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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The hormonal form of vitamin D, 1α,25-dihydroxyvitamin D [1α,25(OH)2D] promotes the differentiation and inhibits the proliferation, invasiveness and metastasis of prostate cells. However, 1α,25(OH)2D is not suitable as a chemopreventive agent because its administration can cause hypercalcemia. Serum levels of 1α,25(OH)2D are tightly regulated by the renal enzyme, 25-hydroxyvitamin D-1α-hydroxylase (1α-OHase), which synthesizes 1α,25(OH)2D 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 now investigated the regulation of the prostate 1α-OHase by the three most important regulators of the renal 1α-OHase: calcium, 1α,25(OH)2D and parathyroid hormone (PTH). The 1α-OHase activity in the primary cultures of prostate epithelial cells was inhibited by 1α,25(OH)2D1 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)2D3. Our findings suggest that, unlike the renal enzyme, the prostate 1α-OHase appears to be 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)2D 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 D2 (ergocalciferol) or vitamin D3 (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)2D, 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 US, 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)2D, in prostate cancer cells. Subsequently, many studies showed that the exposure of prostate cancer cells to 1α,25(OH)2D3 and its analogs resulted in an inhibition of cancer cell proliferation, invasiveness and metastasis of prostate cancer cells, both in vitro and in vivo in animal models (2–5). These findings have led to the active investigation of 1α,25(OH)2D3 and analogs as therapeutic agents for prostate cancer (9).

Despite the encouraging data in the therapeutic setting, 1α,25(OH)2D3 is not suitable as a chemopreventive agent because administration of this hormone systemically can cause hypercalcemia (9). For example, the concentrations of 1α,25(OH)2D3 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)2D3 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)2D3 (1.3–5.9). We demonstrated recently that prostate cells possess 25-hydroxyvitamin D-1α-hydroxylase (1α-OHase) and are capable of synthesizing 1α,25(OH)2D3 intracellularly from 25(OH)D3 (10). In cells that possess 1α-OHase, the antiproliferative effects of 25(OH)D3 were indistinguishable from those of 1α, 25(OH)2D3 (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)D3 is far lower than that for 1α,25(OH)2D3 (13). Thus, it is critical to understand what factors may regulate the conversion of 25(OH)D3 to 1α,25(OH)2D3 within prostate cells. In this report we examined whether the prostate

Abbreviations: EGF, epidermal growth factor; 1α-OHase, 25-hydroxyvitamin D-1α-hydroxylase; 25(OH)D3, 25-hydroxyvitamin D3; 1α,25(OH)2D3, 1α,25-dihydroxyvitamin D3; PTH, parathyroid hormone.
1α-OHase was regulated by calcium, parathormone hormone (PTH) and 1α,25(OH)₂D₃, 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]. A part of the tissue specimen used for cell cultures was 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)₂D₃ 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 Ca²⁺ basal keratinocyte media in the absence of epidermal growth factor (EGF) and other growth factors (15), to which the cells were exposed for an additional 18 h. Cells were then incubated with 0.03, 1.4 or 2.4 mM Ca²⁺ 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)D₃ containing 0.1 μCi [³H]25(OH)D₃ 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 performance liquid chromatography (HPLC) using methylene chloride/isopropanol (19:1) as the mobile phase to prevent 10-oxo-19-nor-25(OH)D₃ contamination as described (10). DPPD, an anti-oxidant, was added during the 2 h incubation with [³H]25(OH)D₃ to prevent the free radical, non-enzymatic auto-oxidation of [³H]25(OH)D₃ to [³H]1,25(OH)₂D₃.

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 reported previously (17). PCR products were sub-cloned into pGL2-Basic vector (Promega, Madison, WI), a firefly luciferase reporter vector lacking eukaryotic promoter and enhancer, by using the KpnI–NheI sites. For transient transfection experiments, cells at 80% confluency grown at 80% confluence were transfected with luciferase reporter gene construct or pGL2-basic vector (negative control) for 4 h 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)D₃ containing 0.1 μCi [³H]25(OH)D₃ and 10 μM 1,2-dianilinoethane (DPPD) and incubated for 2 h for 1α-OHase enzyme activity analysis.

Results
Figure 1 demonstrates the HPLC profiles for the conversion of [³H]25(OH)D₃ to [³H]1α,25(OH)₂D₃ 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 ~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 12-30% 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 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 and 100 nM 1α,25(OH)₂D₃, 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 a 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 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 bp 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 1\(\alpha\),25(OH)\(_2\)D\(_3\) 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% by 100 nM 1\(\alpha\),25(OH)\(_2\)D\(_3\) in PZ-HPV-7 cells (Figure 5).

**Discussion**

Under normal physiologic conditions, circulating, systemic levels of 1\(\alpha\),25(OH)\(_2\)D are tightly regulated by 1\(\alpha\)-OHase present in the epithelial cells of proximal tubules of the kidneys. The renal enzyme is influenced by PTH and 1\(\alpha\),25(OH)\(_2\)D in response to serum levels of calcium (19). Extra-renal 1\(\alpha\)-OHases have now been identified in many cell types, including macrophages, keratinocytes, parathyroid cells, placenta, colon and prostate cells (10,19--23). In contrast to the renal enzyme, extra-renal 1\(\alpha\)-OHases do not appear to be
tightly regulated (19). For example, in peripheral macrophages cultured in serum-free media, synthesis of 1α,25(OH)2D3 was not influenced by PTH even at supraphysiological concentrations (e.g. 5–25 nM), but was inhibited by its own reaction product, 1α,25(OH)2D3, at physiological concentrations (20). Similarly, the production of 1α,25(OH)2D3 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 1α,25(OH)2D3 (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 1α,25(OH)2D3 and virtually no inhibition in response to PTH and calcium (Figures 2 and 3). The mechanism of this inhibition in response to 1α,25(OH)2D3 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 1α,25(OH)2D3-dependent transcriptional repression through a negative regulatory VDRE, as proposed by Murayama et al. (25). Another possibility is that 1α,25(OH)2D3 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)D3 and 1α,25(OH)2D3 (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 h, but not at 24 h after the calcium concentration was increased from 0.5 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 (Figure 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 to 1.2 mM (Figure 3). The higher basal activity at 24 h as compared with 6 h was due to the time-dependent up-regulation of 1α-OHase in PZ-HPV-7 prostate cells induced by EGF (29).

The promoter activity of the 1α-OHase gene was also up-regulated by EGF in PZ-HPV-7 cells. The up-regulation by EGF was inhibited by the mitogen-activated protein kinase 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 up-regulates 1α-OHase to increase 1α,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 (Figure 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 1α,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 1α,25(OH)2D could result in lower risks for clinical prostate cancer. Because serum levels of 1α,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 1α,25(OH)2D (31). The recognition that prostate cells synthesize 1α,25(OH)2D from 25(OH)D (10) has suggested that one way to achieve higher intra-prostatic levels of 1α,25(OH)2D would be to increase serum levels of the prohormone, 25(OH)D (31). However, in order for the prostatic synthesis of 1α,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 1α,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 1α,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 1α,25(OH)2D [the half-life for vitamin D3 and 25(OH)D is 24 h and 3–4 weeks, respectively], versus ~4–6 h in circulation for 1α,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 1α,25(OH)2D (13).

As we have shown, the autocrine synthesis of 1α,25(OH)2D by prostatic cells leads to increased intracellular levels of this hormone. This would probably 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)2D to 1α,24,25-trihydroxyvitamin D, the first step of 1α,25(OH)2D degradative pathway. Although up-regulation of 24-OHase would be expected to decrease the amount of 1α,25(OH)2D 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)D3 for 1 week had anti-proliferative responses that did not differ from their responses to 1α,25(OH)2D3 (11). This suggests that, at least in the short term, up-regulation of 24-OHase was not a significant impediment to the anti-proliferative effects of 25(OH)D. It is probable that even greater anti-proliferative effects could be achieved if the induction of 24-OHase in these cells were minimized. It is intriguing in this regard that several
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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)2D3 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.

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