Calcitrol (1α,25-dihydroxyvitamin D_3) inhibits androgen glucuronidation in prostate cancer cells

Jenny Kaeding,[1,2] Julie Bélanger,[1,2] Patrick Caron,[1,2] Mélanie Verreault,[1,2] Alain Bélanger,[1,3] and Olivier Barbier[1,2]

[1]Molecular Endocrinology and Oncology Research Center, Laval University Hospital Centre Research Center, [2]Faculty of Pharmacy, and [3]Faculty of Medicine, Laval University, Sainte-Foy, Quebec, Canada

Abstract

Calcitriol (1α,25-dihydroxyvitamin D_3), the active metabolite of vitamin D, has recently emerged as a promising therapeutic agent in the treatment of prostate cancer, the second most common cause of cancer death in American males. In the present study, we have analyzed the effects of calcitriol treatment on the expression and activity of the UDP-glucuronosyltransferase (UGT) 2B15 and 2B17 in prostate cancer LNCaP and 22Rv1 cells. These two enzymes share a crucial role in the inactivation of androgens in the human prostate. We report that calcitriol treatment results in lower glucuronide conjugation of the active androgen dihydrotestosterone and its reduced metabolites androstane-3α-diol and androsterone in LNCaP cells. The same treatment also drastically decreased the mRNA and protein levels of UGT2B15 and UGT2B17 in LNCaP and 22Rv1 cells. Using casodex, an androgen receptor (AR) antagonist, and AR-specific small interfering RNA probes, we show that calcitriol requires a functional AR to inhibit the expression of the *UGT2B17* gene in LNCaP cells. By contrast, transient transfection and site-directed mutagenesis experiments revealed that calcitriol down-regulates *UGT2B15* promoter activity through a responsive region between positions -171 and -113 bp. In conclusion, the present study identifies the vitamin D receptor activator calcitriol as a negative regulator of the UGT2B15- and UGT2B17-dependent inactivation of androgens in prostate cancer LNCaP cells. Androgens promote prostate cancer cell proliferation; thus, the reduction of their inactivation could have a limiting effect of the calcitriol antiproliferative properties in prostate cancer cells. [Mol Cancer Ther 2008;7(2):380–90]

Introduction

Prostate cancer is the second most common cause of cancer death in Americans (1). Over the last several years, the active metabolite of vitamin D, 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$, calcitriol], has been identified as a promising anticancer agent (2). In addition to its classic targets (the regulation of calcium homeostasis and bone metabolism), calcitriol exhibits both antiproliferative and prodifferentiating actions in several malignant cells and tumors, including those from prostate cancer (3–5). Furthermore, in clinical trials, calcitriol shows synergistic and/or additive effects with chemotherapy, radiation, or other anticancer treatments (6, 7). The mechanisms underlying the antiproliferative activity of calcitriol are varied and are cell type specific (8). Nevertheless, calcitriol exerts antiproliferative actions in a variety of prostate cancer cell lines (9–11) as well as in primary cultures of normal and cancer cells (12). Most of its biological effects are mediated by direct transcriptional regulation of specific target genes. The vitamin D derivative binds to and activates the nuclear receptor VDR, which in turn regulates the transcriptional activity of responsive genes (13).

The androgens testosterone and dihydrotestosterone (DHT) are required for prostate growth but also play an important role in prostate cancer development. Both experimental and epidemiologic data indicate that androgens are among the main factors controlling the development, maintenance, and progression of prostate cancer (13, 14). Thus, a first-line treatment strategy for advanced prostate cancer is androgen deprivation. DHT is extensively metabolized in the prostate (reviewed in refs. 15, 16) to ultimately form androstane-3α-diol-17glucuronide (3α-diol-17G) and androsterone-3glucuronide (ADT-3G), which are the major androgen metabolic end products found in circulation (17, 18). Glucuronidation is a conjugation reaction, in which the glucuronosyl group from the cofactor UDP-glucuronic acid is transferred to an acceptor molecule catalyzed by enzymes of the UDP-glucuronosyltransferase (UGT) superfamily (19). Among the 18 functional human UGT isoforms identified thus far, only UGT2B7, UGT2B15, and UGT2B17 are able to conjugate androgens and only UGT2B15 and UGT2B17 are expressed in the human prostate (15). The amino acid sequence identity between these two enzymes is very high (95%), but their specificity towards 5α-reduced androgens differs significantly. The

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Requests for reprints: Olivier Barbier, Centre Hospitalier Universitaire de Québec-Laval University Hospital Centre Research Center, 2705, Boulevard Laurier, Sainte-Foy, Quebec G1V 4G2, Canada.
Phone: 418-654-2296; Fax: 418-654-2769.
E-mail: olivier.barbier@pha.ulaval.ca

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conjugating activity of UGT2B15 is limited to the formation of 3α-diol-17G and DHT-17G, whereas UGT2B17 forms 3α-diol-17G, DHT-17G, and ADT-3G (20, 21).

Although several genes regulated by calcitriol have been identified in prostate cancer cells, the particular effect of this active vitamin D metabolite on androgen metabolism has not been investigated. Considering the fact that a microarray study in LNCaP cells revealed a significant reduction of UGT2B15 expression after treatment with calcitriol (22) and that the major role of glucuronidation is the inactivation of androgens, the present study was undertaken to elucidate the effect of calcitriol on the expression of UGT2B15 and UGT2B17 and on their capacity to inactivate 5α-reduced androgens. The androgen-dependent prostate cancer cell line LNCaP is considered a classic model for the study of androgen metabolism in the prostate (18) and was therefore used in this study. LNCaP cells express a functional androgen receptor (AR; ref. 23) and VDR (24, 25) as well as the two androgen-glucuronidating UGT isoforms characterized in the prostate, UGT2B15 and UGT2B17 (26).

Materials and Methods

Materials

$1,25(OH)_2D_3$, UDP-glucuronic acid, poly-L-lysine, cycloheximide (CHX), the anti-actin antibody, and the steroid substrates (3α-diol, ADT, and DHT) were obtained from Sigma. 3α-Diol-17G, ADT-3G, and DHT-17G were purchased from Steraloids and 3α-diol-3G was purchased from Research Plus. Casodex was provided by Endorecherche, and the synthetic AR agonist, R1881, was from Perkin-Elmer. Protein assay reagents were obtained from Bio-Rad Laboratories. Cell culture materials and transfection reagents were obtained from Invitrogen. SYBR Green PCR mix was purchased from Applied Biosystems. The anti-AR antibody was purchased from Santa Cruz Biotechnology, the anti-UGT2B15 and anti-UGT2B17 antibodies were described previously (26, 27), and the secondary antibody against rabbit IgG was purchased from Amersham.

Cell Culture

Human prostate cancer LNCaP, 22Rv1, PC-3, and DU145 cells were obtained from the American Type Culture Collection. LNCaP and 22Rv1 cells were grown in RPMI 1640 supplemented with 10% FBS; PC-3 cells in Ham's F-12K medium supplemented with 10% FBS and DU145 cells were cultured in MEM supplemented with 10% FBS. For RNA and protein analyses, LNCaP cells were seeded in six-well plates and cultured in RPMI 1640 supplemented with 0%, 0.2%, or 10% FBS, as indicated, for 48 h to allow cell attachment. 22Rv1, PC-3, and DU145 cells were seeded in 12-well plates in their respective medium supplemented with 0.2% FBS and cultured overnight. All cell lines were then treated for the indicated duration with vehicle (DMSO or ethanol, 0.1%, v/v), calcitriol at indicated concentrations, R1881 (1 nmol/L), casodex (10 μmol/L), and/or CHX (20 μg/mL) in their respective medium supplemented with 0%, 0.2%, or 10% FBS as indicated. For glucuronidation assays, LNCaP cells were seeded in 10-cm culture dishes and cultured until 70% confluency. After a FBS deprivation period of 24 h in RPMI 1640 supplemented with 0.2% FBS, cells were treated with vehicle (ethanol) or calcitriol (10 nmol/L) for 48 or 96 h in the same medium (medium was changed after 48 h for the 72- and 96-h treatment periods). Finally, cells were harvested and resuspended in PBS (pH 7.4, 50 mmol/L) containing DTT (0.5 mmol/L) and homogenized by pipetting.

Glucuronidation Assay and Determination of C_{19}-Steroid Glucuronides

Glucuronidation assays were conducted using 20 μg total protein from homogenized LNCaP cells as described previously (28). The formation of glucuronide conjugates was analyzed by high-performance liquid chromatography using identification by mass spectrometry as already reported (28, 29).

RNA Extraction, Reverse Transcription, and Real-time PCR

Total RNA isolation and reverse transcription (RT) were done as described previously (28, 30). Real-time PCRs were done using an ABI Prism 7500 instrument from Applied Biosystems. Each 20 μL reaction was composed of 10 μL SYBR Green PCR mix, 2 μL of each primer, and 6 μL RT product (1:500 dilution). The cycling variables for real-time PCR were as follows: initial heating 95°C for 10 min followed by 40 cycles of 95°C for 15 s and the indicated temperature for 60 s. The following primers were used for amplification of the indicated cDNA: UGT2B15 forward 5-GTGTTGGGAATATTATGACTACAGTAAC-3 and reverse 5-GGGTATGTTAAATAGTTCAGCCAGT-3 (125 nmol/L each, 56°C), UGT2B17 forward 5-TGACTTTTGGTTTC-AAGC-3 and reverse 5-TTCCATTTCCTTAGGCAA-3 (300 nmol/L each, 56°C), TMPRSS2 forward 5-GTGATTTCT-CATCCAAATTA-3 and reverse 5-TCCAGCAGAGCTGTT-CTGGC-3 (300 nmol/L each, 60°C), and 28S forward 5-AAACTCTGGTGGAGGTCCGT-3 and reverse 5-CTTAC-CAAAGTGGCCCACTA-3 (200 nmol/L each, 56°C or 60°C). The specific amplification of each UGT cDNA was ensured by testing PCR strategies with all human UGT2B cDNAs (1.0 ng) and by direct sequencing of PCR products. C_T values were analyzed using the comparative C_T ($\Delta\Delta C_T$) method as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta CT}$) was obtained by normalizing to the endogenous reference 28S and was relativized to the vehicle-treated baseline control. For each gene, the amplification efficiency and the accuracies of the $\Delta\Delta C_T$ for UGTs versus 28S rRNA were tested using a 2 to 5 log of concentrations of cDNA produced from LNCaP cell purified mRNA.

Western Blot Analysis

Total protein from LNCaP cells was purified according to the Tri-Reagent acid/phenol protocol. For Western blot experiments, 1 μg human liver microsomes (BD Biosciences Discovery Labware) and 10 μg total protein were size separated by 10% SDS-PAGE and immunoblotted with anti-UGT2B15 or anti-UGT2B17 antibody as reported (26, 27),

an anti-AR antibody (1:4,000 dilution), or an anti-actin antibody (1:1,000 dilution). Relative expression levels were quantified using BioImage Visage 110s (Genomic Solution).

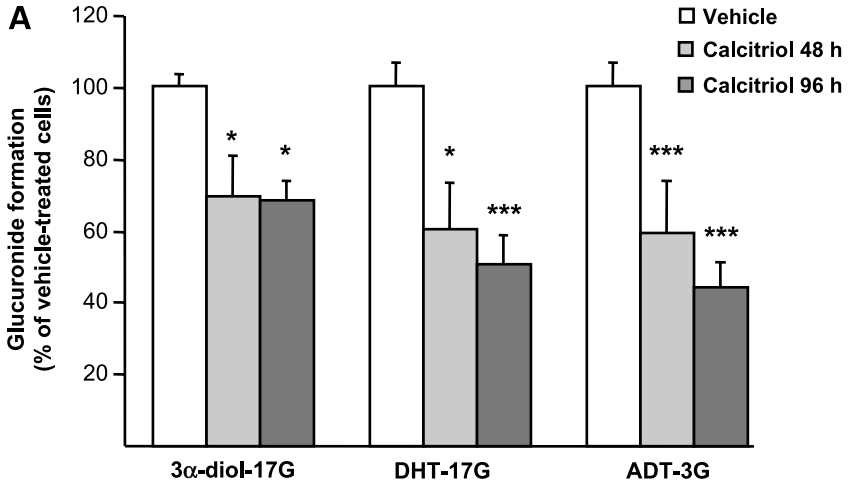
Reporter Constructs and Transient Transfection Assays

The luciferase constructs containing 2,401 bp of the *UGT2B15* and *UGT2B17* promoters and their deletions constructs (1,236, 876, 436, 326, 235, and 109 bp) were described previously (31, 32). To remove the putative 59-bp VDRE (-113 to -171 bp) insert (2B15-i59) from the *UGT2B15* promoter and to introduce it into the *UGT2B17* promoter, the pGL3-based 2.4-kb promoter constructs of *UGT2B15* and *UGT2B17* were digested by *Nde*I and *Nco*I. Fragments were separated by gel electrophoresis, and the digestion product from *UGT2B15* was cloned into the *Nde*I/*Nco*I-digested pGL3-based *UGT2B17* promoter construct and vice versa. In addition to the 59-bp insert, three nonidentical bases (at positions -382/-322, -314/-254, and -214/-154 in the promoters of UGT2B15/UGT2B17) were introduced in reporter constructs during the subcloning processes. Three changes, numbered *mut1-mut3*, were removed afterwards by site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene) and the following PCR primers (mutated nucleotides are italicized): UGT2B15 *mut1* (-382 bp), 5-GATATTAAAAAATG*C*CGTTTGAGTTGTAT-3′; UGT2B15 *mut2* (-314 bp), 5-AGCCTCTCAC*T*TGCCACTGTTCTTA-3′; UGT2B15 *mut3* (-214 bp), 5-GAGTAATTGTAA*C*ATAAAAGAACACC-3′; UGT2B17 *mut1* (-322 bp), 5-GATATTAAAAAATG*G*CGTTTGAGTGTAT-3′; UGT2B17 *mut2* (-254 bp), 5-AGCCTCTCAC*C*TGCCACTGTTCTTA-3′; and UGT2B17 *mut3* (-154 bp), 5-GAGTAATTGTAAA*T*ATAAAAGAACACC-3′. Mutations of the putative VDRE in the UGT2B15 -2,401-bp construct were also introduced using the same kit. The forward primers used for mutagenesis were as follows: UGT2B15-VDRE mutation, 5′-CACACTAAAATAAATATGA*TTTT*ATCAATCTTTTGTTGGTCTCC-3′. DNA sequencing was done for all constructs to verify the correct mutations and to ensure that no spurious mutations occurred elsewhere.

Lipofectin or LipofectAMINE 2000 (Invitrogen) was used as transfection reagent for LNCaP and 22Rv1 cells or PC-3 and DU145 cells, respectively. The indicated pGL3 reporter (100 ng) was cotransfected with 30 ng pCMV-β-GAL as an internal control. All samples were complemented with pBS-SK+ (Stratagene) to an identical amount of 500 ng/well. At 6 h post-transfection, the transfection medium was removed and cells were incubated in the presence of calcitriol at the indicated concentrations or vehicle (0.1%, v/v) in serum-free medium for 24 h. Subsequently, cells were harvested and cell lysates (20 μL) were assayed for luciferase and β-galactosidase activities.

Gene Silencing Using Synthetic Small Interfering RNA

Nontargeting or AR-specific small interfering RNAs (siRNA) were ON-TARGET plus SMART pool reagents from Dharmacon. LNCaP cells were transfected with siRNA using Lipofectin transfection reagent according to the manufacturer's instructions. At 24 h post-transfection, the medium was changed to RPMI 1640 supplemented with 0.2% FBS for 24 h. Cells were then treated with



B

Substrate	Glucuronide formation after 96 hours (ng/μg protein/h)	
	Vehicle	Calcitriol
3α-diol	341 ± 56	232 ± 29
DHT	760 ± 87	383 ± 55
ADT	232 ± 6	102 ± 11

Figure 1. Calcitriol decreases the glucuronidation of androgen metabolites in LNCaP cells. LNCaP cells were treated with vehicle or calcitriol (10 nmol/L) in RPMI 1640 supplemented with 0.2% FBS for 48 or 96 h, and glucuronidation assays were done with 3α-diol, ADT, and DHT (200 μmol/L) using 20 μg cell homogenates. Glucuronide formation was quantified by liquid chromatography/mass spectrometric analysis and corrected for the amount of total protein. Values (mean ± SD) are expressed as percentage of control (**A**) or as glucuronide formation (**B**). *, $P < 0.05$; ***, $P < 0.001$, statistically significant differences between control and calcitriol treatment (Student's *t* test).

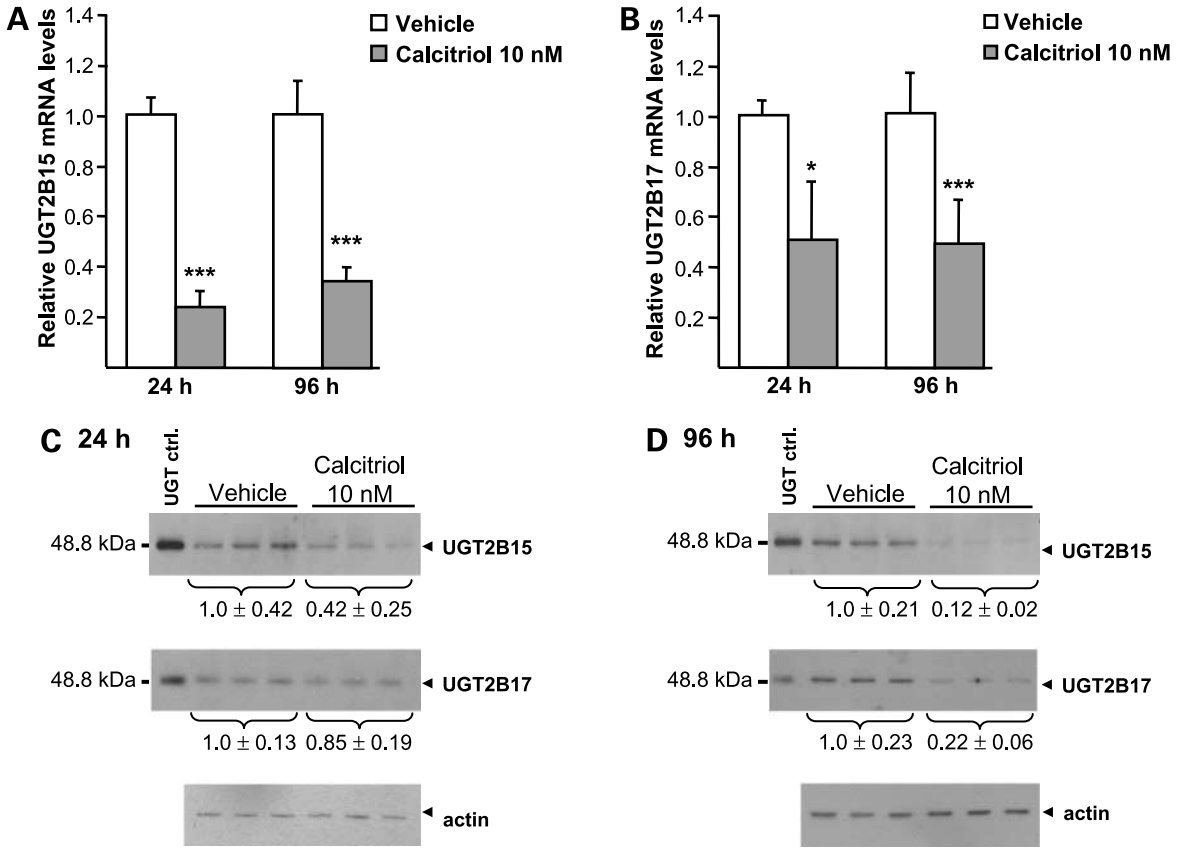


Figure 2. Calcitriol inhibits UGT2B15 and UGT2B17 expression in LNCaP cells. LNCaP cells were treated with vehicle or 10 nmol/L calcitriol in RPMI 1640 supplemented with 0.2% FBS for the indicated time. UGT2B15 (**A**) and UGT2B17 (**B**) mRNA levels were measured by real-time RT-PCR and normalized to 28S rRNA levels. Values are expressed as mean ± SD. *, $P < 0.05$; ***, $P < 0.001$, statistically significant differences between control and calcitriol treatment (Student's *t* test). **C** and **D**, 10 μg total protein for each sample was immunoblotted with anti-UGT2B15 (1:1,500; *top*) or anti-UGT2B17 (1:2,000; *middle*) antibodies. Human liver microsomes (2 μg) were used as a positive control (*UGT ctrl*). The equal loading of each lane was ensured by hybridizing with an anti-actin antibody (1:1,000; *bottom*). Immunoreactions were quantified by densitometry and are expressed as relative quantity of proteins compared with vehicle-treated cells after normalization with actin control.

calcitriol (10 nmol/L) or R1881 (1 nmol/L) in RPMI 1640 supplemented with 0.2% FBS for another 24 h. The AR protein levels and TMPRSS2 and UGT mRNA levels were analyzed as described above.

Statistical Analyses

All data are presented as mean ± SD. Statistical analysis was done using a two-tailored Student's *t* test JMP V4.0.2 software (SAS Institute). In all cases, $P \leq 0.05$ was considered statistically significant.

Results

Calcitriol Decreases Glucuronidation Rates of Androgens in LNCaP Cells

To determine whether calcitriol affects androgen glucuronidation in prostate cancer cells, LNCaP cells were treated with vehicle or calcitriol (10 nmol/L) for 48 or 96 h, and glucuronidation assays were then done with cell homogenates in the presence of 3α-diol, ADT, or DHT (Fig. 1). The formation of all glucuronide conjugates was significantly reduced after 48 h of treatment with calcitriol, and the effect was even more pronounced after 96 h (Fig. 1). The strongest effect was observed for ADT-3G (56% reduction after 96 h) followed by DHT-17G (50% reduction after 96 h). These results show that calcitriol inhibits the glucuronide conjugation of DHT and its 5α-reduced metabolites 3α-diol and ADT in LNCaP cells.

Calcitriol Decreases UGT2B15 and UGT2B17 Expression in LNCaP Cells

UGT2B15 and UGT2B17 are the two major androgen-glucuronidating enzymes in LNCaP cells (15). To investigate whether calcitriol modulates the expression of these two genes, LNCaP cells were treated with 10 nmol/L calcitriol for 24 or 96 h and mRNA levels of UGT2B15 and UGT2B17 were determined by real-time RT-PCR. Both UGT2B15 and UGT2B17 mRNA levels were significantly reduced after 24 and 96 h of calcitriol treatment (Fig. 2A and B); however, the reduction of UGT2B15 was more pronounced (65% reduction after 96 h) than that of UGT2B17 (50% reduction after 96 h). Subsequent analysis of UGT2B15 and UGT2B17 protein levels in calcitriol-treated LNCaP cells confirmed the real-time RT-PCR results

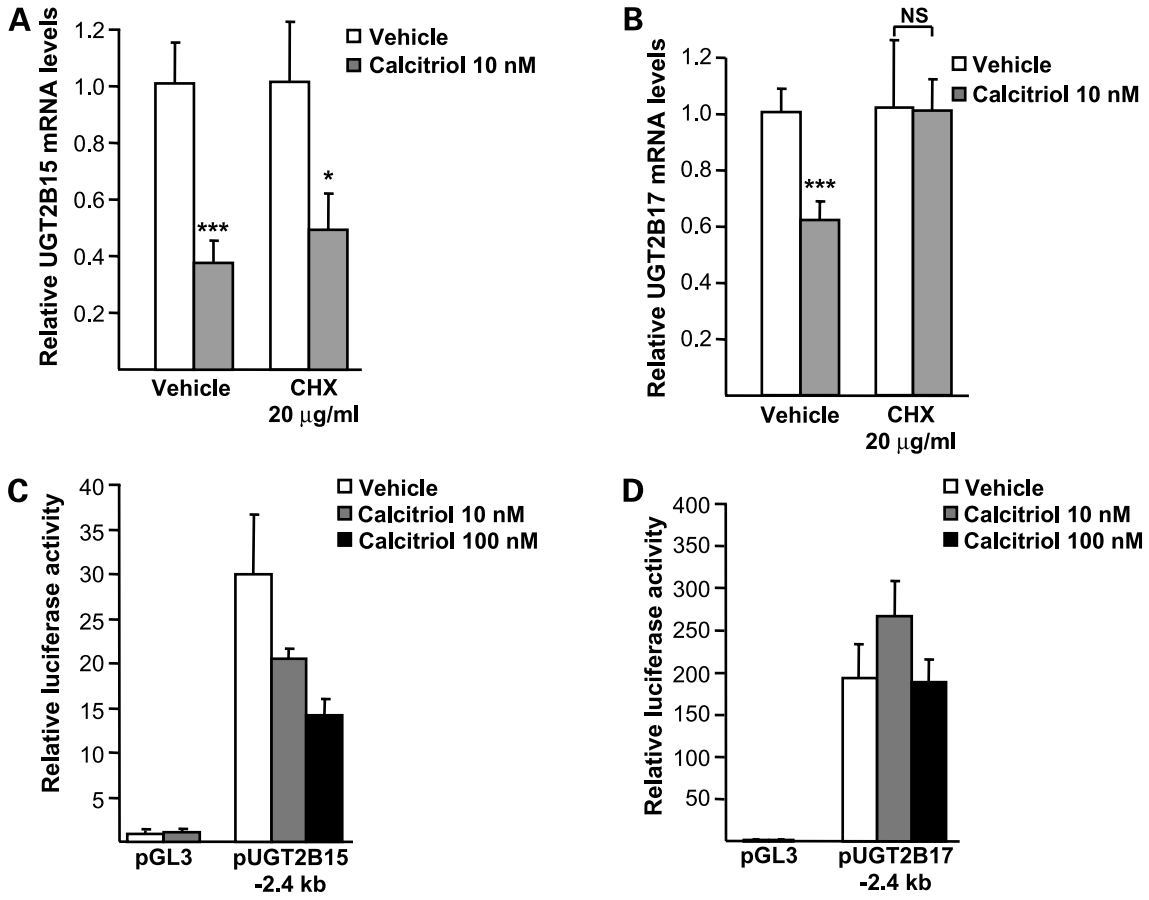


Figure 3. Calcitriol regulation of *UGT2B15* and *UGT2B17* expression is distinct in LNCaP. **A** and **B**, LNCaP cells were treated with vehicle, calcitriol (10 nmol/L), and/or CHX (20 μg/mL) for 24 h in RPMI 1640 supplemented with 0.2% FBS. UGT2B15 (**A**) and UGT2B17 (**B**) mRNA levels were measured by real-time RT-PCR and normalized to 28S rRNA levels. Values are expressed as mean ± SD. *, $P < 0.05$; ***, $P < 0.001$, statistically significant differences between control and calcitriol treatment (Student's *t* test). *NS,* not significant. **C** and **D**, LNCaP cells were transiently transfected with the empty pGL3 luciferase expression plasmid or with pGL3 containing a 2.4-kb fragment of the *UGT2B15* (**C**) or *UGT2B17* (**D**) promoter region directly upstream of the luciferase gene. At 6 h post-transfection, the medium was changed and cells were treated with vehicle or calcitriol (10 or 100 nmol/L) for 24 h. Values are mean ± SD of luciferase activity normalized to β-galactosidase activity and are expressed as fold induction over empty pGL3.

(Fig. 2C and D). Calcitriol treatment resulted in strong inhibition of UGT2B15 mRNA levels and protein expression (88% reduction after 96 h; Fig. 2D) and somewhat weaker inhibition of UGT2B17 mRNA levels and protein expression (78% reduction after 96 h; Fig. 2D).

Additional time-course experiments (6-96 h) revealed that UGT2B15 mRNA levels decreased more rapidly than UGT2B17 (Supplementary Data 1).[4] We also examined whether calcitriol modulated UGT expression in a dose-dependent manner (0.1-100 nmol/L) and found that calcitriol concentrations higher than 10 nmol/L were saturating for both enzymes (Supplementary Data 1).[4] Together, the reduction of UGT2B15 and UGT2B17 mRNA and protein levels is consistent with the reduction of androgen glucuronidation activity observed initially.

Calcitriol Differentially Regulates UGT2B15 and UGT2B17 Expression

The VDR agonist calcitriol has both genomic and nongenomic effects on cancer cells (4). To determine the manner in which VDR affects UGT2B15 and UGT2B17 expression, LNCaP cells were treated with calcitriol (10 nmol/L) in the presence or absence of CHX, an inhibitor of protein synthesis (Fig. 3A and B). Cotreatment with CHX only slightly interfered with the calcitriol-dependent repression of UGT2B15 expression (Fig. 3A) but completely abolished the effect on UGT2B17 (Fig. 3B). This observation indicates that UGT2B17 suppression by calcitriol requires the synthesis of at least one mediator protein, whereas the inhibition of UGT2B15 is independent of *de novo* protein synthesis.

To confirm these observations, transient transfections of LNCaP cells were carried out using plasmid constructs in which luciferase transcription was driven by the 2.4-kb *UGT2B15* or *UGT2B17* promoters (Fig. 3C and D). As

[4] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

expected (33), transfection of the *UGT2B15* and *UGT2B17* promoter plasmids resulted in strong basal luciferase activity. Treatment with increasing concentrations of calcitriol led to a significant dose-dependent decrease of the *UGT2B15* promoter activity (Fig. 3C). However, no reduction of the promoter activity was observed for the *UGT2B17* construct in the presence of calcitriol (Fig. 3D). Taken together, these observations suggest that the suppression of the two UGT genes by calcitriol involves different mechanisms.

AR Is an Obligate Mediator of Calcitriol-Dependent UGT2B17 Gene Repression

Previous studies reported that a series of VDR target genes are affected by calcitriol only in LNCaP cells cultured in the presence of serum (13). Most of the experiments described above (except transient transfections) were done with cells cultured in the presence of 0.2% FBS. To determine the importance of FBS for UGT regulation in prostate cancer cells, LNCaP cells were treated for 24 h with calcitriol (10 nmol/L) in the absence or presence of 0.2% or 10% FBS. UGT2B15 mRNA levels were significantly decreased by calcitriol in all conditions (Supplementary Data 2).[4] However, the more serum the medium contained, the more the expression of *UGT2B15* was inhibited. Likewise, *UGT2B17* expression was more strongly inhibited with 10% FBS than with 0.2% FBS. More interestingly, calcitriol failed to inhibit *UGT2B17* expression in the absence of serum (Supplementary Data 2).[4] Although the presence of serum has a synergistic effect for both *UGT2B15* and *UGT2B17* calcitriol-dependent inhibition, these results indicate that serum is absolutely required for the calcitriol-mediated repression of *UGT2B17* expression.

AR plays an important role in the serum-dependent antiproliferative effects of calcitriol (10), and treatment with AR agonists also significantly reduces UGT mRNA levels in LNCaP cells (29, 34). These observations suggest a role for AR in the regulation of *UGT2B17* expression by calcitriol. To evaluate this hypothesis, LNCaP cells grown in 0.2% FBS were treated with calcitriol in the absence or presence of the AR antagonist casodex. As expected (34), casodex abolished the reduction in the levels of UGT2B15 (Fig. 4A) and UGT2B17 (Fig. 4B) mRNA caused by the synthetic AR agonist, R1881. However, the reduction of UGT2B15 mRNA levels by calcitriol was not significantly affected by casodex (Fig. 4A), whereas it abolished the reduction of UGT2B17 mRNA levels by calcitriol (Fig. 4B).

We confirmed that AR is required for the suppressive effect of calcitriol on *UGT2B17* expression by specific blockage of endogenous AR signaling using an AR-specific siRNA. The AR siRNA was effective in strongly reducing AR protein levels in LNCaP cells in comparison with the nontargeted control siRNA (Fig. 4C). Furthermore, the stimulation of TMPRSS2 expression, a positively regulated AR target gene (35), by R1881 was reduced by 73% by the AR siRNA (Fig. 4D). These data confirm the ability of AR siRNA to interfere with the androgen signaling pathway. Calcitriol and R1881 were fully capable of reducing UGT2B15 (Fig. 4E) and UGT2B17 (Fig. 4F) mRNA levels in LNCaP cells transfected with the nontargeted control siRNA (29, 34). However, the transfection of AR siRNA abolished the inhibitory effect of R1881 on both UGT isoforms (Fig. 4E and F), whereas the effect of calcitriol was only blocked for UGT2B17 (Fig. 4F). In contrast, the calcitriol-induced inhibition of *UGT2B15* expression was maintained even in the presence of AR siRNA (Fig. 4E).

Taken together, these different experimental approaches clearly show that calcitriol essentially requires a functional AR to inhibit the expression of the UGT2B17 gene, whereas it exerts its suppressive effects on the UGT2B15 gene in an AR-independent manner.

A VDR-Responsive Region Is Located within the Proximal UGT2B15 Promoter

Our results using CHX treatment and transient transfections (Fig. 3) indicate that calcitriol directly controls *UGT2B15* expression. To localize the potential VDR-responsive region within the *UGT2B15* promoter, LNCaP cells were transiently transfected with plasmid constructs in which successive 5′ deletions of the *UGT2B15* promoter drive the expression of the luciferase gene. Cells were then treated with calcitriol. Deletion of the promoter to -235 bp sustained promoter activity and the inhibitory response to calcitriol treatment (Fig. 5A; data not shown). However, the deletion of the promoter to -109 bp eliminated basal activation and the subsequent response to calcitriol (Fig. 5A). Thus, a putative VDR-responsive region was identified in the *UGT2B15* promoter region between -235 and -109 bp.

Considering the high identity of the nucleotide composition between the proximal UGT2B15 and UGT2B17 gene promoters (~91% identity for the region between -1,662 and the transcriptional start site; ref. 31), we expected that minor nucleotide changes between the two genes gave rise to a VDRE-like sequence within the *UGT2B15* promoter. Alignment of the sequences revealed the presence of a 59-bp insert (-113 to -171) within the *UGT2B15* promoter (B15-i59) that was absent in the *UGT2B17* promoter (Fig. 5B). Additionally, the distal boundary of this insert (-157 to -177) also contained a highly conserved inverted palindrome-9 motif (AGGTCAtcaatctttTGTTGG), a sequence that has been shown previously to function as a VDRE (36). To test whether the 59-bp insert was responsive to calcitriol, the B15-i59 fragment was cut out of the UGT2B15 -2,401-bp construct and cloned into the UGT2B17 -2,401-bp promoter at the equivalent position. Transfection of the UGT2B15 construct lacking B15-i59 revealed significant reduction of the basal promoter activity (Fig. 5C) to a similar level as the UGT2B15 -235-bp construct (Fig. 5A). However, in contrast to this wild-type promoter, activity of the UGT2B15 construct lacking the 59-bp insert was not affected by the presence of calcitriol (Fig. 5C). Furthermore, insertion of the B15-i59 fragment into the UGT2B17 promoter yielded a stronger basal activation compared with the wild-type construct and a significant response to calcitriol treatment (Fig. 5D), indicating that the B15-i59 insert may be important for the direct negative response of the *UGT2B15* promoter to calcitriol.

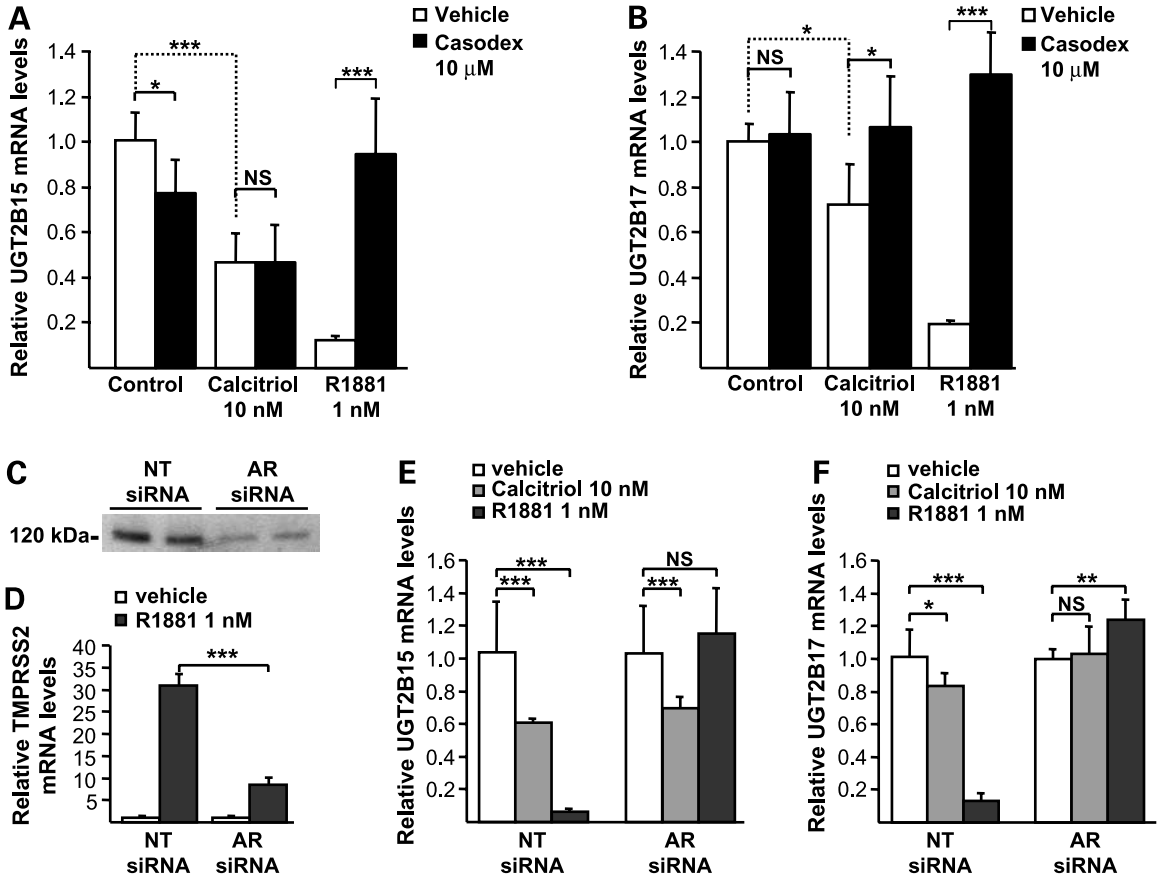


Figure 4. Calcitriol requires AR to regulate *UGT2B17* expression in LNCaP cells. **A** and **B**, LNCaP cells were treated with vehicle, calcitriol (10 nmol/L), R1881 (1 nmol/L), and/or casodex (10 μmol/L) for 24 h in RPMI 1640 supplemented with 0.2% FBS. UGT2B15 (**A**) and UGT2B17 (**B**) mRNA levels were measured by real-time RT-PCR and normalized to 28S rRNA levels. Values are expressed as mean ± SD. *, $P < 0.05$; ***, $P < 0.001$, statistically significant differences (Student's *t* test). *NS,* not significant. **C** to **F**, LNCaP cells were transiently transfected with nontargeting (*NT*) or AR siRNA for 24 h. After a 24-h pretreatment period in RPMI 1640 supplemented with 0.2% FBS, cells were treated for another 24 h with vehicle, calcitriol (10 nmol/L), or R1881 (1 nmol/L). Total protein (10 μg) from vehicle treated samples was immunoblotted (**D**) with an anti-AR antibody (1:4,000). TMPRSS2 (**D**), UGT2B15 (**E**) or UGT2B17 (**F**) mRNA levels were measured by real-time RT-PCR and normalized to 28S rRNA levels. Values are expressed as mean ± SD. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$, statistically significant differences (Student's *t* test). *NS,* not significant.

To further confirm the importance of the putative VDRE distal to B15-i59 in the *UGT2B15* promoter, half of the inverted palindrome-9 sequence (-172 to -177) was modified by site-directed mutagenesis to generate the UGT2B15 -2,401 mutant VDRE construct. As expected, the mutation abolished the response of the *UGT2B15* promoter to calcitriol treatment and also reduced the basal promoter activity (Fig. 5C). Taken together, these results indicate that the UGT2B15 promoter region between -113 and -171 contains a VDR-responsive element that mediates the effects of calcitriol on the expression of *UGT2B15*.

Calcitriol Reduces the UGT2B15 and UGT2B17 Expression in 22Rv1 Cells

The androgen-dependent LNCaP cell line is a widely used classic model for prostate cancer (18), but one knows that this type of cancer is a heterogeneous and multistage disease. Thus, to determine whether calcitriol affects androgen-conjugating UGT enzymes in other cell models, we have compared its effects on UGT2B15 and UGT2B17 gene expression in (*a*) 22Rv1 cells: an AR-positive, androgen-independent cell line that responds to androgen treatment (14, 37), and (*b*) PC-3 and DU145 cells: two AR-negative, androgen-independent cell lines (37). All these cell lines have been reported to express VDR (10, 24, 38).

First of all, these experiments have revealed that UGT2B15 and UGT2B17 transcripts are absent from PC-3 or DU145 cells (data not shown), indicating that these enzymes are not expressed in these AR-negative prostate cancer cells. By contrast, 22Rv1 cells exhibited elevated levels of UGT2B15 and UGT2B17 mRNA, and treatment with calcitriol resulted in a significant reduction of these levels (Fig. 6). Interestingly, and similarly to what we observed with LNCaP cells (Supplementary Data 1),[4] the reduction of UGT2B17 expression was delayed when compared with the response of the UGT2B15 gene. Indeed,

the reduction of UGT2B15 mRNA concentration was significant after 24 h of treatment (Fig. 6A), whereas UGT2B17 mRNA levels were decreased only when cells were incubated with calcitriol for 72 h (Fig. 6B). In addition, the effect of calcitriol on the UGT promoters was also investigated in 22Rv1 through transient transfection experiments. The two 2.4-kb UGT promoter constructs displayed strong basal activity (Fig. 6C and D), and as observed in LNCaP cells (Fig. 3C), calcitriol treatment strongly reduced UGT2B15 promoter activity (by >50%; Fig. 6C), whereas UGT2B17 was affected only at lower levels (reduction of 20% and 28% in the presence of 10 and 100 nmol/L calcitriol, respectively; Fig. 6D). In the PC-3 and DU145 cells, no basal activity was observed with these constructs, which is consistent with the absence of UGT2B15 and UGT2B17 gene expression (data not shown).

Overall these observations indicate that calcitriol is able to reduce androgen glucuronidation in prostate cancer cells where UGT2B15 and UGT2B17 enzymes are expressed.

Discussion

In this study, human *UGT2B15* and *UGT2B17* were identified as negatively regulated target genes of the endogenous vitamin D metabolite, calcitriol, in prostate cancer LNCaP and 22Rv1 cells. We have also established that this negative regulation causes a drastic reduction of androgen glucuronidation. These observations are of clinical importance because vitamin D derivatives are considered to be promising therapeutic compounds for prostate cancer treatment (6), whereas androgens play a major role in the pathogenesis of this disease (16).

The observation that calcitriol drastically reduces androgen glucuronidation in UGT-expressing prostate cancer cells suggests that treatment with this molecule may have profound consequences for androgen homeostasis and activity in androgen-sensitive prostate cancer cells. Indeed, reduction of glucuronidation in LNCaP cells causes an accumulation of free androgens and higher cell proliferation rates (28, 39). Thus it is reasonable to speculate that, by

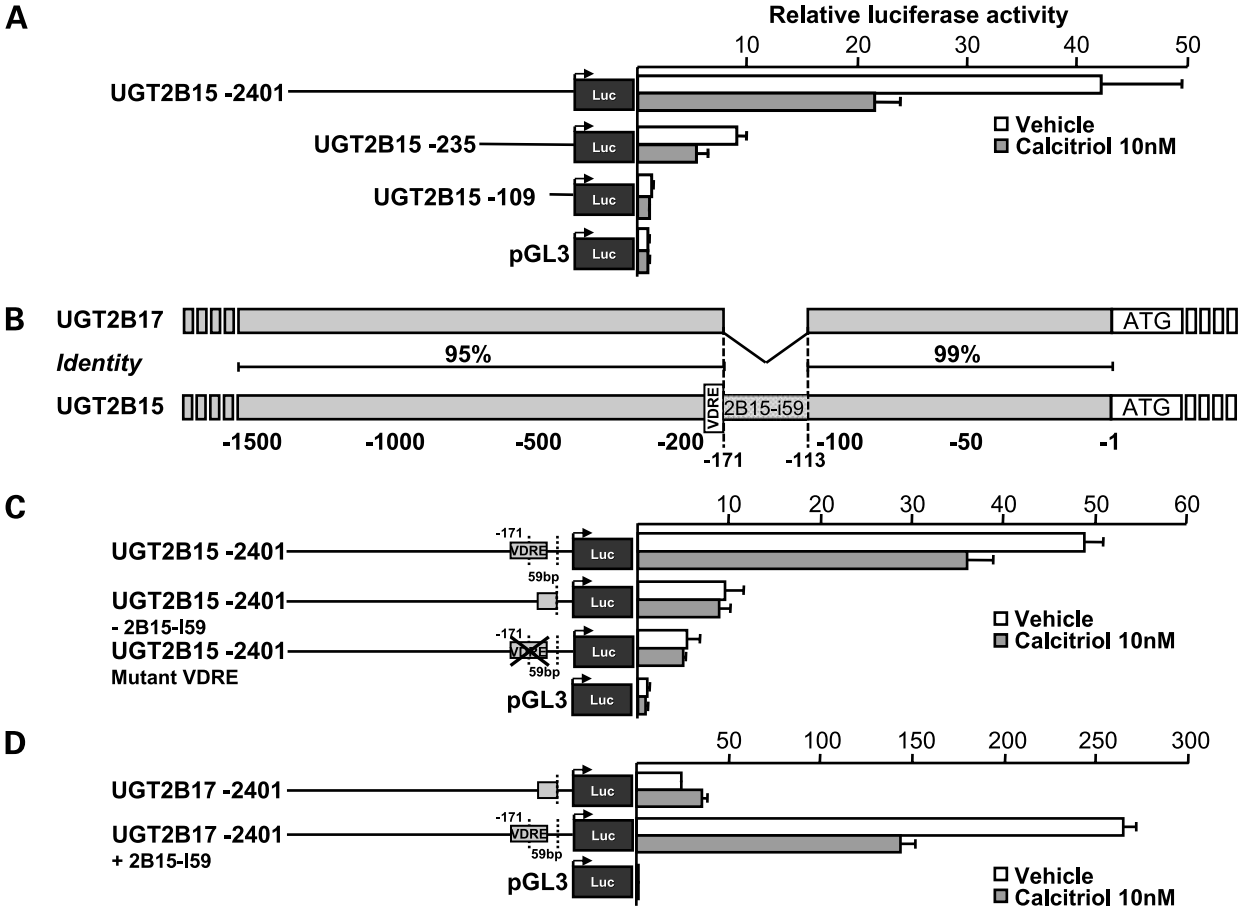


Figure 5. A calcitriol-responsive element is located in the region between -113 and -171 within the *UGT2B15* promoter. LNCaP cells were transiently transfected with the empty pGL3 or with pGL3 containing serial deletions (**A**) or mutated sequences of the 2.4-kb *UGT2B15* (**C**) or *UGT2B17* (**D**) promoters. At 6 h post-transfection, the medium was changed and cells were treated with vehicle or calcitriol (10 nmol/L) for 24 h. Values are mean ± SD of luciferase activities normalized to β-galactosidase and are expressed as fold induction over empty pGL3. **B,** schematic representation of the proximal promoters of *UGT2B15* and *UGT2B17* indicating the position of the 2B15-i59 fragment (-113 to -171 bp) and the putative VDR-responsive element within the *UGT2B15* promoter relative to the transcription initiation site.

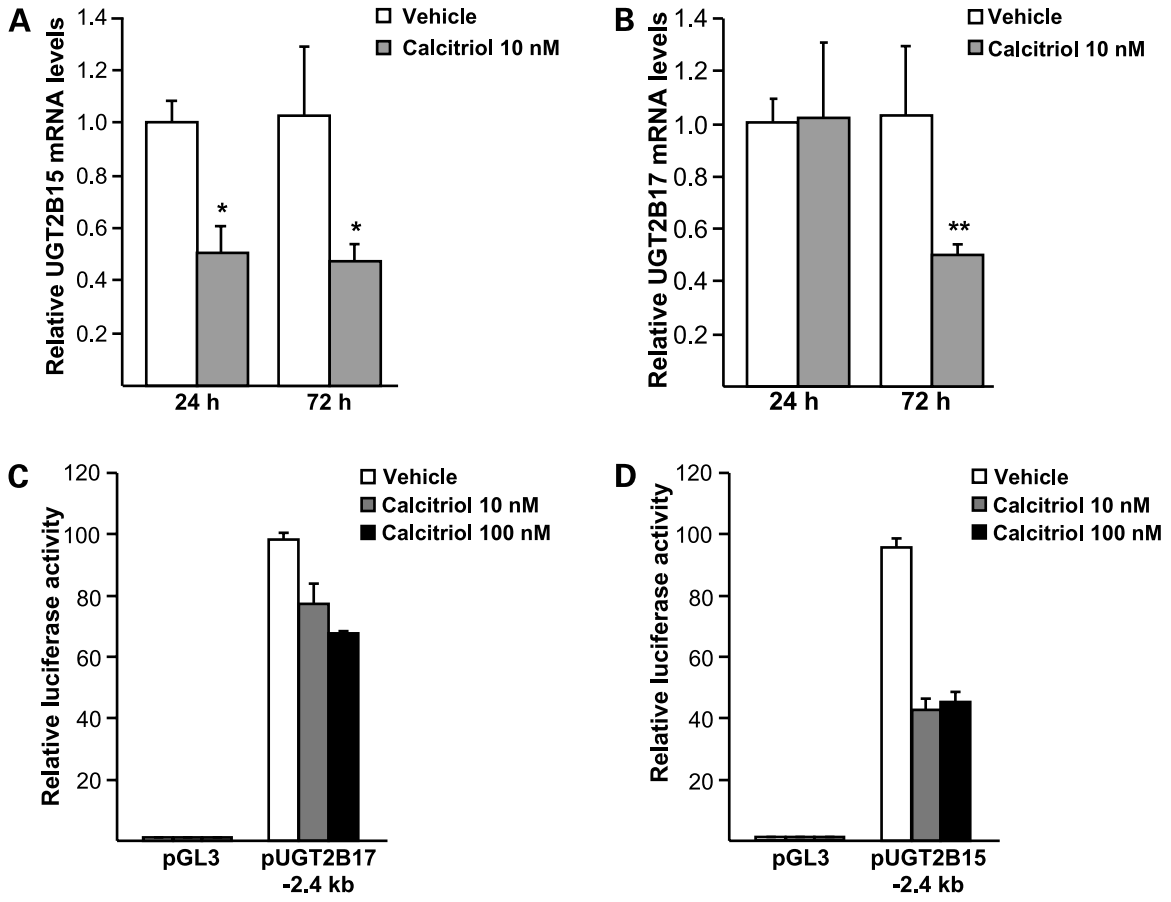


Figure 6. Calcitriol also affects androgen-conjugating UGT2B enzymes in 22Rv1 cells. **A** and **B**, prostate cancer 22Rv1 cells were treated with vehicle or calcitriol (10 nmol/L) in RPMI 1640 supplemented with 0.2% FBS for 24 and 72 h. UGT2B15 (**A**) and UGT2B17 (**B**) mRNA levels were determined by real-time RT-PCR and normalized to 28S rRNA levels. Values are expressed as mean ± SD. *, $P < 0.05$; **, $P < 0.005$, statistically significant differences between control and calcitriol treatment (Student's *t* test). **C** and **D**, 22Rv1 cells were transiently transfected with the empty pGL3 luciferase expression plasmid or with pGL3 containing a 2.4-kb fragment of the *UGT2B15* (**C**) or *UGT2B17* (**D**) promoter region directly upstream of the luciferase gene. At 6 h post-transfection, the medium was changed and cells were treated with vehicle or calcitriol (10 or 100 nmol/L) for 24 h. Values are mean ± SD of luciferase activity normalized to β-galactosidase activity and are expressed as fold induction over empty pGL3.

reducing *UGT* expression, calcitriol will also result in DHT accumulation. In addition, DHT is a potent inhibitor of *UGT2B15* and *UGT2B17* expression and of its own glucuronidation (26, 28, 29). Therefore, the reduction of DHT glucuronidation caused by calcitriol may be further amplified through this negative autoregulatory loop. However, calcitriol also exerts numerous androgen-independent antiproliferative effects in prostate cancer cells and has been shown to be efficient for dampening prostate cancer cell proliferation (4, 7, 40, 41). Nevertheless, the reduction of UGT gene expression and activity may be interpreted as a plausible limitation of the antineoplastic properties of calcitriol in androgen-sensitive prostate cancer cells. These observations also suggest that other vitamin D derivatives having androgen-independent antiproliferative effects that do not modulate UGT enzymes may display stronger antiproliferative effects in LNCaP cells than calcitriol. Interestingly, a recent study revealed that vitamin D_5, a 1α-hydroxylated metabolite of vitamin D, showed significantly different molecular effects in bone and prostate cells (40, 42, 43). In particular, this compound is a stronger reducer of LNCaP cell proliferation than calcitriol, and it fails to activate AR in this cell line (43). These data suggest that vitamin D_5 may not affect *UGT2B17* expression, and it will therefore be of importance to determine the effect of such novel vitamin D derivatives on androgen glucuronidation in prostate cancer cells.

When the effects of calcitriol treatment on UGT expression was investigated in other prostate cancer cell lines, our primary observation was that UGT2B15 and UGT2B17 enzymes are not expressed in the AR-negative PC-3 and DU145 cells. While this observation impairs to show the AR requirement for the calcitriol-induced *UGT2B17* gene repression in recurrent prostate cancers, it is consistent with the previously reported absence of androgen glucuronidation in these cells (44). Interestingly, a similar pattern of response to calcitriol treatment was observed in LNCaP and 22Rv1 cells, thus indicating that the inhibitory effect of

calcitriol on androgen glucuronidation is not limited to LNCaP cells but may correspond to a more general phenomenon in UGT-positive prostate cancer cells.

In addition to androgen inactivation, UGT2B15 and/or UGT2B17 also exerts important roles for drug metabolism (45). The nonsteroidal anti-inflammatory drug ibuprofen is metabolized by both UGT isoforms (46), and the antiestrogen tamoxifen is potently glucuronidated by UGT2B15 (47). Interestingly, ibuprofen cooperates with calcitriol in an additive manner to inhibit LNCaP cell growth (6). Likewise, tamoxifen cooperates to inhibit mammary carcinoma MCF7 cell growth (6). It is therefore likely that calcitriol, by reducing *UGT* expression, also affects the metabolism of these drugs and thereby reinforces their beneficial properties. Taken together, these observations suggest that the calcitriol-dependent inhibition of UGT2B15 and UGT2B17 gene expression could be a therapeutic advantage when calcitriol is used concomitantly with other drugs conjugated by one or both enzymes.

Using several experimental approaches, we have shown that the calcitriol-dependent reduction of UGT2B15 and UGT2B17 gene expression occurs at the transcriptional level. However, the two genes are differentially affected in the presence of the VDR activator: calcitriol down-regulates *UGT2B17* expression in an AR-dependent manner, whereas it interferes more directly with the UGT2B15 gene through a VDR-responsive promoter region. The androgen-dependent manner by which *UGT2B17* is regulated is consistent with the previous observations that VDR up-regulates *AR* (48) and that AR reduces *UGT2B17* expression (29, 32). In addition to VDR and AR, this regulatory cascade may also involve other unidentified factors because both the regulation of *AR* by VDR (48) and *UGT2B17* by AR (34) have been shown to be indirect. The implication of such a multifactor regulatory process is further supported by the delay observed for *UGT2B17* down-regulation when compared with *UGT2B15*. Furthermore, the AR-dependent manner in which calcitriol regulates *UGT2B17* expression is also consistent with previous observations that many regulatory and antiproliferative effects of vitamin D compounds in LNCaP cells are mediated through AR (13).

Contrariwise, the modulation of *UGT2B15* appears to occur in a simple manner through a VDR-responsive region in the *UGT2B15* promoter that is directly modulated in the presence of calcitriol. This region contains a highly conserved inverted palindrome-9 sequence, reported previously to function as a negative element for regulation of transcription in the presence of the ligand-activated VDR (36). Site-directed mutagenesis of this sequence abolishes the calcitriol-dependent reduction of the *UGT2B15* promoter activity. However, electrophoretic mobility shift assays and chromatin immunoprecipitation experiments failed to show the formation of such a complex (data not shown). Together, these data suggest that activation of VDR may initiate the formation of a protein-protein complex comprising VDR and an unidentified positive regulator of UGT2B15. In such case, VDR sequesters the positive factor(s), which is no more available to bind to and activate the VDR-responsive region within the *UGT2B15* promoter. Similar direct protein-protein interactions between VDR and other transcription factors have been reported in case of VDR negatively regulated genes, such as human parathyroid hormone or the osteoblast endopeptidase PHEX (49, 50).

In conclusion, the present study shows that, through differential regulation of *UGT2B15* and *UGT2B17* expression, calcitriol treatment results in reduced inactivation of androgens in LNCaP cells, an effect that potentially limits its antineoplastic properties in androgen-sensitive prostate cancer cells, such as LNCaP and 22Rv1 cells.

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