Prostate 25-Hydroxyvitamin D-1α-Hydroxylase is Up-regulated by Suberoylanilide Hydroxamic Acid (SAHA), A Histone Deacetylase Inhibitor

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Abstract. Prostatic 25-hydroxyvitamin D-1α-hydroxylase (1α-OHase) is up-regulated by epidermal growth factor (EGF) and down-regulated by 1α,25-dihydroxyvitamin D [1α,25(OH)2D] at the promoter level in an autocrine/paracrine fashion, suggesting that local production of 1α,25(OH)2D could provide an important cell growth regulatory mechanism. Gene expressions depend on the acetylation status of the histone tails of chromatin, which is regulated by histone acetyltransferases and histone deacetylases (HDAC). A number of HDAC inhibitors, including suberoylanilide hydroxamic acid (SAHA), can inhibit tumor growth in vitro and in vivo. Moreover, SAHA increases the expression of genes which modulate cell cycle progression, tumor suppression, differentiation and apoptosis. Therefore, whether SAHA might also regulate 1α-OHase activity in PZ-HPV-7 prostate cells was investigated. SAHA at 10 μM up-regulated 1α-OHase activity approximately two-fold as analyzed by the formation of 2H-1α,25(OH)2D3 from 2H-25-hydroxyvitamin D3 using high performance liquid chromatography. SAHA (10 μM) also stimulated 1α-OHase mRNA expression as measured by real-time polymerase chain reaction, and promoter activity determined by luciferase reporter gene assay. The findings suggest that another important action of SAHA may be to up-regulate the expression of the 1α-OHase gene that controls the synthesis of 1α,25(OH)2D which in turn regulates prostate growth and differentiation in an autocrine/paracrine fashion.

Abundant epidemiological and experimental evidence suggests that one precipitating factor for prostate cancer might be vitamin D insufficiency (1). It is well established that vitamin D plays important roles in regulating cellular proliferation and differentiation (2). To become biologically active, vitamin D must be hydroxylated first at the C-25 position to form 25-hydroxyvitamin D [25(OH)D], catalyzed by vitamin D-25-hydroxylase (25-OHase), and then at the 1α-position catalyzed by 1α-OHase, to form 1α,25-dihydroxyvitamin D [1α,25(OH)2D], the active form (3). Both 25(OH)D and 1α,25(OH)2D can also be hydroxylated to form corresponding 24-hydroxylated metabolites catalyzed by vitamin D-24R-hydroxylase (24R-OHase), an enzyme which is mainly responsible for the clearance of excess 25(OH)D and 1α,25(OH)2D in the circulation (3, 4). These three enzymes, namely 25-OHase, 1α-OHase and 24R-OHase, have been cloned (4) and are expressed in prostate cells (5). Data from our laboratory demonstrate that, unlike the renal enzyme, 1α-OHase in prostate cells appears to be regulated in an autocrine/paracrine fashion (6, 7). Our data also suggest that prostatic 1α-OHase is regulated by both protein kinase A (PKA) and mitogen activated protein kinase (MAPK) signaling pathways at the gene promoter level and its expression is greatly diminished in prostate cancer cells. Therefore, the results suggest that a defect in the regulation of 1α-OHase activity and/or expression which lead to insufficient synthesis of 1α,25(OH)2D could contribute to growth dysregulation of human prostate cancer cells, indicating that prostatic 1α-OHase may play an important role in the natural history of prostate cancer (1).

Histone acetyltransferases (HAT) and histone deacetylase (HDAC) regulate the acetylation of histones, which can modify the structure and function of histones and proteins in transcription factor complexes and thereby modulate gene expression.
expression (8). These two enzymes can also reversibly acetylate nonhistone proteins that are involved in the regulation of cell proliferation and apoptosis. The inhibitors of HDACs have been found to cause growth arrest, differentiation and apoptosis of many tumor cells by altering the transcription of genes (9). Because of these properties, HDAC inhibitors have become a new class of targeted anticancer agents. The HDAC inhibitors are a structurally diverse group of molecules and can be classified into several structural groups including hydroxamates, cyclic peptides, aliphatic acids and benzamides. Similar to trichostatin A (TSA), sulforaphane hydroxamic acid (SAHA) belongs to the hydroxamate group (Figure 1).

The biological activities of SAHA have been extensively studied. SAHA has been shown to be a potent inducer of apoptosis and cell arrest in a variety of cancer cell types (10). For example, the expression of cyclin dependent kinase (CDK) inhibitor, p21waf1, can be induced by HDAC inhibitor and its expression correlates with an increase in the acetylation of histones associated with the p21waf1 promoter region, which is a direct target for SAHA (11). In the present study, we assessed the effect of SAHA on the expression of 1α-OHase, an enzyme responsible for the autocrine synthesis of the potent antiproliferative 1α,25(OH)2D3, in prostate cells.

Materials and Methods

Cell cultures. The transformed PZ-HPV-7 cell line (CRL-2221), derived from epithelial cells of the peripheral zone of the normal prostate tissue, was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA).

Treatment of prostate cells for enzyme activity analysis. PZ-HPV-7 prostate cells were grown on 6-well plates in MCDB-153 medium supplemented with L-histidine (50 mg/L), L-isoleucine (99 mg/L), L-methionine (14 mg/L), L-phenylalanine (15 mg/L), L-tryptophan (10 mg/L), L-tyrosine (14 mg/L), bovine pituitary extract (2 ml/L), epidermal growth factor (EGF) (25 μg/L), insulin (5 mg/L), L-histidine (50 mg/L), L-isoleucine (99 mg/L), L-leucine (50 mg/L), L-phenylalanine (15 mg/L), L-proline (99 mg/L), L-tyrosine (14 mg/L), L-tryptophan (10 mg/L), and prostaglandin E1 (PGE1) (50 μg/L) (12).

1α-OHase enzyme activity. Monolayer cultures of PZ-HPV-7 cells were incubated for 2 hours at 37°C with 50 nM 25(OH)D3 containing 0.1 μCi of 3H-25(OH)D3 as the enzyme substrate and 1,2-dianilinoethane (DPPD), an antioxidant and a known inhibitor of free radical-generated 1α,25(OH)2D. At the end of incubation, cultures were placed on ice, and media were removed. Immediately afterward, 1 ml of methanol was added to cells to extract both radioactive and nonradioactive 25(OH)D3 and 1α,25(OH)2D. After extraction at room temperature for 15 minutes, the methanol extract was transferred to a glass tube and the cells were washed with an additional 0.5 ml of methanol. The extract and wash were combined, dried down with a stream of nitrogen, and re-dissolved in 0.2 ml of mobile phase solvent containing methylene chloride:isopropanol (19:1) for high-performance liquid chromatographic (HPLC) analysis. The HPLC system used was a Hewlett Packard series 1100 high performance liquid chromatography (HPLC) (Agilent Technologies, Santa Clara CA, USA) attached with an in-line flow Scintillation Analyzer (Packard BioScience Company, Downers Grove, IL, USA) for the detection of radioactivity. The retention time for 25(OH)D3 and 1,25(OH)2D3 was calibrated by applying radioactive and nonradioactive standard 25(OH)D3 and 1,25(OH)2D3 to the HPLC column before, during and after unknown sample application. For the analysis of unknown samples, a 100-μl aliquot of extracts was applied to a normal phase silica column (5-μm particle size, 250×4.6 mm) with a flow rate of 1 ml/min using a methylene chloride:isopropanol (19:1) solvent system as the mobile phase. The radioactive 25(OH)D3 and 1,25(OH)2D3 peaks from the unknown samples as determined by Packard Scintillation Analyzer were identified according to the retention time of the radioactive standards. The concentration of 3H-25(OH)D3 produced was calculated from the specificity of 3H-25(OH)D3 added to cells during the incubation. The protein concentration in each 35-mm dish was measured by Bradford method (13) to determine the enzyme activity expressed as pmol of 1,25(OH)2D3 produced/mg protein/h.

Transfection of luciferase reporter plasmids into prostate cells for promoter assay. Luciferase reporter plasmid containing the human 1α-OHase promoter designated as AN2 was transiently transfected into PZHPV-7 cells as described elsewhere (14). Cells were treated with SAHA (Sigma, St Louis, MO, USA) dissolved in absolute ethanol or absolute ethanol (control group) for 24 hours. After treatment, cell lysates obtained were subjected to luciferase assay in triplicate using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA, USA) (6).

Real-time qPCR analysis. Total RNA from PZ-HPV-7 cells was isolated and cDNA was generated from the total RNA with random hexamer primers as described elsewhere (15). Sequence specific primers were designed against coding sequences of human CYP2B1 with Primer Express software (Applied Biosystems, Forest City, CA, USA). For each real-time qPCR reaction, 20 ng of single-stranded cDNA was mixed with 2X SYBR Green PCR Master Mix (Applied Biosystems) and an optimal concentration of sequence specific primers. Samples were analyzed on an ABI Prism 7700 sequence detection system (Applied Biosystems). To
normalize the amount of sample cDNA added to the reaction, Taqman PDAR eukaryotic 18S rRNA (Applied Biosystems) was used as the endogenous control. Relative quantitation of gene expression was calculated using the ΔΔCt method (User Bulletin publication #2, Applied Biosystems).

**Results**

Since 1α-OHase can be regulated by EGF and 1α,25(OH)2D at the promoter level in PZ-HPV-7 cells, we were interested in finding out whether SAHA can also affect the expression of 1α-OHase in the same cell line. Two concentrations of SAHA, 1 μM and 10 μM were tested for their effect on the promoter activity of 1α-OHase after transient transfection of PZ-HPV-7 cells with luciferase reporter plasmid containing the human 1α-OHase AN2 promoter sequence. No effect was observed with 1 μM of SAHA after 24 hours of treatment, while 10 μM SAHA caused a two-fold increase in 1α-OHase promoter activity (p<0.01) (Figure 2).

The effect of SAHA on the 1α-OHase mRNA expression in PZ-HPV-7 cells was determined by real-time PCR using 18S rRNA as internal control. Treatment with 10 μM SAHA caused more than a 30-fold stimulation in 1α-OHase mRNA expression (Figure 3).

Because of the stimulatory effect of SAHA on the promoter activity and mRNA expression of 1α-OHase in PZ-HPV-7 cells, we examined the effect of SAHA on the conversion of ³H-25(OH)D₃ to ³H-1α,25(OH)₂D₃ in PZ-HPV-7 cells by HPLC analysis as described in the Materials and Methods section. In the presence of 10 μM of SAHA, a greater than two-fold increase in the formation of ³H-1α,25(OH)₂D₃ from ³H-25(OH)D₃ was observed after 24 hours of SAHA treatment as compared to the control (Figure 4).

**Discussion**

The enzyme, 1α-hydroxylase or CYP27B1, plays a central role in the vitamin D endocrine system by converting 25(OH)D, the pro-hormone, to the active form of vitamin D or 1α,25(OH)₂D. 1α,25(OH)₂D is known to regulate over 200 genes, including those associated with calcium homeostasis, immune responses, blood pressure control, cell proliferation, differentiation and apoptosis (16). However, until the mid 1980’s, it was generally accepted that under normal conditions the 1α-hydroxylation of 25(OH)D only took place in the kidneys. Local synthesis of 1α,25(OH)₂D from 25(OH)D was first reported by Bikle et al. (17) in cultured keratinocytes, where 1α,25(OH)₂D acts locally to regulate cellular proliferation and differentiation. Later, 1α-OHase activity was demonstrated in several other cell types.
and tissues, including the primary cultured prostate cells obtained from normal and cancerous prostate tissues, and immortalized prostate cancer cell lines (16, 18).

Interestingly, we observed a lower 1α-OHase enzyme activity in the primary cultured BPH and prostate cancer cells than in the cultures of normal cells (5). Two commonly used prostate cancer cell lines, PC-3 and DU145, had even lower enzyme activity than the primary cultured prostate cancer cells. No detectable 1α-OHase activity was found in LNCaP prostate cancer cell line. The importance of 1α-OHase for the antiproliferative effects of vitamin D was further demonstrated in experiments in which LNCaP cells were transfected with either 1α-OHase cDNA plasmid or vector only (5). When LNCaP cells, which were either transient or stably transfected with 1α-OHase cDNA, were treated with 25(OH)D3, the cells were growth inhibited, whereas 25(OH)D3 had no effect on the LNCaP cells which were transfected with vector only. The results clearly indicate a critical role this enzyme plays in mediating the actions of vitamin D that can be obtained either through cutaneous synthesis or from dietary sources.

Our previous finding of a marked decrease in 1α-OHase activity in cancer cells compared to normal cells led us to investigate the possibility of whether this could be due to dysregulation of the enzyme at the promoter level (14). To study the regulation of gene promoter, we cloned and sequenced the promoter region from PZ-HPV-7, DU145, PC-3 and LNCaP prostate cells. The sequence of the 1α-OHase gene promoter from these four cell lines was found to be identical to that of the human keratinocytes (21) with a length of 1.4 kb. Using the promoter/luciferase reporter gene constructs, we investigated the 1α-OHase promoter activity in the presence and absence of calcium, parathyroid hormone, EGF and other agents in PZ-HPV-7 cells. We found that the promoter activity of 1α-OHase in prostate cells was not regulated by calcium and parathyroid hormone, two primary endocrine regulators of renal 1α-OHase, whereas it was regulated by EGF at the promoter level in an autocrine/paracrine manner (7). When the reporter gene construct was transfected into DU145, PC-3 and LNCaP cells, we found a substantially lower basal promoter activity in PC-3 and DU145 cells, compared to that found in PZ-HPV-7 cells. A complete loss of promoter activity in LNCaP cells was observed (14).

Our present results showing that the prostate 1α-OHase promoter can also be up-regulated by SAHA (Figure 2), indicate that the balance between the acetylated and deacetylated states of chromatin in the prostate cells may play a major role in regulating 1α-OHase gene expression. Up-regulation of the promoter activity led to the up-regulation of mRNA expression (Figure 3) and enzyme activity (Figure 4). We also tested another class of HDAC inhibitor, sodium butyrate and found that the effects induced by SAHA were not reproduced with sodium butyrate (unpublished data). It is possible that the short-chain fatty acids, such as butyrate, are less effective because of their short side-chains, limiting their binding with the catalytic pocket of HDACs (19). In contrast, compounds containing hydroxamic acid residues, including TSA and SAHA, have high affinity for the HDAC catalytic site and, thereby, effectively block the access of the substrate to the zinc ion, preventing the deacetylation of histones. SAHA, a simple hydroxamic acid derivatives, is considered as a second-generation polar-planar compound, and has been shown to induce growth arrest, differentiation and apoptosis in vitro and in vivo xenograft mouse models (20). In summary, we demonstrated here that 1α-OHase promoter activity, mRNA expression and the enzyme activity were up-regulated upon treatment with micromolar concentrations of SAHA. The findings suggest that another important action of SAHA may be to up-regulate the expression of the 1α-OHase gene that controls the synthesis of 1α,25(OH)2D, which in turn regulates prostate growth and differentiation in an autocrine/paracrine fashion.

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References


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