et al.* 2004) and that its expression can be regulated indirectly by estrogen receptor-mediated effects (Petz *et al.* 2002). Recent studies have demonstrated that the *PGR* is regulated by the activity of the androgen receptor, which interacts with an ERE in the *PGR* promoter (Peters *et al.* 2009).

The kidney is a site for *PGR* expression in humans, and both *PGR* transcripts and protein are detected in the renal cortex (Bumke-Vogt *et al.* 2002). To further define the role of *PGR* in renal function, we used an immortalized mouse PCT cells, which is responsible for activation of sex steroid hormones (Meseguer & Catterall 1987) or sex hormone-binding globulin (Hong *et al.* 2011). After treatment with *Pgr*-specific siRNA in PCT cells, the corresponding *Pgr* deficiency was also accompanied by decreased *Cyp24a1* mRNA levels when compared to those seen with control siRNA treatment. The correlation between *Pgr* and *Cyp24a1* levels in PCT cells was evaluated following transfection with the *PRa* or *PRb* expression plasmids. In the context of vitamin D metabolism, the presence of the *PGR* was found to enhance *Cyp24a1* expression, which encodes the vitamin D-inactivating enzyme, 24-hydroxylase. Reduced levels of *Cyp24a1* mRNA were observed in

the kidney of DHT-treated orchidectomized mice and in DHT-treated PCT cells. DHT has been previously linked to inhibit *Cyp24a1* expression and $1\alpha,25$ -dihydroxyvitamin D_3 catabolism (Lou *et al.* 2005), and physiological concentration of DHT has been shown to suppress the anti-proliferation effect of $1\alpha,25$ -dihydroxyvitamin D_3 in LNCaP cells (Zhao *et al.* 1997). Since DHT did not alter the basal level of *Cyp24a1* mRNA in LNCaP cells and the 24-hydroxylase gene does not have androgen response element (ARE) in promoter region, DHT indirectly suppresses in *Cyp24a1* expression through DNA-independent inhibition (Lou *et al.* 2005). While *Cyp24a1* expression in PCT cells was significantly decreased by treatment with DHT, the relative amount of the *PGR* was consistently lower in DHT-treated PCT cells. The latter is important because *PGR*, which represents genes directly inhibited by DHT, is involved in the regulation of transcription and cellular function (Li *et al.* 2003). These observations address the obvious question of whether progesterone receptor in the kidney restricts or enhances renal functions such as mineral absorption and hormone production.

Like other steroid hormone receptors, *PGR* also contains a DNA-binding domain for specific progesterone-responsive element DNA sequences on putative target genes (Mangelsdorf *et al.* 1995). *Cyp24a1* expression is controlled by suppression of *PGR* levels *via* DHT. We provide evidence that the loss of *PGR* transcripts results in reduced renal 24-hydroxylase, which catabolizes 25-dihydroxyvitamin D_3 . Recent ChIP sequencing studies have reported *PGR*-binding events in the regulatory regions of various transcriptional factors in the murine uterus, as well as the presence of a canonical palindromic steroid receptor hormone response element, AGAACAnnnTGGTCT (Umesono & Evans 1989, Rubel *et al.* 2012). Using ChIP sequencing in renal PCT cells, we confirmed that *PGR* was indeed recruited to the gene *Cyp24a1*, or more specifically, to the regulatory region of this gene that contained the consensus progesterone response element, AGAATACTGCATGTTCT. Accordingly, we observed that DHT inhibited the expression of *Cyp24a1* mRNA and 24-hydroxylase by PR-mediated action. Although we considered physiological relevance like estrogen-induced *PGR*, it is clear that androgen predominantly exists over estrogen in male. *PGR* and *ESR1* under condition of androgenic inhibition could be linked with bone strength by activated vitamin D (Vanderschueren *et al.* 2014).

Calcitriol regulates its own metabolism by stimulating renal *Cyp24a1* while it suppresses renal *Cyp27b1* (Bikle

2014). Since surgical orchiectomy reduced plasma $1\alpha,25$ -dihydroxyvitamin D_3 and testosterone restored orchiectomy effects (Nyomba *et al.* 1987), the reduced calcitriol was related with low *CYP24A1* expression (Jones *et al.* 1998). In present study, orchidectomized mice exhibited lower renal *Cyp24a1* mRNA levels and higher *Cyp27b1* mRNA levels when compared to sham-operated animal. Based on the results, it is possible that the orchidectomized mice might have low calcitriol level, and that the reduced calcitriol might reflect low levels of *Cyp24a1* mRNA. In orchiectomy condition with low levels of calcitriol, DHT treatment additionally suppressed the renal *Cyp24a1* mRNA levels. This is a particularly interesting evidence that the level of 25-hydroxyvitamin D_3 increased under conditions where the supply for DHT is present, and that the increased levels was restored by bicalutamide. As reported in several previous studies, the serum concentrations of calcitriol decreased after orchiectomy in male rats and were restored by treatment with testosterone (Tanaka *et al.* 1976, Pike *et al.* 1978). Accordingly, the possibility exists that the DHT-induced 25-hydroxyvitamin D_3 might be converted to calcitriol because of DHT-mediated 24-hydroxylase suppression. This is also relevant with previous findings that have shown male kidneys in other species preferentially hydroxylate 25-hydroxyvitamin D_3 using 24-hydroxylase rather than 25-hydroxyvitamin D_3 -1- α hydroxylase (Pike *et al.* 1978). While androgen action suppressed the 24-hydroxylase expression, the androgen withdrawal could preferentially hydroxylate 25-hydroxyvitamin D_3 to 24,25-hydroxyvitamin D_3 .

24-hydroxylase has been previously implicated in vitamin D homeostasis, which is evident from the low levels of vitamin D seen in *Cyp24a1* transgenic rat (Hosogane *et al.* 2003) and the slow clearance of exogenous 25-dihydroxyvitamin D_3 in *Cyp24a1*-null mice (Masuda *et al.* 2005). In agreement with several other studies (Hosogane *et al.* 2003, Lou *et al.* 2005, Masuda *et al.* 2005), we also expect androgen to enhance vitamin D-mediated functions by suppressing *CYP24A1* expression. Using the vitamin D-response gene including *Calbindin-9k* and *Calbindin-28K*, we confirm the bioconversion into calcitriol in DHT-induced 25-hydroxyvitamin D_3 increased condition. Although it has recently become apparent that both protein are not required for the uptake of calcium absorption (Koo *et al.* 2012), the evidence for the vitamin D-dependent uptake of calcium are well described as indicator of plasma vitamin D level (Belkacemi *et al.* 2005, Hong & Jeung 2013). This is demonstrated in induction of vitamin D-response genes by DHT exposure.

The results presented in our report indicate that androgen deficiency within renal function acts to enhance 24-hydroxylase expression and suppresses the actions of vitamin D, especially under conditions of limited supply of androgens. These findings uncover an important role for androgen in vitamin D homeostasis and suggest that therapeutic modulation of progesterone receptor may be used to treat vitamin D deficiency and related disorders.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-17-0187>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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