Androgen suppress the level of Cyp24a1 expression

Sang R Lee1, Mi-Young Park1, Hyun Yang2, Geun-Shik Lee2 Beum-Soo An4, Bae-kuen Park1, Eui-Bae Jeung5 and Eui-Ju Hong1

1College of Veterinary Medicine, Chungnam National University, Daejeon, Republic of Korea
2Korean Institute of Oriental Medicine, Daejeon, Republic of Korea
3College of Veterinary Medicine, Kangwon National University, Chuncheon, Gangwon, Republic of Korea
4Department of Biomaterials Science, College of Natural Resources & Life Science, Pusan National University, Miryang, Republic of Korea
5College of Veterinary Medicine, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea

Correspondence should be addressed to E-J Hong: ejhong@cnu.ac.kr

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 5α-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-alpha 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.

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
Androgens suppress the level of Cyp24a1 expression

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 1α,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 D₃-1α hydroxylase (CYP27B1) at the 1α position in the renal cortex, which gives rise to the active form of vitamin D, 1α,25-dihydroxyvitamin D₃ (Jones et al. 1998). Both 25-hydroxyvitamin D₃ and 1α,25-dihydroxyvitamin D₃ can be further hydroxylated by the vitamin D-inactivating enzyme, 24-hydroxylase (CYP24A1). 1α,25-dihydroxyvitamin D₃ plays a major role in regulating calcium homeostasis. Low levels of 1α,25-dihydroxyvitamin D₃ increase the release of parathyroid hormone that stimulates CYP27B1 expression, while high levels of 1α,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, 5α-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 Krap 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 orchectomy. 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/mL) 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/mL) and streptomycin (100 µg/mL).

Gene knockdown and gene overexpression assay

Briefly, 4 × 10⁵ 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 5% 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/mL) and streptomycin (100 µg/mL).

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

http://jme.endocrinology-journals.org
https://doi.org/10.1530/JME-17-0187
© 2018 Society for Endocrinology
Published by Bioscientifica Ltd.
Printed in Great Britain

Downloaded from Bioscientifica.com at 11/20/2018 11:37:39AM via free access
Androgen suppress the level of Cyp24a1 expression

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 60 min using 1 µg 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 immunosorbed 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 450 nm 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 (orchiectomy) on male mice. After removal of the principal source for endogenous androgen production, mice were treated with DHT (50 μg/day) or DHT-bicalutamide-treated (1 mg/day) for 5 days. We observed clear decreases in Kap mRNA levels in the kidneys of all orchiectomy 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.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Upper primer (5′–3′)</th>
<th>Lower primer (5′–3′)</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kap</td>
<td>TCTCTGTGGTCTGACTGTGGCC</td>
<td>CGTGAAGTCCATGATCTGCT</td>
<td>Mouse</td>
</tr>
<tr>
<td>Ar</td>
<td>CTGGGAAGGCTTACCACAC</td>
<td>GGTTGCTATGTAGGCGCCTC</td>
<td>Mouse</td>
</tr>
<tr>
<td>ESR1</td>
<td>TGTGTCAGCTACAAAGCAATG</td>
<td>CATCATGCCACCTTCGTAACA</td>
<td>Mouse</td>
</tr>
<tr>
<td>PGR</td>
<td>CTCGGGAACCGCAACAGAATG</td>
<td>ACACAAACCCTTTGAGTACAG</td>
<td>Mouse</td>
</tr>
<tr>
<td>VDR</td>
<td>ACCCTGGTCTCTTGGCAGCC</td>
<td>GGCATCTCCATAGAAAGG</td>
<td>Mouse</td>
</tr>
<tr>
<td>Cyp24a1</td>
<td>CTGCCCTCTTGGACAGAGTC</td>
<td>CCTACCGTCTGTCATAGC</td>
<td>Mouse</td>
</tr>
<tr>
<td>Cyp27b1</td>
<td>GCCAGATTTACGGTGCGAC</td>
<td>CTGGATCAGCTAGGTCGTC</td>
<td>Mouse</td>
</tr>
<tr>
<td>Gapdh</td>
<td>GTCACTGATCTTACGTCGTC</td>
<td>GCCGCCCTATGCAATGACG</td>
<td>Human</td>
</tr>
<tr>
<td>AR</td>
<td>GCAGCACAGATGCTCCTATT</td>
<td>CGTCGATTACTGAATTGGTC</td>
<td>Human</td>
</tr>
<tr>
<td>ESR1</td>
<td>CCCACTGCAACAGCCTGTTCTC</td>
<td>AGGACACATAAGACATCAC</td>
<td>Human</td>
</tr>
<tr>
<td>PGR</td>
<td>ACCCGCCCTCTCTCAACTACC</td>
<td>GCCCTTGTCTCCTTCTTGGCA</td>
<td>Human</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGAGCCGAGATCCCCTCAAAAT</td>
<td>AGCTCGTCTACCTCAGATT</td>
<td>Human</td>
</tr>
<tr>
<td>Cyp24a1</td>
<td>GCCAGACCTCGTAGTGGTCTC</td>
<td>AGCTCTGGAGCTCTCAGAT</td>
<td>Human</td>
</tr>
<tr>
<td>Pparg1a</td>
<td>CCGGCAAGTACATGCCTGCT</td>
<td>CAAGTACTGTCGTAGCTT</td>
<td>Human</td>
</tr>
<tr>
<td>Sox7</td>
<td>TCTACCGTGCCTTCTGCT</td>
<td>TCCCAGGTTTATTTTGGG</td>
<td>Mouse</td>
</tr>
</tbody>
</table>

Downloaded from Bioscientifica.com at 11/20/2018 11:37:39AM via free access
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 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 FBS) and supplemented with 10nM DHT. After 24h, 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 EAr-negative HEK 293 cells that had been transfected with cDNA constructs encoding the human androgen receptor or estrogen receptor α. As expected, EAr-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 17β-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
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 expression 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...
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, Pparγ1a 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/TGGTCT (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, Pparγ1a and Sox17 were introduced as positive controls in the PGR ChIP experiment (Fig. 4B and C). Our finding suggests that PGR regulates 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-
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 10nM DHT for 24 h. Using mouse glycerinaldehyde-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, F and G) 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, Lechnt 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 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 (Liberto 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 1α,25-dihydroxyvitamin D₃ catabolism (Lou et al. 2005), and physiological concentration of DHT has been shown to suppress the anti-proliferation effect of 1α,25-dihydroxyvitamin D₃ 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 (Mangelsdorff 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 D₃. 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, AGAACAnnTGTTC (Umeseono & 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, AGAATGCATGTTC. 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...
Since surgical orchectomy reduced plasma 1α,25-dihydroxyvitamin D₃ and testosterone restored orchectomy 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 orchectomy 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 D₃ 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 orchietomy 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 D₃ 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 D₃ using 24-hydroxylase rather than 25-hydroxyvitamin D₃-1-alpha hydroxylase (Pike et al. 1978). While androgen action suppressed the 24-hydroxylase expression, the androgen withdrawal could preferentially hydroxylate 25-hydroxyvitamin D₃ to 24,25-hydroxyvitamin D₃.

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 D₃ 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 D₃ 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.

Funding
This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2013R1A1A2057648 and 2015R1D1A1A01060335 to E-J H). This work was also supported by research fund of Chungnam National University.

References
Bieche I, Narjoc C, Asselah T, Vacher S, Marcellin P, Lidereau R, Beaune P & de Waziers I 2007 Reverse transcriptase-PCR quantification of mRNA levels from cytochrome (CYP)1, CYP2 and CYP3 families in 22 different human tissues. Pharmacogenetics and Genomics 17 731–742. (https://doi.org/10.1097/FPC.0b013e32810f2e58)


Deeb KK, Trump DL & Johnson CS 2007 Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. *Nature Reviews Cancer* **7** 684–700. ([https://doi.org/10.1038/nrc2196](https://doi.org/10.1038/nrc2196)).


Iida K, Shinki T, Yamaguchi A, DeLuca HF, Kurokawa K & Suda T 1995 A placenta in the knockout mice of calbindin-D(9k) and -D(28k).


Liberato MH, Sonohara S & Brentani MM 1993 Effects of androgens on proliferation and progesterone receptor levels in T47D human breast cancer cells. *Tumor Biology* **14** 38–45. ([https://doi.org/10.1159/0000217823](https://doi.org/10.1159/0000217823)).


Meseguer A & Catterall JF 1987 Mouse kidney androgen-regulated protein messenger ribonucleic acid is expressed in the proximal convoluted tubules. *Molecular Endocrinology* **1** 535–541. ([https://doi.org/10.1210/mend-1-8-535](https://doi.org/10.1210/mend-1-8-535)).

Meseguer A & Catterall JF 1990 Cell-specific expression of kidney androgen-regulated protein messenger RNA is under multihormonal control. *Molecular Endocrinology* **4** 1240–1248. ([https://doi.org/10.1210/mend-4-8-1240](https://doi.org/10.1210/mend-4-8-1240)).


Nardulli AM, Greene GL, O’Malley BW & Katzenellenbogen BS 1988 Androgen action in proximal tubule epithelial cells. *Molecular Endocrinology* **2** 269–281. ([https://doi.org/10.1210/mend-2-4-269](https://doi.org/10.1210/mend-2-4-269)).

Nyomba BL, Bouillon R & De Moor P 1987 Evidence for an interaction of insulin and sex steroids in the regulation of vitamin D metabolism in the rat. *Journal of Endocrinology* **115** 295–301. ([https://doi.org/10.1677/joe.0.1150295](https://doi.org/10.1677/joe.0.1150295)).


Tanaka Y, Castillo I & DeLuca HF 1976 Control of renal vitamin D hydroxylases in birds by sex hormones. PNAS 73 2701–2705. (https://doi.org/10.1073/pnas.73.8.2701)


Yu S, Kim T, Yoo KH & Kang K 2017 The T47D cell line is an ideal experimental model to elucidate the progesterone-specific effects of a luminal A subtype of breast cancer. Biochemical and Biophysical Research Communications 466 752–758. (https://doi.org/10.1016/j.bbrc.2017.03.114)


Zhu JG, Ochalek JT, Kaufmann M, Jones G & Deluca HF 2013 CYP2R1 is a major, but not exclusive, contributor to 25-hydroxyvitamin D production in vivo. PNAS 110 15655–15655. (https://doi.org/10.1073/pnas.1315006110)


Received in final form 10 December 2017
Accepted 22 December 2017