Carcinogenesis Advance Access published January 16, 2004

The Prostate 25-Hydroxyvitamin D-1α-Hydroxylase is not Influenced by Parathyroid Hormone and Calcium: Implications for Prostate Cancer Chemoprevention by Vitamin D

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Running Title: Regulation of 1α-Hydroxylase and Prostate Cancer

Key Words: Vitamin D, calcium, parathyroid hormone.

The abbreviations used are: 25(OH)D_3, 25-hydroxyvitamin D_3; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1α-OHase, 25-hydroxyvitamin D-1α-hydroxylase; VDR, vitamin D receptor; FBS, fetal bovine serum; DPPD, 1,2-dianilinoethane; PTH, parathyroid hormone; EGF, epidermal growth factor

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Abstract

The hormonal form of vitamin D, 1α,25-dihydroxyvitamin D [1α,25$(OH)_2$D] promotes the differentiation and inhibits the proliferation, invasiveness and metastasis of prostate cells. However, 1α,25$(OH)_2$D is not suitable as a chemopreventive agent because its administration can cause hypercalcemia. Serum levels of 1α,25$(OH)_2$D are tightly regulated by the renal enzyme, 25-hydroxyvitamin D-1α-hydroxylase [1α-OHase], which synthesizes 1α,25$(OH)_2$D from the prohormone, 25-hydroxyvitamin D [25(OH)D]. Normal prostate epithelial cells in primary culture, as well as several prostate cancer cell lines, also express 1α-OHase and synthesize the hormone intracellularly. We investigated the regulation of the prostate 1α-OHase by the three most important regulators of the renal 1α-OHase: calcium, 1α,25$(OH)_2$D, and parathyroid hormone (PTH). The 1α-OHase activity in the primary cultures of prostate epithelial cells was inhibited by 1α,25$(OH)_2D_3$ at 10 and 100 nM, whereas PTH at 10 and 100 nM had no significant effect. Calcium at 1.2 and 2.4 mM had no significant effect on the enzyme activity in the PZ-HPV-7 cell line, a prostate epithelial cell line derived from normal prostate tissue. Conversely, 1.2 or 2.4 mM calcium markedly inhibited 1α-OHase activity in a human kidney cell line used as a positive control. Furthermore, PTH at 100 nM and calcium at 1.2 and 2.4 mM had no effect on the 1α-OHase gene promoter activity in prostate cells, whereas the promoter activity was inhibited 48±5 % by 100 nM 1α,25$(OH)_2D_3$. Our findings suggest that, unlike the renal enzyme, the prostate 1α-OHase is largely unregulated by serum levels of PTH and calcium. These findings support the hypothesis that vitamin D or 25(OH)D may be useful as chemopreventive agents for prostate cancer because their administration should cause an increased synthesis of 1α,25$(OH)_2$D within prostate cells.

Introduction

Vitamin D is synthesized in the skin in response to sunlight or is ingested from the diet in the form of vitamin D_2 (ergocalciferol) or vitamin D_3 (cholecalciferol). Vitamin D is subsequently hydroxylated in the liver to 25-hydroxyvitamin D [25(OH)D], the major circulating metabolite of vitamin D. 25(OH)D undergoes a second hydroxylation at the 1α-position in the kidney, to form 1α,25$(OH)_2$D, the active hormonal metabolite (1).

Considerable epidemiologic, experimental, and clinical observations support the hypothesis that vitamin D may prevent the development of clinical prostate cancer (2-5). In 1990, Schwartz and Hulka (6) suggested that the major features of the descriptive epidemiology of prostate cancer, i.e., increasing incidence with age, Black race, and residence at northern latitudes, resembled the descriptive epidemiology of vitamin D deficiency. Subsequently, the same group (7) demonstrated that the geographic pattern of prostate cancer mortality in the U.S., at the level of the county, was inversely related to the availability of ultraviolet radiation, the principal source of vitamin D. The same year, Miller and colleagues (8) demonstrated the existence of the specific receptor (VDR) for the hormonal form of vitamin D, 1α,25$(OH)_2$D, in prostate cancer cells. Subsequently, many studies showed that the exposure of prostate cancer cells to 1α,25$(OH)_2D_3$ and its analogs resulted in an inhibition of cancer cell proliferation, invasiveness and metastasis, both in vitro and in vivo in animal models (2-5). These findings have led to the active investigation of 1α,25$(OH)_2D_3$ and analogs as therapeutic agents for prostate cancer (9).

Despite these encouraging data in the therapeutic setting, 1α,25$(OH)_2D_3$ is not suitable as a chemopreventive agent because administration of this hormone systemically can cause hypercalemia (9). For example, the concentrations of 1α,25$(OH)_2D_3$ required to inhibit prostate cancer cell proliferation in tissue culture are in the range of 10-100 nM, i.e., 1000 times higher than the 20-150 pM concentration of 1α,25$(OH)_2D_3$ normally found in the systemic circulation. Thus, in order to use vitamin D metabolites in prostate cancer chemoprevention, a method must be devised to safely expose prostate cells to 1α,25$(OH)_2D_3$ (1,3-5,9). We recently demonstrated that prostate cells possess 25-hydroxyvitamin D-1α-hydroxylase [1α-OHase] and are capable of synthesizing 1α,25$(OH)_2D_3$ intracellularly from 25(OH)D_3 (10). In cells that possess 1α-OHase, the antiproliferative effects of 25(OH)D_3 were indistinguishable from those of 1α,25$(OH)_2D_3$ (11,12). This finding has important implications for prostate cancer chemoprevention because the risk of hypercalcemia associated with the systemic administration of vitamin D and 25(OH)D_3 is far lower than that for 1α,25$(OH)_2D_3$ (13). Thus, it is critical to understand what factors may regulate the conversion of 25(OH)D_3 to 1α,25$(OH)_2D_3$ in prostate cells. In this report we examined whether the prostate 1α-OHase was regulated by calcium, parathyroid hormone and 1α,25$(OH)_2D_3$, three major regulators of the renal 1α-OHase.

Materials and Methods

Cell cultures

Primary cultures of human prostate epithelial cells were prepared from the peripheral zone of normal prostate tissue as described previously (11). Primary prostate epithelial cells were cultured in a serum-free defined growth medium [Prostate Epithelial Growth Medium BulletKit (PEGM), Clonetics/ Biowhitekar, San Diego, CA, USA]. A part of the tissue specimen used for cell cultures were independently evaluated by histology to confirm the stated pathology (normal, BPH, or cancer) (11).

The transformed PZ-HPV-7 cell line (CRL-2221) was obtained from ATCC (Manassas, VA) and was derived from epithelial cells of the peripheral zone of the normal prostate tissue by transfecting with HPV18 DNA (14). The PZ-HPV-7 cells were grown in keratinocyte growth medium as described previously (15). As a positive control, the human kidney cell (HKC-8) line was grown as described (16) and was kindly provided by Dr. Martin Hewison (University of Birmingham, Birmingham, UK).

Treatment of Prostate Cells for Enzyme Activity Analysis

The second passage primary cultured cells were sub-cultured in the PEGM medium into 35 mm dishes for the enzyme activity studies. Two days after the initial plating, triplicate plates of cells were incubated with fresh PEGM media with and without PTH or 1α,25$(OH)_2D_3$ for 24 h prior to 1α-OHase enzyme activity analysis.

PZ-HPV-7 human prostate and HKC-8 human kidney cell cultures were grown on their respective growth media until they reached 70-80% confluency. The medium was then changed to 0.03 mM Ca^{2+} basal keratinocyte media in the absence of EGF and other growth factors (15), to which the cells were exposed for an additional 18 h. Cells were then incubated with 0.03, 1.2 or 2.4 mM Ca^{2+} in the presence of EGF (25 ng/ml) for a period of time as indicated in the figure legends. At the end of incubation, the media were removed from cultures and replaced with basal medium plus 50 nM of 25$(OH)D_3$ containing 0.1 μCi ^{3}H-25$(OH)D_3$ and 10 μM 1,2-dianilinoethane (DPPD) and incubated for 2 h for 1α-OHase enzyme activity analysis.

Analysis of 1α-OHase enzyme activity

The 1α-OHase enzyme activity was determined by high performace liquid chromatography (HPLC) using methylene chloride/isopropanol (19:1) as the mobile phase to prevent 10-oxo-19-nor-25$(OH)D_3$ contamination as described (10). DPPD, an anti-oxidant, was added during the 2 h incubation with ^{3}H-25$(OH)D_3$ to prevent the free radical, non-enzymatic auto-oxidation of ^{3}H-25$(OH)D_3$ to ^{3}H-1α,25$(OH)_2D_3$.

Construction of Reporter Plasmids and Transfection into Prostate Cells

PCR was used to generate a truncated form of the human 1α-OHase promoter designated as AN2 as previously reported (17). PCR products were sub-cloned into pGL2-Basic vector (Promega, Madison, WI, USA), a firefly luciferase reporter vector lacking eukaryotic promoter and enhancer, by using the Kpn I-Nhe I sites. For transient transfection experiments, cells at 80% confluency grown on 35 mm^2 dishes were transfected with 2 μg of luciferase reporter gene construct or pGL2-basic vector (negative control) for 4 h in the absence of additives or fetal calf serum (FCS) with 12.5 μl of

LipofectAmine 2000 (Life Technologies, Grand Island, NY, USA). Media with growth factors were added and cells were grown for 18 h. Cells were then treated with human PTH (1-34) (Bachem, Inc, King of Prussia, PA, USA), 1α,25$(OH)_2D_3$, $CaCl_2$ or appropriate vehicle controls as indicated for an additional 4 h in appropriate medium with growth factors added.

Luciferase Assays

After treatment, cells were harvested in 100 μl lysis buffer (Promega, Madison, WI, USA). The protein concentration of the cell lysates were determined by Bradford assay using the Bio-Rad assay kit (Bio-Rad, Hercules, CA, USA). Fifty μg of cell lysates were subjected to luciferase assay in triplicate using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA, USA).

Results

Figure 1 demonstrates the HPLC profiles for the conversion of [^{3}H]-25$(OH)D_3$ to [^{3}H]-1α,25$(OH)_2D_3$ in HKC-8 human kidney cells and primary cultured prostate epithelial cells derived from normal prostate tissue. In the HKC-8 cell line, we obtained about 4% conversion when cells were maintained in the presence of 0.03 mM calcium, whereas 20-30% conversion could be achieved in primary cultured kidney epithelial cells, such as rat renal proximal tubular cells (18). We also routinely observed 12 to 30% conversion in primary cultured normal prostate cells and in PZ-HPV-7 cell line in the presence of EGF. Treatment of primary prostate epithelial cells for 24 h with PTH at 10 nM and 100 nM had no significant effect on the 1α-OHase activity (Figure 2). Conversely, enzyme activity decreased to 66±4 and 20±11% of the control in the presence of 10 nM and 100 nM 1α,25$(OH)_2D_3$, respectively.

Next, we examined whether calcium, which is known to inhibit renal 1α-OHase activity, influenced prostate 1α-OHase activity in the transformed non-cancerous PZ-HPV-7 cells. Human HKC-8 kidney cells were maintained in the same culture media (containing EGF) and were used as positive control. We observed no significant changes in 1α-OHase activity at either 6 or 24 h after media calcium concentration was changed from 0.03 mM to 1.2 mM with EGF (Figure 3). In contrast, the 1α-OHase activity in HKC-8 kidney cells was inhibited 40% in the presence of 1.2 or 2.4 mM calcium (Figure 4).

In order to confirm these results, we next transfected a luciferase reporter gene construct containing 1100 base pairs of the proximal promoter sequence (AN2) of the human 1α-OHase gene into PZ-HPV-7 cells to examine the effect of PTH, calcium and 1α,25$(OH)_2D_3$ on the promoter activity. Neither PTH at 100 nM (Figure 5), nor calcium at 1.2 and 2.4 mM (Figure 6) had any influence on the AN2 promoter activity. Conversely, the promoter activity was reduced 48±5 % by 100 nM 1α,25$(OH)_2D_3$ in PZ-HPV-7 cells (Figure 5).

Discussion

Under normal physiologic conditions, circulating, systemic levels of $1\alpha,25(OH)_2D$ are tightly regulated by 1α-OHase present in the epithelial cells of proximal tubules of the kidneys. The renal enzyme is influenced by PTH and $1\alpha,25(OH)_2D$ in response to serum levels of calcium (19). Extra-renal 1α-OHases have now been identified in many cell types, including marcrophages, keratinocytes, parathyroid cells, placenta, colon and prostate cells (10, 19-23). In contrast to the renal enzyme, extra-renal 1α-OHases do not appear to be tightly regulated (19). For example, in peripheral macrophages cultured in serum-free media, synthesis of $1\alpha,25(OH)_2D_3$ was not influenced by PTH even at supraphysiological concentrations (e.g. 5-25 nM), but was inhibited by its own reaction product, $1\alpha,25(OH)_2D_3$ at physiological concentrations (20). Similarly, the production of $1\alpha,25(OH)_2D_3$ by alveolar macrophages from patients with sarcoidosis is not regulated by calcium (21). In keratinocytes, the 1α-OHase is unaffected by PTH (17) and calcium (22) but is inhibited by $1\alpha,25(OH)_2D_3$ (22). Like keratinocytes and unlike renal cells (24), prostate cells do not appear to have PTH/PTHrP type I receptors (unpublished observation). Therefore, it would be expected that PTH (1-34) should have no effect on the promoter activity of the 1α-OHase gene (Figure 5) and on its enzymatic activity (Figure 2).

We observed a dose-dependent inhibition of 1α-OHase activity in response to pharmacologic doses of $1\alpha,25(OH)_2D_3$ and virtually no inhibition in response to PTH and calcium (Figures 2 and 3). The mechanism of this inhibition in response to $1\alpha,25(OH)_2D_3$ is presently unknown. Through its interaction with vitamin D response element (VDRE), the VDR is essential for the hormone's action in target genes (3-5,9). Since there are no known VDRE nucleotide sequences in the promoter region of 1α-OHase gene, this effect must occur through other means. One mechanism could be a $1\alpha,25(OH)_2D$-dependent transcriptional repression through a negative regulatory VDRE, as proposed by Murayama et al. (25). Another possibility is that $1\alpha,25(OH)_2D_3$ may mediate its inhibitory action on 1α-OHase by an autocrine inhibitory loop through impairing ligand-dependent EGF receptor nuclear signaling similar to that reported by Cordero et al. (26).

Like primary cultures of normal prostate epithelial cells, PZ-HPV-7 cells have high 1α-OHase activity and they show a similar inhibition of proliferation in response to $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ (unpublished observation). As we noted in our previous reports (10, 27), among the three best characterized prostate cancer cell lines, LNCaP, PC-3 and DU 145 cells, LNCaP cells do not express this enzyme, and very low levels are expressed in PC-3 and DU 145 cells. Thus, these cell lines would not be the best choices to study the normal regulation of 1α-OHase. More importantly, in the context of chemoprevention, the key question is whether normal prostate cells are regulated by calcium and PTH. For this reason, PZ-HPV-7 cells and primary cultured prostate epithelial cells are the optimum choices.

Bland et al. (16) studied the effects of calcium on 1α-OHase activity in a transformed human kidney cell line, HKC-8. 1α-OHase activity was attenuated 80-90% at 10 hrs, but not at 24 hrs after the calcium concentration was increased from 0.5 mM to 2 mM in the culture media. The authors suggested that changes in the local calcium

concentrations might stimulate the expression of calcium-sensing receptors in the kidney cells and thereby regulate 1α-OHase activity by a mechanism independent of PTH (28). Unlike human kidney cells (Fig. 4) in which 1α-OHase activity was inhibited by 40 %, we found that 1α-OHase activity in prostate epithelial cells was not affected by increasing the media calcium concentration from 0.03 mM to 1.2 mM (Fig. 3). The higher basal activity at 24 h as compared to 6 h was due to the time-dependent upregulation of 1α-OHase in PZ-HPV-7 prostate cells induced by EGF (29). The promoter activity of 1α-OHase gene was also upregulated by EGF in PZ-HPV-7 cells. The upregulation by EGF was inhibited by mitogen-activated protein kinase kinase (MAPKK) inhibitor, PD 98059. In the prostate, EGF regulates the proliferation of prostate cells via autocrine and paracrine loops (30). Thus, EGF is likely to have a dual role in growth regulation of normal prostate cells: in addition to stimulating cell proliferation, EGF upregulates 1α-OHase to increase $1\alpha,25(OH)_2D$ synthesis to inhibit cell growth. However, EGF has no effect on 1α-OHase activity in HKC-8 cells (unpublished observation). Moreover, we did not observe calcium to have any effects on the promoter activity of 1α-OHase even at 2.4 mM calcium concentration (Fig. 6). These findings suggest that the prostate 1α-OHase resembles the 1α-OHase of the sarcoid macrophages and keratinocytes.

Our findings may have important implications for the use of vitamin D and 25(OH)D for prostate cancer chemoprevention. It is now clear that $1\alpha,25(OH)_2D$ regulates the differentiation of prostate cells and inhibits events associated with the metastatic cascade. These findings suggest that exposure of prostate cells to $1\alpha,25(OH)_2D$ could result in lower risks for clinical prostate cancer. Because serum levels of $1\alpha,25(OH)_2D$ are tightly regulated by the kidney, large increases in 25(OH)D in normal individuals do not result in increased serum levels $1\alpha,25(OH)_2D$ (31). The recognition that prostate cells synthesize $1\alpha,25(OH)_2D$ from 25(OH)D (10) has suggested that one way to achieve higher intra-prostatic levels of $1\alpha,25(OH)_2D$ would be to increase serum levels of the prohormone, 25(OH)D (31). However, in order for the prostatic synthesis of $1\alpha,25(OH)_2D$ to be useful in cancer chemoprevention, the prostate 1α-OHase must not be under the same tight control as is the renal 1α-OHase. Our demonstration that the intra-prostatic synthesis of $1\alpha,25(OH)_2D$ in cultures is unaffected by PTH and calcium confirms that the prostate 1α-OHase is distinct from the renal enzyme.

In conclusion, we suggest that the lack of regulation by PTH and calcium make both vitamin D and 25(OH)D potentially useful chemopreventive agents for prostate cancer because their administration should cause an increased synthesis of $1\alpha,25(OH)_2D$ within prostate cells. Furthermore, vitamin D and 25(OH)D each have desirable properties as chemopreventive agents. Both compounds have much longer half-lives than $1\alpha,25(OH)_2D$ (the half-life for vitamin D_3 and $25(OH)D_3$ is 24 hrs and 3-4 weeks, respectively), vs. about 4-6 hrs in circulation for $1\alpha,25(OH)_2D$ (32-34). Moreover, the therapeutic window (i.e., the interval between therapeutic and toxic doses) of vitamin D and 25(OH)D is far wider than that of $1\alpha,25(OH)_2D$ (13).

As we have shown, the autocrine synthesis of $1\alpha,25(OH)_2D$ by prostatic cells leads to increased intracellular levels of this hormone. This likely would result in the up-regulation of 25(OH)D-24-hydroxylase (24-OHase), which, in turn, would result

in the hydroxylation of 1α,25$(OH)_2$D to 1α,24,25-trihydroxyvitamin D, the first step in the 1α,25$(OH)_2$D degradative pathway. Although upregulation of 24-OHase would be expected to decrease the amount of 1α,25$(OH)_2$D available to the cell, prostate cells in culture show great variability in 24-OHase expression (35). We demonstrated previously that prostate cells derived from primary cultures of non-cancerous human prostate tissue that were exposed to 25$(OH)D_3$ for one week had antiproliferative responses that did not differ from their responses to 1α,25$(OH)_2D_3$ (11). This suggests that, at least in the short term, up-regulation of 24-OHase was not a significant impediment to the antiproliferative effects of 25-OHD. It is likely that even greater antiproliferative effects could be achieved if the induction of 24-OHase in these cells were minimized. It is intriguing in this regard that several naturally-occurring compounds, such as phytoestrogens present in soy, have been shown to inhibit the production of 24-OHase in prostate cells or interact synergistically with 1α,25$(OH)_2D_3$ to inhibit prostate cell growth (36, 37). These findings should encourage the further development of nutritionally-based models for prostate cancer chemoprevention using vitamin D.

Acknowledgements

The authors thank Kelly Persons for maintaining the cell cultures used in the study. This work was supported in part by grants MO1RR00533 from NIH, 4118PP1017 and 41211159016 from The Commonwealth of Massachusetts, and US Army DAMD (to T.C.C.), RO1CA68565 and R03CA85750 from NIH (to G.G.S.), and R01 CA061038 from NIH and US Army DAMD 17/98/8526 (to B.L.L.).

References

1. DeLuca, H. F. The transformation of a vitamin into a hormone: the vitamin D story. Harvey Lect., 75: 333-79, 1979.
2. Schwartz, G.G. Prostate cancer and vitamin D: From concept to clinic. A 10-year update. In: A.W., Norman, R. Bouillon, Thomasset M., Vitamin D Endocrine System. University of California Press, 445-452, 2000.
3. Zhao, X.Y. and Feldman, D. The role of vitamin D in prostate cancer. Steroids, 66: 293-300, 2001.
4. Polek, T.C. and Weigel, N.L. Vitamin D and prostate cancer. J. of Andrology, 23: 9-17, 2002.
5. Chen, T.C. and Holick, M.F. Vitamin D and prostate cancer prevention and treatment. Trends Endocrinol. Metab., 14:423-430, 2003.
6. Schwartz, G.G. and Hulka, B.S. Is vitamin D deficiency a risk factor for prostate cancer? (hypothesis). Anticancer Res., 10:1307-1311, 1990.
7. Hanchette, C.L. and Schwartz, G.G. Geographic patterns of prostate cancer mortality: Evidence for a protective effect of ultraviolet radiation. Cancer, 70: 2861-2869, 1992.
8. Miller, G.J., Stapleton, G.E., Ferrara, J.A., Lucia, M.S., Pfister, S., Hedlund, T.E., Upadhya, P. The human prostatic cancinoma cell line LNCaP expresses biologically active, specific receptors for 1,25 Dihydroxyvitamin D_3. Cancer Res., 52:515-520, 1992.
9. Miller, G. Vitamin D and prostate cancer: biologic interactions and clinical potentials. Cancer Met. Rev., 17:353-360, 1999.
10. Schwartz, G.G. Whitlatch, L.W., Chen, T.C., Lokeshwar, B.L., Holick, M.F. Human prostate cells synthesize 1,25-dihydroxyvitamin D_3 from 25-hydroxyvitamin D_3. Cancer Epidemiol. Biomark. & Prev., 7:391-395, 1998.
11. Chen, T.C., Schwartz, G.G., Burnstein, K.L., Lokeshwar, B.L., Holick, M.F. The in vitro evaluation of 25-hydroxyvitamin D_3 and 19-nor-1α,25-dihydroxyvitamin D_2 for prostate cancer therapy. Clin. Cancer Res., 6: 901-908, 2000.
12. Barreto, A., Schwartz, G.G., Woodruff, P., Cramer, S.D. 25-Hydroxyvitamin D_3, the prohormone of 1,25-dihydroxyvitamin D3, inhibits the proliferation of primary prostatic epithelial cells. Cancer Epidemiol. Biomark. Prev., 9: 265-270, 2000.
13. Vieth, R. Vitamin D supplementation, 25-hydroxyvitamin D concentrations, and safety. Am. J. Clin. Nutr., 69:842-56, 1999.
14. Weijerman, P.C., Konig, J.J., Wong, S.T., Niesters, H.G., Peehl, D.M. Lipofection-mediated immortalization of human prostatic epithelial cells of normal and malignant origin using human papillomavirus type 18 DNA. Cancer Res., 54:5579-83, 1994.
15. Chen, T.C., Persons, K, Liu, W-W, Chen, M.L., Holick, M.F. The antiproliferative and differentiative activities of 1,25-dihydroxyvitamin D_3 are potentiated by epidermal growth factor and attenuated by insulin in cultured human keratinocytes. J. Invest. Dermatol., 104:113-117, 1995.
16. Bland, R., Walker, E.A., Hughes, S.V., Stewart, P.M., Hewison, M. Constitutive expression of 25-hydroxyvitamin D_3-1 alpha-hydroxylase in a transformed human

proximal tubule cell line: evidence for direct regulation of vitamin D metabolism by calcium. Endocrinology, 140:2027-2034, 1999.
17. Kong, X. F., Zhu, X.H., Pei, Y.L., Jackson, D.M., Holick, M.F. Molecular cloning, characterization, and promoter analysis of the human 25-hydroxyvitamin D_3-1α-hydroxylase gene. Proc. Natl. Acad. Sci., 96:6988-6993, 1999.
18. Chen, T.C., Curthoys, N.P., Lagenaur, C.F. and Puschett, J.B. Characterization of primary cell cultures derived from rat renal proximal tubules. In Vitro, 25:714-722, 1989.
19. Bell, N.H. Renal and nornrenal 25-hydroxyvitamin D-1α-hydroxylases and their clinical significance. J. Bone Miner. Res., 13:350-353, 1998.
20. Dusso, A.S., Finch, J., Brown, A., Ritter, C., Delmez, J., Schreiner, G., Slatopolsky, E. Extrarenal production of calcitriol in normal and uremic humans. J. Clin. Endocrinol. Metab., 72:157-164, 1991.
21. Adams, J.S. Extrarenal production of active vitamin D metabolites in human lymphoproliferative diseases. In Feldman D, Glorieux FH, Pike JW (eds.) Vitamin D. Academic Press, San Diego, CA, U.S.A., pp. 903-921, 1997.
22. Bikle, D.D., Nemanic, M.F., Gee, E., and Elias, P. 1,25$(OH)_2D_3$ production by human keratinocytes. Kinetics and regulation. J. Clin. Invest., 78:557-566, 1986.
23. Cross, H.S., Peterlik, M., Reddy, G.S., Schuster, I. Vitamin D metabolism in human colon adenocarcinoma-derived Caco-2 cells: Expression of 25-hydroxyvitamin D_3-1α-hydroxylase activity and regulation of side-chain metabolism. J. Steroid. Biochem. Mol. Biol., 62: 21-28, 1997.
24. Hanafin NM, Chen TC, Heinrich G, Segre GV, Holick MF. Cultured human fibroblasts and not cultured human keratinocytes express a PTH/PTHrP receptor mRNA. J. Invest. Dermatol., 105:133-137, 1995.
25. Murayama, A., Kim, M., Unno, K., Takeyama, K., Kato, S. A novel mechanism of the vitamin D dependent transcriptional repression through the human 25-hydroxyvitamin D_3 1 alpha-hydroxylase in nVDRE. J. Bone Miner. Res., 16:S229, 2001.
26. Cordero, J.B., Cozzolino, M., Lu, Y., Vidal, M., Slatopolsky, E., Stahl, P.D., Barbieri, M.A., and Dusso, A. 1,25-Dihydroxyvitamin D down-regulates cell membrane growth- and nuclear growth-promoting signals by the epidermal growth factor receptor. J. Biol. Chem., 277: 38965-38971, 2002.
27. Whitlatch, L.W., Young, M.V., Schwartz, G.G., Flanagan, J.N., Burnstein, K.L., Lokeshwar, B.L., Rich, E.S., Holick, M.F. and Chen, T.C. 25-Hydroxyvitamin D-1α-hydroxylase activity is diminished in human prostate cancer and is enhanced by gene transfer. J. Steroid Biochem. Mol. Biol., 81:135-140, 2002.
28. Brown, E. M. and MacLeod, R. J. Extracellular calcium sensing and extracellular calcium signaling. Physiol. Rev., 81:239-297, 2001.
29. Wang, L. Flanagan, J.N., Whitlatch, L.W., Jamieson, D.P., Holick, M.F. and Chen, T.C. Regulation of 25-hydroxyvitamin D-1α-hydroxylase by epidermal growth factor in prostate cells. J. Steroid Biochem. Mol. Biol., (2004, in press).
30. Arteaga, C.L. Epidermal growth factor receptor dependence in human tumors: More than just expression? The Oncologist 7 (Supple 4):31-39, 2002.
31. Chesney, R.W., Rosen, J.F., Hanstra, A.J., Smith, C., Mahaffey, K., DeLuca, H.F. Absence of seasonal variations in serum concentrations of 1,25-Dihydroxyvitamin

D despite a rise in 25-hydroxyvitamin D in summer. J. Clin. Endocrinol. Metab., 53:139-143, 1981.
32. Clemens, T.L., Adams, J.S. & Holick, M.F. Measurement of circulating vitamin D in man. Clin. Chim. Acta., 121: 301-308, 1982.
33. Gray, R.W., Weber, H.P., Dominquez, J.H., Lemann, J. Jr. The metabolism of vitamin D_3 and 25-hydroxyvitamin D_3 in normal and anephric humans. J. Clin. Endocrinol. Metab., 39:1045-1056, 1974.
34. Gray, R.W., Caldas, A.E., Wilz, D.R., Lemann, J. & Smith, G.A. Excretion of ^{3}H-1,25-dihydroxyvitamin D_3 in health adults. J. Clin. Endocrinol. Metab., 46:756-765, 1978.
35. Miller, G.J., Stapleton, G.E., Hedlund, T.E., Moffat, K.A. Vitamin D receptor expression, 24-hydroxylase activity, and inhibition of growth by 1alpha,25-dihydroxyvitamin D_3 in seven human prostatic carcinoma cell lines. Clin Cancer Res., 1:997-1003. 1995.
36. Cross, H.S., Kallay, E., Farhan, H., Weiland, T., Manhardt, T. Regulation of extrarenal vitamin D metabolism as a tool for colon and prostate cancer prevention. Recent Results Cancer Res., 164:413-25, 2003.
37. Rao, A., Woodruff, R.D., Wade, W.N., Kute, T.E., Cramer, S.D. Genistein and vitamin D synergistically inhibit human prostatic epithelial cell growth. J Nutr., 132:3191-4, 2002.

Figure Legends

Figure 1. HPLC elution profile of tritium activity of lipid extracts from cultured HKC-8 human kidney cell line and cultured normal human prostate primary cultures incubated with [^{3}H]25(OH)D_3 and 10 μM DPPD at 37°C for 2 hr.

Figure 2. 1α-OHase activity in the primary culture of normal prostate cells was inhibited by 1α,25$(OH)_2D_3$, but not by PTH. *$p<0.05$, **$p<0.01$. Primary cultured cells grown to 60% confluency were treated with 1α,25$(OH)_2D_3$ or PTH for 24 hrs in the presence of EGF prior to enzyme analysis. Data are means ± SD of 3-6 independent determinations.

Figure 3. Effect of calcium on 1α-OHase activity in PZHPV-7 prostate cells. Media for PZHPV-7 cells grown to 70-80% confluency in a growth factor supplemented medium were changed to 0.03 mM Ca^{2+} basal media in the absence of growth factors for 18 h, and then cells were incubated with basal (0.03 mM Ca^{2+}) or 1.2 mM Ca^{2+} and EGF (25 ng/ml) for 6 or 24 hrs before the enzyme activity was determined. Data are means ± SD of 3 dishes. The experiment was repeated 3 times with similar results.

Figure 4. Effect of calcium on 1α-OHase activity in HKC-8 human kidney cells. Media for HKC-8 cells grown to 70-80% confluency in a serum supplemented medium were changed to 0.03 mM Ca^{2+} basal media in the presence of growth factors for two days, and then cells were incubated with basal (0.03 mM Ca^{2+}), 1.2 or 2.4 mM Ca^{2+} for 6 hrs before the enzyme activity was determined. Data are means ± SD of 3 dishes. The experiment was repeated 3 times with similar results.

Figure 5. Effect of PTH and 1α,25$(OH)_2D_3$ on the promoter activity of 1α-OHase gene in PZHPV-7 prostate cells. PZHPV-7 cells grown to 70-80% confluency in a growth factor supplemented medium were transfected with AN2-1α-OHase gene promoter fragment/luciferase reporter gene construct and then treated with 100 nM PTH, or 100 nM 1α,25$(OH)_2D_3$ for 24 hrs before cell lysate was recovered for the determination of luciferase activity. *$p<0.05$. Data are means ± SD of 3 separate dishes. The experiment was repeated 3 times with similar results.

Figure 6. Effect of calcium on the promoter activity of 1α-OHase gene in PZHPV-7 cells. PZHPV-7 cells grown to 70-80% confluency in a growth factor supplemented medium were transfected with AN2-1α-OHase gene promoter fragment/luciferase reporter gene construct and then treated with calcium chloride for 24 hrs before cell lysate was recovered for the determination of luciferase activity. Data are means ± SD of 3 dishes. The experiment was repeated 3 times with similar results.

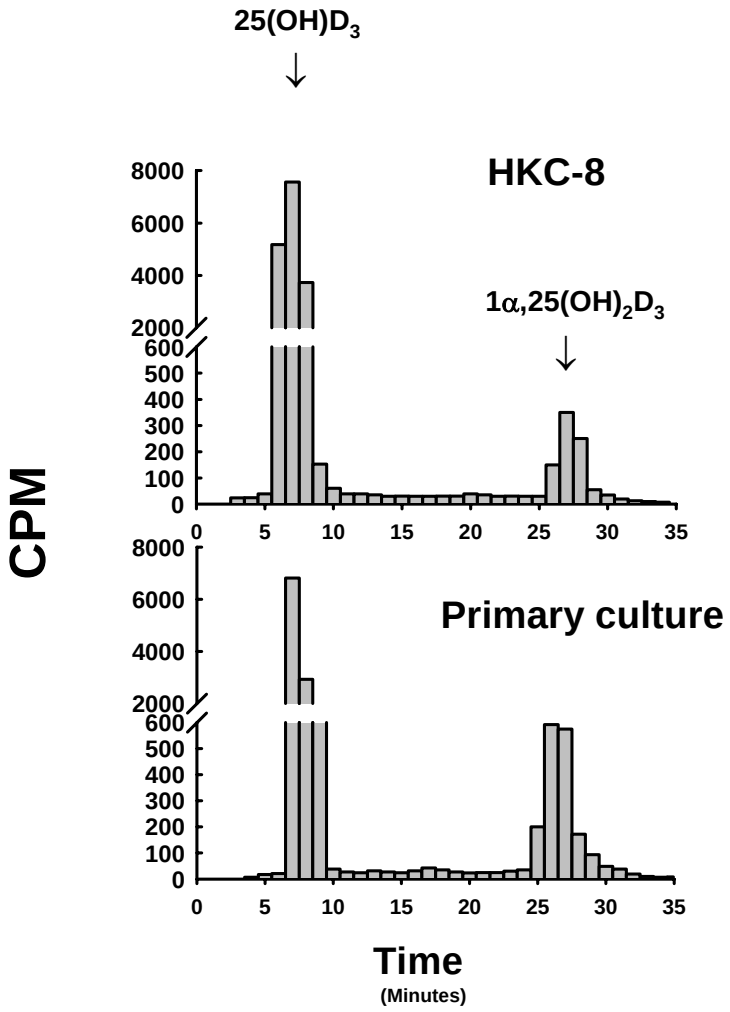
25(OH)D3
HKC-8
1α,25(OH)2D3
Primary culture
CPM
Time
(Minutes)
8000
6000
4000
2000
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35

Figure 1

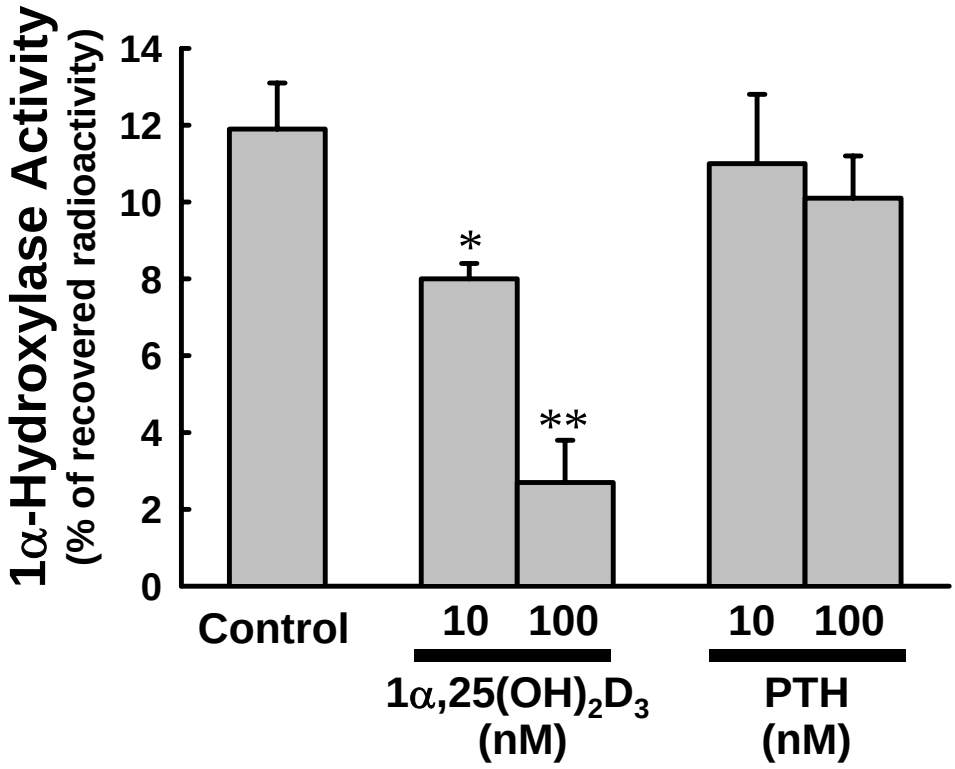
1α-Hydroxylase Activity
(% of recovered radioactivity)
14
12
10
8
6
4
2
0
*
**
Control
10
100
10
100
1α,25(OH)2D3
(nM)
PTH
(nM)

Figure 2

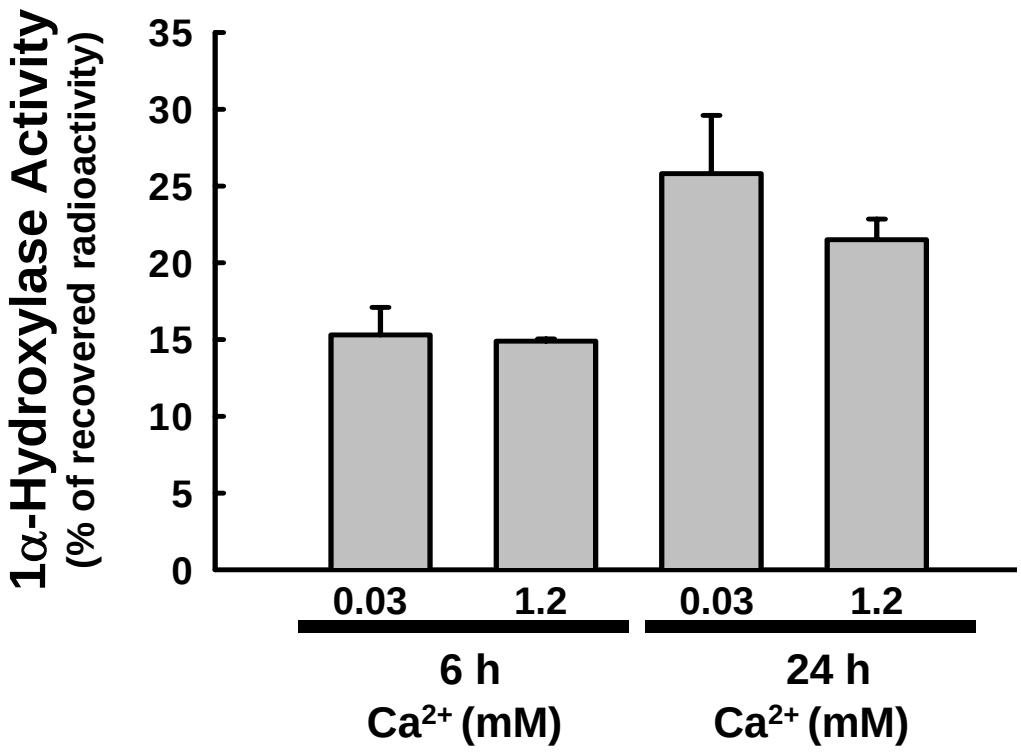
1α-Hydroxylase Activity
(% of recovered radioactivity)
35
30
25
20
15
10
5
0
0.03
1.2
0.03
1.2
6 h
Ca2+ (mM)
24 h
Ca2+ (mM)

Figure 3

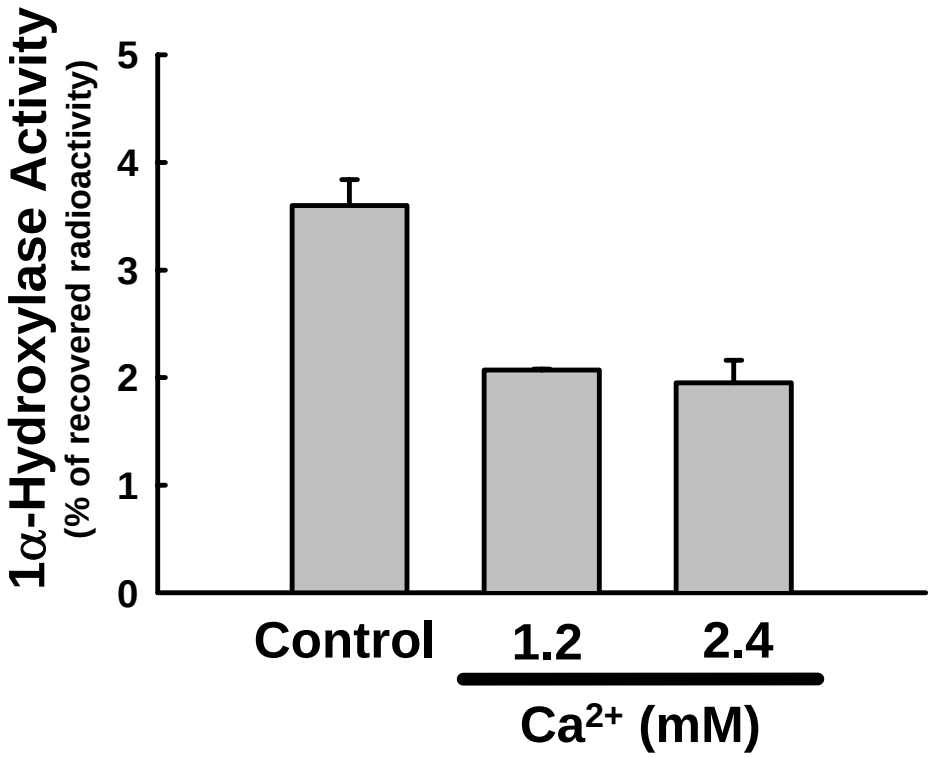
1α-Hydroxylase Activity
(% of recovered radioactivity)
5
4
3
2
1
0
Control
1.2
2.4
Ca2+ (mM)

Figure 4

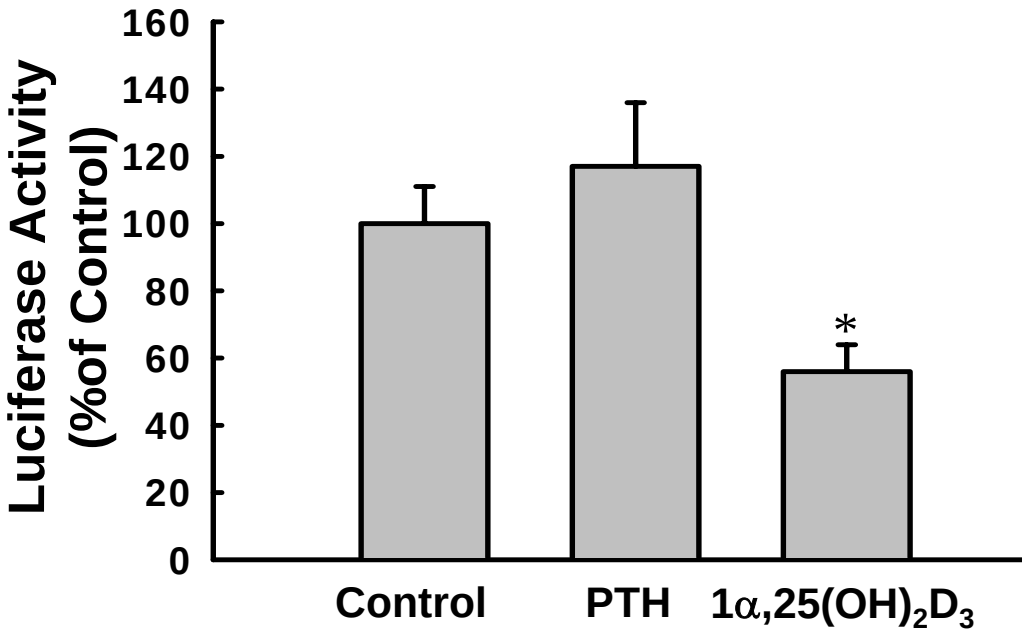
Luciferase Activity
(%of Control)
160
140
120
100
80
60
40
20
0
*
Control
PTH
1α,25(OH)2D3

Figure 5

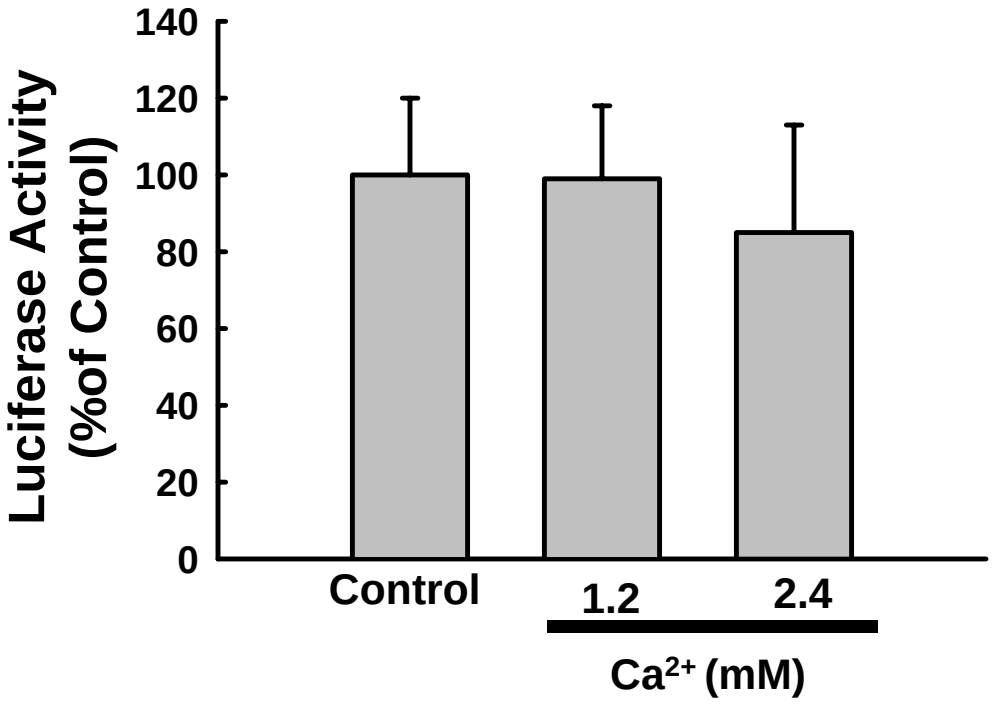
Luciferase Activity
(%of Control)
140
120
100
80
60
40
20
0
Control
1.2
2.4
Ca2+ (mM)

Figure 6