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Regulation of cholesterol 25-hydroxylase expression by vitamin D_3 metabolites in human prostate stromal cells ☆

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Abstract

Vitamin D_3 plays an important role in the control of cell proliferation and differentiation. Cholesterol 25-hydroxylase (CH25H) is an enzyme converting cholesterol into 25-hydroxycholesterol. Vitamin D_3 as well as 25-hydroxycholesterol has been shown to inhibit cell growth and induce cell apoptosis. Here we show that 10 nM $1\alpha,25(OH)_2D_3$ and 500 nM $25OHD_3$ upregulate CH25H mRNA expression in human primary prostate stromal cells (P29SN). Protein synthesis inhibitor cycloheximide does not block $1\alpha,25(OH)_2D_3$ mediated upregulation of CH25H mRNA. Transcription inhibitor actinomycin D blocks basal level as well as $1\alpha,25(OH)_2D_3$ induced CH25H mRNA expression. $1\alpha,25(OH)_2D_3$ has no effect on CH25H mRNA stability. 25-Hydroxycholesterol significantly decreased the P29SN cell number. A CH25H enzyme inhibitor, desmosterol, increases basal cell number but has no significant effect on vitamin D_3 treated cells. Our data suggest that *ch25h* could be a vitamin D_3 target gene and may partly mediate anti-proliferative action of vitamin D_3 in human primary prostate stromal cells.
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The vitamin D_3 receptor is a nuclear receptor and that binds with high affinity to calcitriol, $1\alpha,25(OH)_2D_3$ [1] and with a significantly lower affinity to calcidiol, $25OHD_3$ [2]. Both metabolites appear to regulate gene expression [3]. Vitamin D_3 metabolites appear to control cell growth via regulation of cell cycle, cell differentiation, and cell apoptosis [4,5]. It is possible that fatty acid and lipid metabolism are involved in vitamin D_3 action. Moreno reported that regulation of prostaglandin metabolism and biological actions constitutes a novel pathway of calcitriol action that may contribute to its anti-proliferative effects in prostate cells [6]. Qiao et al. [7] found that $1\alpha,25(OH)_2D_3$ inhibited fatty acid synthase expression in prostate cancer LNCaP cells while inhibition of the LNCaP cell growth also occurred. Vitamin D_3 may affect cholesterol metabolism. Vitamin D_3 deficiency has been reported to be associated with increased blood cholesterol concentrations [8]. A negative correlation of $25OHD_3$ concentration with total and LDL cholesterol has also been observed [9]. The mechanism of the relationship between vitamin D_3 and cholesterol metabolism is not known.

CH25H is an important enzyme of cholesterol metabolism. It belongs to a family of enzymes that utilize diiron cofactors to catalyse the hydroxylation of hydrophobic substrates such as cholesterol to 25-hydroxycholesterol [10]. 25-Hydroxycholesterol is one of the natural oxysterols. 25-Hydroxycholesterol inhibits the activation of SREBPs [11], which are transcription factors that activate genes involved in the synthesis of cholesterol and other lipids in animal cells [12]. Thus, 25-hydroxycholesterol causes

☆ *Abbreviations:* CH25H, cholesterol 25-hydroxylase; SREBP, sterol-regulatory element binding protein; $1\alpha,25(OH)_2D_3$,1α,25-dihydroxyvitamin D_3; $25OHD_3$,25-hydroxyvitamin D_3.

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a decreased cholesterol synthesis. Furthermore, 25-hydroxycholesterol seems to inhibit the growth of tumour as well as normal cells [13,14]. In addition, 25-hydroxycholesterol induces apoptosis through an inhibition of c-myc [15].

Our microarray data suggested that both $1\alpha,25(OH)_2D_3$ and $25OHD_3$ may up-regulate CH25H, therefore we were interested to study the relationship of CH25H and vitamin D_3 in more detail. We studied whether CH25H participates in the anti-proliferative action of vitamin D_3 in human primary prostate stromal cells.

Methods

Reagents. $1\alpha,25(OH)_2D_3$, $24R,25(OH)_2D_3$, and $25OHD_3$ were obtained from Leo Pharmaceuticals (Ballerup, Denmark) and DHT was from Merck (Darmstadt, Germany). Cycloheximide, actinomycin D, desmesterol (5, 24-cholestadien-3β-ol), 25-hydroxycholesterol (cholest-5-ene-3β, 25-diol), and RPMI-1640 medium were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). FBS was purchased from Gibco-BRL (Life Technology, Paisley, Scotland). TRIzoL reagent was purchased from Invitrogen (Carlsbad, USA). High Capacity DNA Archive Kit and SYBR Green PCR Master Mix Kit were purchased from Applied Biosystems (Foster City, USA). One-Cycle Target Labelling Assay Kit and Human Genome U133 Plus 2.0 GeneChip were purchased from Affymetrix (Affymetrix, Inc. Santa Clara, CA).

Cell treatment and RNA isolation. Human primary prostate stromal cells termed P29SN [3] were maintained in phenol red-free DMEM/F12 medium, supplemented with 10% FBS, 3 mM L-glutamine, 5 μg/ml insulin, and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml) at 37 °C in a humid atmosphere with 5% CO_2. Twenty four hours before treatment the medium was changed to 10% FBS-DCC (10% FBS treated with dextran-coated charcoal) supplemented DMEM/F12 medium. Cells were treated with vitamin D_3 metabolites or/and other reagents, which were diluted in 100% ethanol. Total cellular RNA was isolated with TRIzoL reagent following the instructions from the manufacturer. The RNA concentration was calculated from absorbance at 260 nm in a GeneQuant II (Pharmacia Biotech, USA) and A280/A260 was measured to verify the purity of the RNA. The ratio of all the RNA samples fell in 1.9–2.1. Randomly selected RNA samples were subjected to denaturing-gel electrophoresis. The ratio of the intensity of the 28S band and that of the18S band was 1.5–2.0.

Affymetrix microarray analysis. cRNA preparation was performed following the instructions of the manufacturer. Labelled cRNA was hybridized on GeneChip Human Genome U133 Plus 2.0 Array representing over 47,000 transcripts. Scanned images were processed using Affymetrix GeneChip Operating Software Server 1.0 (GCOS Server) (Affymetrix, Santa Clara, CA) and raw data were normalized and analysed using GENESPRING software (Version 7.2, Silicon Genetics, Redwood City, CA).

Real-time PCR analysis and primer design. Real-time PCR was performed in ABI PRISM 7000 Detection System (Applied Biosystems, USA). Total cellular RNA (3–5 μg) was used to synthesize cDNA by using High Capacity Archive Kit (Applied Biosystems, USA) in a final volume of 100 μl. Undiluted cDNA (1–2 μl) reactions or 4–8 μl of 1:10 diluted cDNA reactions was used as input for each of the real-time quantitative PCR reactions by using SYBR Green PCR Master Mix kit (Applied Biosystems, USA). Primers were designed by using Primer Express v2.0 software (Perkin-Elmer Applied Biosystems, Foster City, CA). BLASTn searches were performed to ensure that the primers were gene specific. Since human CH25H gene spans one exon, real-time PCR using unreverse-transcribed RNA as template was performed to confirm that the experimental results were not compromised through genomic DNA contamination. CH25H (NM_003956) forward primer was 5′-TCCTGTTC TGCCTGCTACTCTTC-3′ and its reverse primer was 5′-GGTACAGC CAGGGCACCTT-3′. CYP24 (NM_000782) forward primer was 5′-GCC CAGCCGGGAACTC-3′ and its reverse primer was 5′-AAATACCA CCATCTGAGGCGTATT-3′. CYP27A1(M62401) forward primer was 5′-GAGTGGACACGACATCCAACAC-3′ and its reverse primer was 5′-CTCCTGGATCTCAGGGTCCTT-3′. Human housekeeping gene RPLP0 (NM_001002) was used as endogenous control. Primers for RPLP0 were as follows, forward: 5′-AATCTCCAGGGGCACCATT-3, reverse: 5′-CGCTGGCTCCCACTTTGT-3′.

Cell growth assay. P29SN cells were treated with hormones and other reagents for 0, 3, 6, and 9 days. Eight experiments for each treatment were repeated three times. Cell growth was analysed with crystal violet staining as described earlier [16]. Briefly, cells were fixed with 11% gultaraldehyde for 15 min by shaking at 500 rpm, washed, and air-dried completely. Crystal violet solution (0.1%) was added to stain the fixed cells for 20 min with shaking at 500 rpm. Excess dye was removed by extensive washing with tap water. The plates were air-dried. 10% acetic acid was used to withdraw cell-bound dye. The optical density of extracted dye was measured in plates at 590 nm by Microplate Reader (Wallac, victor 1420 multilabel counter, Turku, Finland).

Statistical analysis. Data of real-time PCR are expressed as mean values ± SD. Significance was assessed by using Student's paired t test otherwise indicated. $^{*}P < 0.05$ was considered as significant, $^{**}P < 0.001$ as highly significant and $P > 0.05$ as not significant (NS).

Results

Vitamin D_3 increases CH25H mRNA expression

To investigate vitamin D_3 regulated genes in human primary prostate stromal cells, Affymetrix microarray analysis was performed using Human Genome U133 Plus 2.0 Array chip representing over 47,000 transcripts, which include 38,500 well-characterized human genes. Cholesterol 25-hydroxylase was shown to be upregulated by 10 nM $1\alpha,25(OH)_2D_3$ and 500 nM $25OHD_3$ in human primary prostate stromal cells. Quantitative real-time PCR results showed that 10 nM $1\alpha,25(OH)_2D_3$ and 500 nM $25OHD_3$ treatment for 24 h increased CH25H mRNA expression

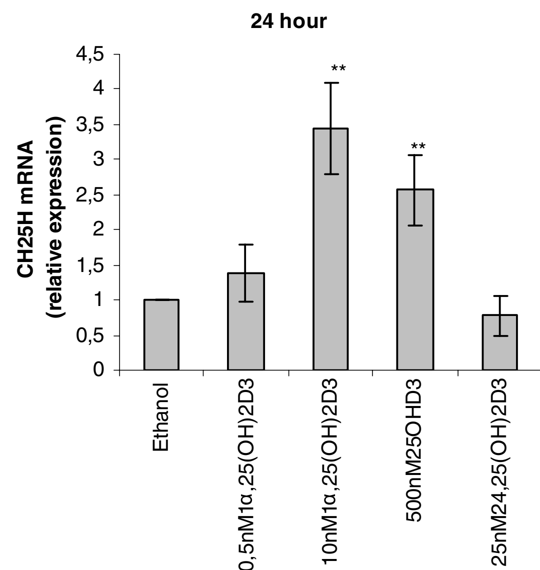

Fig. 1. Up-regulation of CH25H mRNA expression by vitamin D_3 metabolites in human primary prostate stromal cells. Cells were treated with 0.1% ethanol and different Vitamin D_3 metabolites at various concentrations as indicated for 24 h. Relative CH25H mRNA expression was analysed by quantitative real-time PCR. ($n = 4$, $^{**}P < 0.001$).

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