Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Steroid Biochemistry & Molecular Biology 89–90 (2004) 409–412
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Effects of $1,25(OH)_2D_3$, $25OHD_3$, and EB1089 on cell growth and Vitamin D receptor mRNA and 1α-hydroxylase mRNA expression in primary cultures of the canine prostate☆

S. Kunakornsawat [a], T.J. Rosol [a], C.C. Capen [a], J.L. Omdahl [b], B.E. Leroy [a], N. Inpanbutr [a,*]

[a] *Department of Veterinary Biosciences, The Ohio State University, 1900 Coffey Road, Columbus, OH 43210, USA*
[b] *Department of Biochemistry and Molecular Biology, School of Medicine, University of New Mexico, Albuquerque, NM 87131, USA*

Abstract

The aim of this study was to investigate effects of $1,25(OH)_2D_3$ (calcitriol), $25OHD_3$, and EB1089 on cell growth and on Vitamin D receptor (VDR) mRNA and 1α-hydroxylase (1α-OHase) mRNA expression in normal canine prostatic primary cultures. Canine prostatic epithelial cells were isolated, cultured, and treated with vehicle (ethanol), calcitriol, $25OHD_3$, and EB1089 at 10^{-9} and 10^{-7} M. The VDR was present in epithelial and stromal cells of the canine prostate gland. $1,25(OH)_2D_3$, $25OHD_3$, and EB1089 inhibited epithelial cell growth at 10^{-7} M compared to vehicle-treated controls [calcitriol ($P < 0.01$), EB1089 ($P < 0.01$), and $25OHD_3$ ($P < 0.05$)]. Epithelial cells treated with calcitriol and EB1089 at 10^{-7} M had slightly increased VDR mRNA expression (0.2–0.3-fold) at 6 and 12 h compared to controls. There was no difference in 1α-OHase mRNA expression in epithelial cells treated with these three compounds. $1,25(OH)_2D_3$ and its analogs may be effective antiproliferative agents of epithelial cells in certain types of prostate cancer.
© 2004 Elsevier Ltd. All rights reserved.

Keywords: Canine prostate primary culture; $1,25(OH)_2D_3$; Calcitriol; $25OHD_3$; EB1089; Antiproliferative effects; Vitamin D receptor; 1α-Hydroxylase; Primary culture

1. Introduction

Low plasma levels of $1,25(OH)_2D_3$ (calcitriol) and its analogs have been correlated to increased risk of human prostate cancer [1]. Several studies have demonstrated that $1,25(OH)_2D_3$ and its analogs could affect the regulation of growth and differentiation in human prostate cancer mediated through the $1,25(OH)_2D_3$-receptor (VDR) [2]. Although increasing data indicate a role for $1,25(OH)_2D_3$ in prostate cancer, little is known about the role of this hormone in the canine prostate cells. The canine prostate is a valuable animal model for the study of prostate pathobiology and for pharmaceutical research due to its high incidence of benign prostatic hyperplasia. Spontaneous prostatic intraepithelial neoplasia also has been reported in dogs [3]. Prostatic intraepithelial neoplasia is the most likely precursor of human prostate cancer. The dog prostate can serve as a useful model for examining factors that modulate prostatic intraepithelial neoplasia and prostate cancer progression. However, the effects of calcitriol and its analogs has not been reported on the canine prostate. The objective of this study was to investigate effects of $1,25(OH)_2D_3$, $25OHD_3$, and EB1089 on cell growth and on VDR mRNA and 1α-OHase mRNA expression in normal canine prostatic primary cultures.

2. Materials and methods

2.1. Vitamin D and its analogs

$1,25(OH)_2D_3$, $25OHD_3$, and EB1089 were gifts from Dr. L. Binderup (Leo Pharmaceutical Products, Ballerup, Denmark).

2.2. Plasmid DNA of VDR and 1-OHase

Plasmid DNA of human VDR and pig 1α-OHase were from Dr. Omdahl's laboratory. PRSV-hVDR was full-length cDNA encoding human VDR, in pRSVN vector. The full length was released with EcoR I and Bam HI. P1OHpcDNA3 was full-length cDNA encoding pig 1α-OHase, in pcDNA3 vector. The full length was released with Not I and Xho I.

☆ Presented at the 12th Workshop on Vitamin D (Maastricht, The Netherlands, 6–10 July 2003).

* Corresponding author. Tel.: +1-614-292-8641; fax: +1-614-292-7599.
E-mail address: inpanbutr.1@osu.edu (N. Inpanbutr).

0960-0760/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2004.03.009

2.3. Cell culture

Tissue samples were obtained from normal adult male beagles. Following 3–4 h of 0.5% type I collagenase digestion of the minced tissue in DMEM/F12 with 10% FBS. Epithelial cells were derived by changing medium to low calcium Keratinocyte-SFM (Gibco BRL, NY) supplemented with 10 ng/ml of epidermal growth factor (EGF), 10 μg/ml of bovine pituitary extract (BPE), and 50 μg/ml of gentamicin to selectively enrich the epithelial cell population [4].

2.4. Cell growth assay

Cells were seeded at density of 10^5 cells per well in 6-well culture plates (Becton Dickinson, NJ) and grown for 24 h before starting experiments (day 0). Medium containing vehicle (ethanol), $1,25(OH)_2D_3$, $25OHD_3$, and EB1089 were added at concentrations 10^{-9} and 10^{-7} M and was changed every day for up to a week. Each experiment was run in triplicate. At the end of experiments cellular DNA was isolated with 4 M guanidine isothiocyanate (GITC) containing 0.5% sodium lauroyl sarcosine and 25 mM sodium citrate.

2.5. RNA assay

Epithelial cells (2×10^6 cells/ml) were plated in 100-mm tissue culture dishes (Becton Dickinson, NJ) and grown to near confluence in Keratinocyte-SFM medium containing 10 ng/ml of EGF and 10 μg/ml of BPE. Cells were treated with vehicle, $1,25(OH)_2D_3$, $25(OH)D_3$, and EB1089 at 10^{-7} M for 0, 1, 3, 6, 12, 24 h. Cells were washed (PBS), trypsinized, and stored at −80 °C until assayed by Northern blot analysis.

2.6. Fluorescence DNA analysis

DNA content of cell lysates was measured at 450 nm on an IDEXX fluorimeter (IDEXX Laboratories Inc., ME) using a 96-well black U-bottom Microfluor plate reader (Dynatech Labs, UK) and Hoechst 33258 dye (Hoefer Scientific Instruments, CA). Samples were aliquoted into 100 μl TNE buffer (10 mM Tris pH 8.0, 0.5 mM EDTA, 0.25 NaCl), calf thymus DNA (100 μg/ml) served as a calibration control [5].

2.7. Total RNA isolation and Northern blot analysis

Total RNA was isolated by using the Purescript® RNA isolation kit (Gentra systems, MN). Equal amounts of each RNA sample (20 μg) were separated on a 1% formaldehyde–agarose gel, transferred to a nylon membrane (Poll Biosupport, East Hills, NY), and crosslinked with UV light (Stratalinker, CA). Northern blots were hybridized with ^{32}P-labeled human VDR or pig 1α-OHase cDNA probes (NEN Life Science Products Inc., MA). The radioactivity was measured and quantitated with a Phosphorimager (425F–120, Molecular Dynamics Inc., CA), the membrane was then stripped and hybridized with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe to normalize for RNA loading [5].

2.8. Immunohistochemistry

According to our protocol [5], the staining was done by incubating with 5% normal goat serum (NGS) in PBS for 30 min, primary antibody—rat monoclonal antibody against VDR (Chemicon International Inc., CA) 1:100 at 4 °C overnight, secondary antibody—biotinylated goat anti-rat immunoglobulin G at 1:20 in PBS for 30 min, avidin–biotin–peroxidase complex (Kirkegaard & Perry Laboratories, MD) 1:100 in 1% NGS in PBS for 30 min, and color developed with 0.05% DAB and 0.01% hydrogen peroxide in 0.05 M Tris buffer for 5 min, washed, mounted with aqua-Mount and visualized by light microscopy.

2.9. Statistical analysis

Numerical data for the DNA concentrations were analyzed by one-way analysis of variance (ANOVA) and Turkey's mean separation test with the level of significance at $P < 0.05$, $P < 0.01$, or $P < 0.001$ using the Instat program (Graph PAD software (version 3.01) Inc., San Diego, CA). The results were expressed as the mean ± standard error of the mean (S.E.M.) ($n = 3$). Experiments were performed multiple times (as noted in Section 3), each time with cells from separate dogs.

3. Results

The VDR was present in epithelial and stromal cells of the canine prostate gland (Fig. 1). There was a significant decrease in the proliferation of epithelial cells treated with 10^{-7} M $1,25(OH)_2D_3$, $25OHD_3$, and EB1089 compared to vehicle-treated controls (Fig. 2). Epithelial cells treated with $1,25(OH)_2D_3$ and EB1089 at 10^{-7} M had slightly

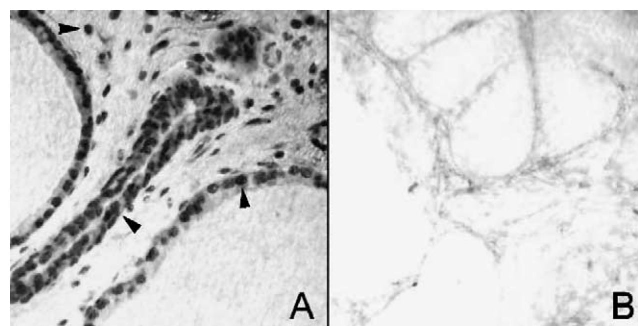

Fig. 1. Immunoperoxidase labeling of $1,25(OH)_2D_3$ receptor (VDR) with monocolonal anti-VDR (A) demonstrated positive labeling of all nuclei of normal canine prostatic epithelial and stromal cells (arrowheads), (B) control section was treated with non-specific antiserum in place of a specific primary antibody. Magnification: 300×.

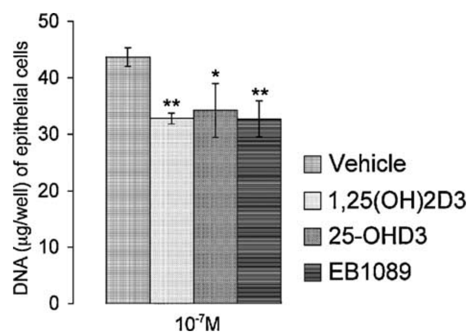

Fig. 2. Growth of normal canine prostate epithelial cells. Cells were treated with 1,25(OH)$_2$D$_3$, 25OHD$_3$, and EB1089 at 10^{-7} M. DNA content of epithelial cells was determined after 7 days. The mean values of triplicate samples ± S.E.M. are presented. Significant differences marked with $^{*}P < 0.05$, $^{**}P < 0.01$ as compared to vehicle-treated controls.

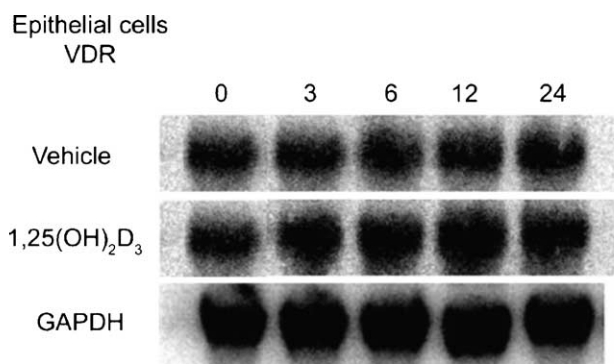

Fig. 3. VDR mRNA expression. VDR mRNA was detectable at all time points (0, 3, 6, 12, and 24 h) in epithelial cells treated with vehicle, 1,25(OH)$_2$D$_3$, 25OHD$_3$, and EB1089. Epithelial cells treated with 1,25(OH)$_2$D$_3$ and EB1089 at 10^{-7} M had slightly increased VDR mRNA expression at 6 and 12 h compared to vehicle-treated controls.

increased VDR mRNA expression at 6 and 12 h compared to vehicle-treated controls (Fig. 3). There was no difference in the expression of 1α-OHase mRNA in epithelial cells treated with these three compounds (Fig. 4).

4. Discussion

We have demonstrated that 1,25(OH)$_2$D$_3$, EB1089, and 25OHD$_3$ inhibited epithelial cell growth of the normal canine prostate. The inhibitory effect of 1,25(OH)$_2$D$_3$ on epithelial cells also have been reported in normal human prostate primary cultures [6]. The greater inhibition of cell growth by EB1089 has been correlated with the higher affinity for VDR-binding compared to 1,25(OH)$_2$D$_3$ in human prostatic cancer cells [7]. These data suggest that the potency of these compounds on inhibiting cell growth is related to the level of VDR and/or the affinity of 1,25(OH)$_2$D$_3$ for VDR-binding. A slight increase in VDR mRNA level was found at 6 and 12 h in epithelial cells treated with 1,25(OH)$_2$D$_3$ and EB1089. Our results support other studies [2] that the inhibitory action of 1,25(OH)$_2$D$_3$ and its analogs on primary culture of canine prostatic epithelial cells is mediated through the VDR.

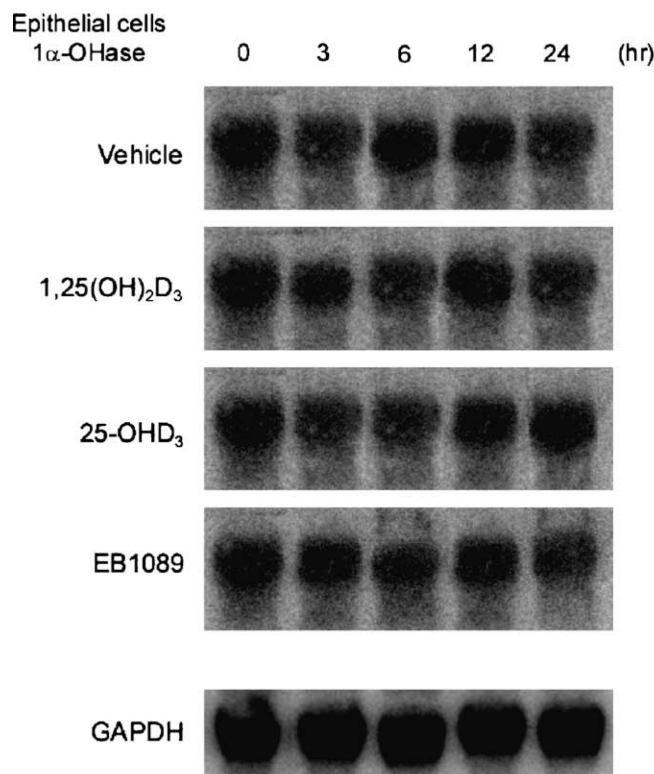

Fig. 4. 1α-OHase mRNA expression. 1α-OHase mRNA was detectable at all time points (0, 3, 6, 12, and 24 h) in both epithelial cells treated with vehicle, 1,25(OH)$_2$D$_3$, 25OHD$_3$, and EB1089. There was no difference in the expression of 1α-OHase mRNA in epithelial cells treated with these three compounds at 10^{-7} M compared to vehicle-treated controls.

Human prostate cell lines (DU 145, PC-3) exhibited 1α-OHase activity and synthesized 1,25(OH)$_2$D$_3$ intracellularly from 25OHD$_3$ [8]. The extra-renal production of this hormone points to an autocrine/paracrine role for 1,25(OH)$_2$D$_3$ where it could locally modulate cell growth. In human prostate primary culture, 25OHD$_3$ has been shown to inhibit the proliferation of epithelial cells [9]. Similarly, we demonstrated that 25OHD$_3$ inhibited the growth of normal canine prostatic epithelial cells. These data suggest that the inhibitory effect of 25OHD$_3$ on prostatic epithelial cells may be due to local conversion of 25OHD$_3$ to 1,25(OH)$_2$D$_3$.

Our results were consistent with other studies [10] that reported 1,25(OH)$_2$D$_3$ and its analogs did not affect the levels of extrarenal 1α-OHase. This suggests that the resistance of canine prostate 1α-OHase to 1,25(OH)$_2$D$_3$ occurs at the mRNA level. Therefore, the most likely explanation for the inhibition of extrarenal 1α-OHase involves different regulatory pathways from renal 1α-OHase, which are less sensitive to autoregulation by 1,25(OH)$_2$D$_3$ [11]. Our results demonstrated that canine prostate epithelial cells' response to calcitriol, 25OHD$_3$, and EB1089 is similar to that of human prostate epithelial cells. This supports our suggestion that canine prostate can be a useful animal model for the study of diseases of the prostate gland.

5. Conclusion

The VDR was present in epithelial and stromal cells of the normal canine prostate gland. There was a significant decrease in the proliferation of epithelial cells treated with $1,25(OH)_2D_3$, $25OHD_3$, and EB1089 at 10^{-7} M compared to vehicle-treated controls. Epithelial cells treated with $1,25(OH)_2D_3$ and EB1089 at 10^{-7} M had slightly increased VDR mRNA expression at 6 and 12 h compared to vehicle-treated controls. There was no difference in the expression of 1α-OHase mRNA in epithelial cells treated with these three compounds.

Acknowledgements

This work was supported by State of Ohio Canine Research Funds Grant No. 520122 and the Royal Thai Government Scholarship. We thank Dr. L. Binderup (Leo Pharmaceutical Products, Ballerup, Denmark) for the gifts of $1,25(OH)_2D_3$, $25OHD_3$, and EB1089.

References

[1] C.L. Hanchette, G.G. Schwartz, Geographic patterns of prostate cancer mortality: evidence for a protective effect of ultraviolet radiation, Cancer 70 (1992) 2861–2869.

[2] G.J. Miller, G.E. Stapleton, J.A. Ferrara, M.S. Lucia, S. Pfister, T.E. Hedlund, P. Upandhya, The human prostate carcinoma line LNCaP expresses biologically active, specific receptors for 1α,25-dihydroxyvitamin D_3, Cancer Res. 52 (1992) 515–520.

[3] D.J. Waters, D.G. Bostwick, Prostatic intraepithelial neoplasia occurs spontaneously in the canine prostate, J. Urol. 157 (1997) 713–716.

[4] E.A.G. Blomme, Y.L.K. Sugimoto, Y.C. Lin, C.C. Capen, T.J. Rosol, Stromal and epithelial cells of the canine prostate express parathyroid hormone-related protein, but not the PTH/PTHrP receptor, Prostate 36 (1998) 110–120.

[5] S. Kunakornsawat, C.C. Capen, T.J. Rosol, R. Middleton, S.S. Hanah, N. Inpanbutr, Effects of 1,25-dihydroxyvitamin D_3, EB1089, and analog V on PTHrP production, PTHrP mRNA expression, and cell growth in SCC S/88 cells, Anticancer Res. 21 (5) (2001) 10–18.

[6] D. Krill, J. Stoner, B.R. Konety, M.J. Becich, R.H. Getzenberg, Differential effects of Vitamin D on normal human prostate epithelial and stromal cells in primary culture, Urology 54 (1999) 171–177.

[7] X. Wang, X. Chen, J. Akhter, D.L. Morris, The in vitro effect of Vitamin D_3 analogue EB-1089 on a human prostate cancer cell line (PC-3), Br. J. Urol. 80 (1997) 260–262.

[8] G.G. Schwartz, L.W. Whitlatch, T.C. Chen, B.L. Lokeshwar, M.F. Holick, Human prostate cells synthesize 1,25-dihydroxyvitamin D_3 from 25-hydroxyvitamin D_3, Cancer Epidemiol. Biomarkers Prev. 7 (1998) 391–395.

[9] A.M. Barreto, G.G. Schwartz, R. Woodruff, S.D. Cramer, 25-Hydroxyvitamin D_3, the prohormone of 1,25-dihydroxyvitamin D_3, inhibits the proliferation of primary prostatic epithelial cells, Cancer Epidemiol. Biomarkers Prev. 9 (2000) 265–270.

[10] G. Jones, H. Ramshaw, A. Zhang, R. Cook, V. Byford, J. White, M. Petkovich, Expression and activity of Vitamin D-metabolizing cytochrome P450s (CYP1alpha and CYP24) in human nonsmall cell lung carcinomas, Endocrinology 140 (1999) 3303–3310.

[11] M. Hewison, D. Zehnder, R. Bland, P.M. Stewart, 1α-hydroxylase and the action of Vitamin D, J. Mol. Endocrinol. 25 (2000) 141–148.