1α,25-dihydroxyvitamin D_3 (Calcitriol) inhibits hypoxia-inducible factor-1/vascular endothelial growth factor pathway in human cancer cells

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Abstract

In vitro **and** ***in vivo*** **studies have shown that 1α,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] inhibits angiogenesis in cancer. We now examined whether the antiangiogenic effects of 1,25(OH)$_2$D$_3$ are mediated by the hypoxia-inducible factor (HIF)-1 pathway. Our results showed that 1,25(OH)$_2$D$_3$ reduces the protein expression of both the regulated HIF-1α subunit and the vascular endothelial growth factor (VEGF) in various human cancer cells. 1,25(OH)$_2$D$_3$ also inhibited HIF-1 transcriptional activity (measured by reporter gene assay) as well as HIF-1 target genes, including *VEGF, ET-1*, and *Glut-1*. We also showed that 1,25(OH)$_2$D$_3$ inhibits cell proliferation under hypoxia. Using HIF-1α knockout colon cancer cells, we show that the inhibition of the hypoxia-induced VEGF by 1,25(OH)$_2$D$_3$ is mediated through a HIF-dependent pathway. Because HIF-1 is a major positive contributor in human tumorigenesis and angiogenesis, we believe that its inhibition by 1,25(OH)$_2$D$_3$ strengthens the rationale to use vitamin D and its low-calcemic analogues in cancer chemoprevention and therapy. [Mol Cancer Ther 2007;6(4):1433–9]**

Introduction

Vitamin D has traditionally been associated with systemic calcium hemostasis (1), but it also has important non-calcemic biological action. It is now recognized that 25-hydroxyvitamin D-1α-hydroxylase, which synthesizes 1α,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$], the active form of the vitamin D, and vitamin D receptor are present in many normal and cancer tissues (2–5). There are increasing lines of evidence that 1,25(OH)$_2$D$_3$ regulates cell proliferation, differentiation, apoptosis, immune responses, and angiogenesis in an autocrine/paracrine fashion (1, 4, 6, 7). Indeed, studies on prostate, colon, breast, lung, and other cancers indicate that 1,25(OH)$_2$D$_3$ prevents cancer progression by reducing cell proliferation, by increasing cell differentiation and apoptosis, and by inhibiting angiogenesis (1, 4–7).

Hypoxia is the major pathophysiologic condition that regulates angiogenesis. Increased angiogenesis in response to hypoxia is part of the cellular adaptation mediated by the key transcription factor, hypoxia-inducible factor (HIF)-1 (8). HIF-1 is composed of the oxygen-regulated subunit HIF-1α and the constitutively expressed HIF-1β subunit. HIF-1α is produced and rapidly degraded under normoxic conditions due to posttranslational oxygen-dependent hydroxylation on specific proline residues (402 and 564) (review in ref. 8). The hydroxylated protein is ubiquitinated by the von Hippel Lindau protein E3 ligase complex and targeted to proteasomal degradation. Under hypoxia, HIF-1α is stabilized and heterodimerizes with HIF-1β to bind to an enhancer element called the 'hypoxia response element' (HRE) in target genes. HIF-1 drives the transcription of >70 survival genes, including the glycolytic enzymes, *glucose transporter-1* (*Glut-1*), *endothelin-1* (*ET-1*), *vascular endothelial growth factor* (*VEGF*), *VEGF receptor-1* (*Flt-1*), *carbonic anhydrase 9* (*CA9*), and *erythropoietin* (see ref. 8 for complete list). The hypoxic response pathway has also been recognized as an important contributor to a wide range of human cancers, including breast, prostate, brain, lung, colon, and head and neck (9). Increased levels of HIF-1 activity are often associated with increased tumor aggressiveness, therapeutic resistance, and mortality (9). Therefore, we tested the hypothesis that the antiangiogenic effects of 1,25(OH)$_2$D$_3$ in cancer are mediated through the HIF pathway. Our data showed that 1,25(OH)$_2$D$_3$ inhibits HIF-1α protein expression and HIF-1 target genes. Furthermore, we showed that 1,25(OH)$_2$D$_3$ failed to suppress VEGF expression in HIF-1α knockout human cancer cells. These observations emphasize the role of the HIF pathway in 1,25(OH)$_2$D$_3$ inhibition of angiogenesis in cancer.

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Table 1. Primer sequences and PCR conditions

Gene	Primer sequence	Cycling conditions (°C, s)		
		Annealing	Elongation	Acquisition
VEGF	5′-TCTTCAAGCCATCCTGTGTG-3′ (forward); 5′-TCTCTCCTATGTGCTGGCCT-3′ (reverse)	62, 10	72, 10	83, 5
HIF-1α	5′-GGACAAGTCACCACAGGACA-3′ (forward); 5′-GGGAGAAAATCAAGTCGTGC-3 (reverse)	62, 10	72, 10	79, 5
ET-1	5′-CCATGAGAAACAGCGTCAAA-3′ (forward); 5′-AGTCAGGAACCAGCAGAGGA-3′ (reverse)	62, 10	72, 10	79, 5
Glut-1	5′-GGGCATGTGCTTCCAGTATGT-3′ (forward); 5′-ACCAGGAGCACAGTGAAGAT-3′ (reverse)	62, 10	72, 10	79, 5
Niemann-Pick	5′-TGGGCGCGATATTTCTGGTG-3′ (forward); 5′-CTCCACGCGGCTGCCTTTC-3′ (reverse)	68, 5	72, 9	87, 5

Materials and Methods

Reagents and Antibodies

1,25(OH)$_2$D$_3$ was a generous gift from Dr. Zeev Mazor (Teva Pharmaceuticals Industries, Pitach Tikva, Israel). Cycloheximide was purchased from Sigma-Aldrich (St. Louis, MO). Primary antibodies were mouse monoclonal anti-HIF-1α (BD Biosciences, San Diego, CA), goat polyclonal anti-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and polyclonal human antibody to human topoisomerase I (TopoGEN, Columbus, OH). Secondary antibodies were conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA). The chemiluminescence reagent was from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel).

Cell Lines and Hypoxic Treatment

PC-3 and LNCaP cells were cultured in RPMI 1640. CL-1, MCF-7, and SW-480 cells were maintained in DMEM. Parental HCT116 and HCT116$^{HIF\text{-}1\alpha-/-}$ were maintained in McCoy's 5A medium and passed in parallel to preserve the same passage number (10). All media were supplemented with 10% FCS and antibiotics. All cells were cultured at 37°C in a humidified atmosphere and 5% CO_2 in air. For hypoxic exposure, the cells were placed in a sealed modular incubator chamber

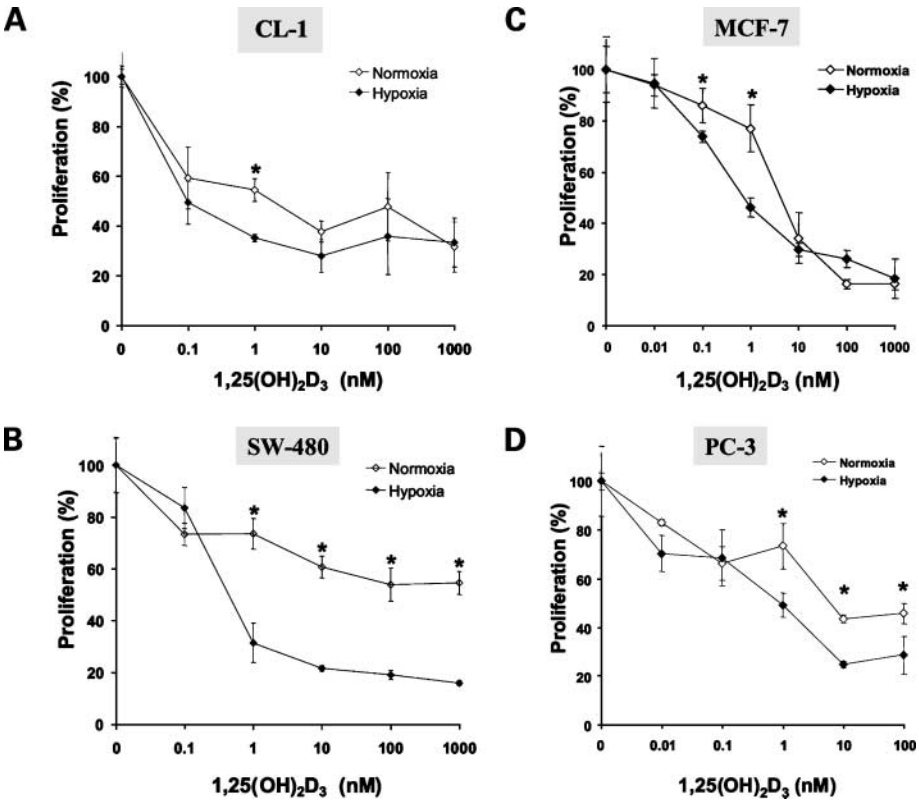

Figure 1. 1,25(OH)$_2$D$_3$ inhibits proliferation in human cancer cells under normoxia and hypoxia. CL-1 (**A**), SW-480 (**B**), MCF-7 (**C**), and PC-3 (**D**) cells were treated with the indicated concentrations of 1,25-(OH)$_2$D$_3$ under normoxic and hypoxic conditions. All conditions included 0.1% ethanol as the vehicle control (concentration ''0''). After 48 h (CL-1) or 4 d (SW-480, MCF-7, and PC-3), the cells were processed for 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt proliferation assay. Proliferation was expressed as decrease in percentage of the initial absorbance that was measured in untreated cells (100%). *Points,* proliferation (n = 3); *bars,* SD. *, $P < 0.01$ between normoxia and hypoxia at the same concentration.

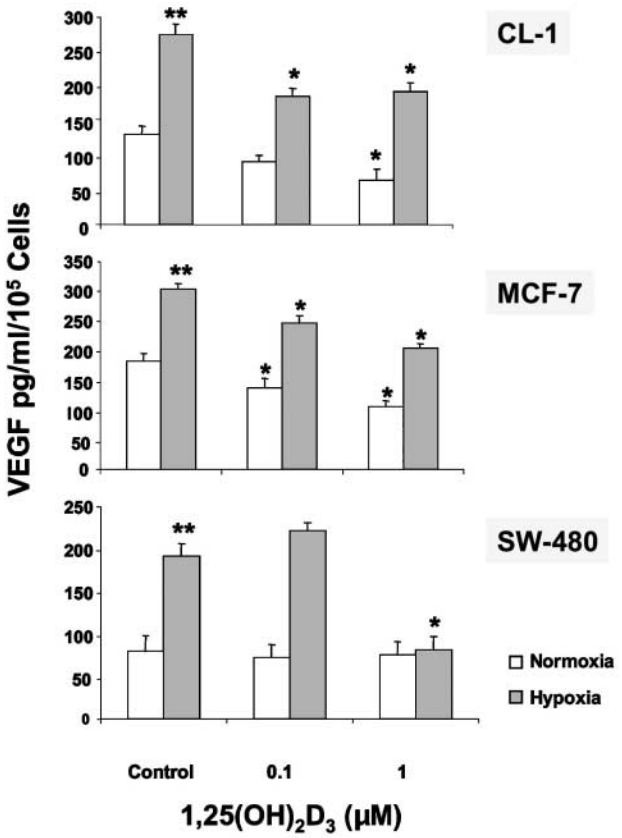

Figure 2. $1,25(OH)_2D_3$ inhibits VEGF secretion in human cancer cells under normoxia and hypoxia. Conditioned media from CL-1 (*top*), MCF-7 (*middle*), and SW-480 (*bottom*) cells treated with $1,25(OH)_2D_3$ under normoxic and hypoxic conditions for 24 h were analyzed for VEGF levels expressed as picogram per milliliter per 10^5 cells. *Columns,* mean ($n = 2$); *bars,* SD; *, $P < 0.05$ between the indicated concentrations and normoxia or hypoxia control, respectively; **, $P < 0.01$ between hypoxia and normoxia controls.

(Billups-Rothenberg, Del Mar, CA) flushed with 1% O_2, 5% CO_2, and 94% N_2.

$1,25(OH)_2D_3$ Treatment and Cell Proliferation Assay

Cells (2,000–5,000 per well) were seeded in 96-well plates in a volume of 200 μL for cell proliferation assay using a 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt kit (Biological Industries). On the next day, the cells were treated with increasing concentrations of $1,25(OH)_2D_3$ (in triplicates) and cultured under either normoxic or hypoxic conditions for 2 to 4 days. $1,25(OH)_2D_3$ was solubilized in 100% ethanol at 1 mmol/L concentrations as a stock solution. All samples, including controls, contained 0.1% ethanol. After the indicated time of treatment, the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt reagent was added and the cells were processed as described previously (11).

Protein Extraction and Immunoblot Analysis

For Western blotting, the cells were seeded in six-well or 6-cm plates. After 24 h, the cells were treated overnight with $1,25(OH)_2D_3$ under either normoxic or hypoxic conditions. They were then harvested, and whole-cell or nuclear extracts were prepared and processed for Western blotting as described previously (12).

Reporter Gene Assay and Luminescence Measurements

For HIF-1 transcriptional activity, we used a reporter expression plasmid (pBI-GL V6L) containing the luciferase gene under the control of HRE of the *VEGF* as described previously (12, 13).

Isolation and Analysis of RNA

Total RNA was extracted from cells using RNeasy Mini kit (Qiagen, Inc., Valencia, CA), reverse transcribed into cDNA

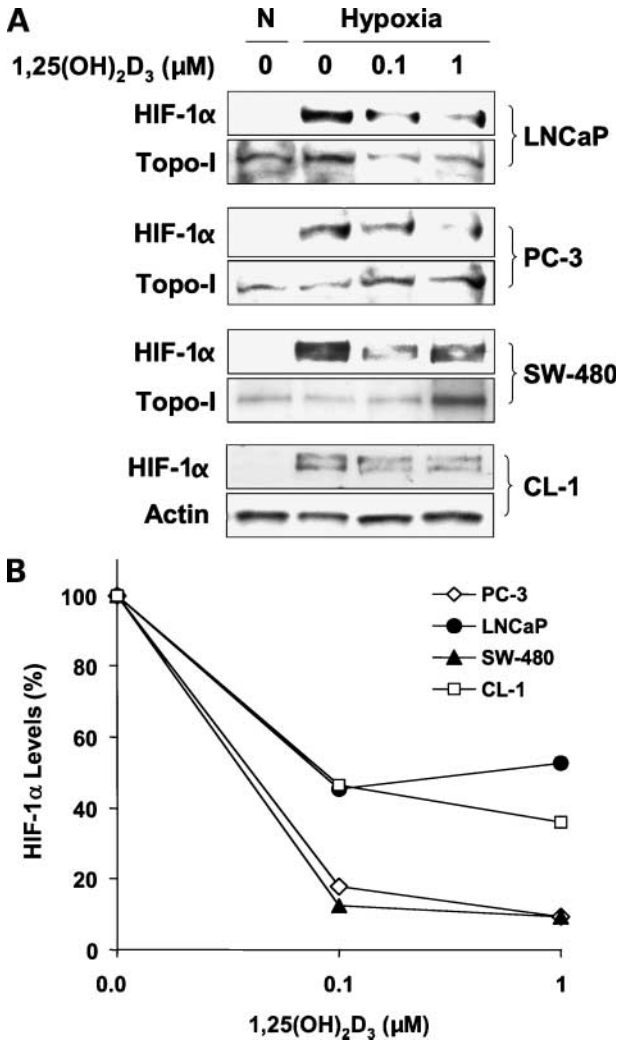

Figure 3. $1,25(OH)_2D_3$ inhibits HIF-1α protein expression in cancer cells. **A,** LNCaP, PC-3, SW-480, and CL-1 cells were treated with increasing concentrations of $1,25(OH)_2D_3$ under normoxic (*N*) or hypoxic conditions for 24 h. Nuclear (LNCaP, PC-3, and SW-480) or whole-cell (CL-1) extracts were prepared, analyzed by SDS-PAGE, and immunoblotted with antibodies to HIF-1α and reprobed with antibodies to topoisomerase I (*Topo-I*) or actin, respectively. **B,** quantification of the HIF-1α levels under hypoxia in (**A**) were analyzed by densitometry and normalized to topoisomerase I (PC-3, LNCaP, and SW-480) or to actin (CL-1) levels. HIF-1α levels from vehicle controls under hypoxia were given the value of 100% from each cell line.

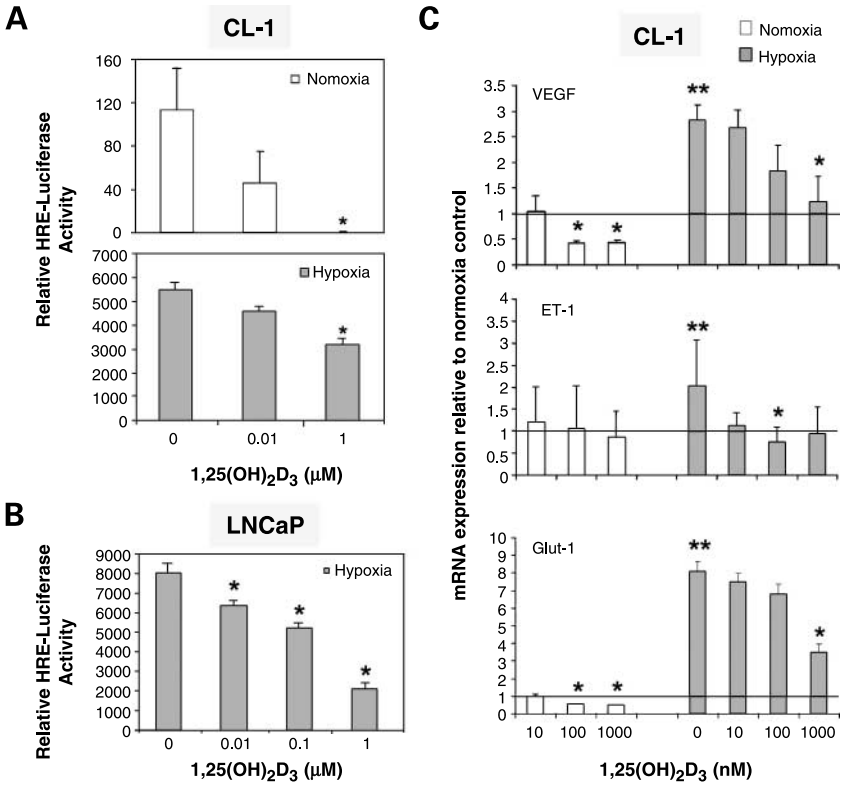

Figure 4. 1,25(OH)$_2$D$_3$ inhibits HIF-1 transcriptional activity. CL-1 (**A**) or LNCaP (**B**) cells were transiently transfected with a plasmid expressing luciferase under the control of HRE. After 24 h of transfection, the cells were treated with 1,25-(OH)$_2$D$_3$ under normoxia or hypoxia for overnight and then analyzed for luciferase luminescence assay. Relative luciferase activity represents arbitrary units per milligram of protein at each assay point. *Columns,* mean (n = 3); *bars,* SD. *, P < 0.05 compared with control. No luciferase activity was detected in LNCaP cells under normoxia. **C,** total RNA was isolated from CL-1 cells treated with 1,25(OH)$_2$D$_3$ under normoxic or hypoxic conditions for 24 h and analyzed by quantitative real-time PCR using primers specific to VEGF (*top*), ET-1 (*middle*), and Glut-1 (*bottom*). The results were normalized to Niemann-Pick mRNA expression levels. *Columns,* mean of independent repetitions (n = 2); *bars,* SD. *, P < 0.05 between the indicated concentrations and normoxia or hypoxia control, respectively; **, P < 0.01 between hypoxia and normoxia controls.

using Reverse-iT 1st Strand Synthesis kit (ABgene, Epsom, Surrey, United Kingdom), and analyzed using quantitative real-time PCR to determine the expression of *VEGF*, *ET-1*, *Glut-1*, and *HIF-1α* genes as described previously (11). The expression of the each gene was normalized to *Niemann-Pick* mRNA expression levels. Sequences of the PCR primers, annealing, elongation, and acquisition temperatures for each gene are outlined in Table 1.

ELISA Assay of VEGF

VEGF was measured using a quantitative, solid-phase, ELISA (Quantikine human VEGF Immunoassay, R&D Systems, Minneapolis, MN) as described previously (12). The results were expressed as concentrations of VEGF (picogram per milliliter) per the total protein amount (milligrams) or number of cells in each well.

Data Analysis

The experiments presented in the figures are representative of three or more different repetitions. Quantification of band densities was done using the public domain NIH Image (version 1.61). Statistical analysis was done using a one-way ANOVA or t test. P < 0.05 was considered statistically significant.

Results

1,25(OH)$_2$D$_3$ Inhibits the Proliferation of Prostate and Colon Cancer Cells under Normoxia and Hypoxia

We first examined the effects of 1,25(OH)$_2$D$_3$ on cell proliferation under hypoxic conditions in various cancer cells (Fig. 1). We used androgen-insensitive prostate cancer cells (CL-1 and PC-3), colon cancer cells (SW-480), and breast cancer cells (MCF-7; Fig. 1). CL-1 cells are highly aggressive androgen-insensitive prostate cancer cells originally derived from LNCaP cells (14). Compared with LNCaP, these cells represent a higher proliferative and malignant potential phenotype and express significantly higher levels of VEGF (14). The IC$_{50}$ of 1,25(OH)$_2$D$_3$ on proliferation was significantly lower (10-fold) under hypoxia compared with normoxia in all cell lines tested (Fig. 1). In the SW-480 cells, the difference in the potency of 1,25(OH)$_2$D$_3$ on proliferation was even more pronounced under hypoxia (IC$_{50}$, 0.4 nmol/L) than under normoxia (IC$_{50}$, >10 nmol/L; Fig. 1B). Similar antiproliferative effects of 1,25(OH)$_2$D$_3$ were also observed in the androgen-sensitive LNCaP cells (data not shown). Our data show that the inhibitory effects of 1,25(OH)$_2$D$_3$ on cell proliferation were also apparent under hypoxia as they were under normoxia. Interestingly, the effect of 1,25(OH)$_2$D$_3$ on cell proliferation was even more potent under hypoxic conditions (Fig. 1).

1,25(OH)$_2$D$_3$ Inhibits VEGF Secretion in Human Cancer Cells

1,25(OH)$_2$D$_3$ was shown to have antiangiogenic effects *in vitro* and *in vivo* (6). We therefore studied the effects of 1,25(OH)$_2$D$_3$ on VEGF in various human cancer cells, including prostate (CL-1), breast (MCF-7), and colon (SW-480), under both normoxic and hypoxic conditions (Fig. 2). VEGF was significantly induced (~2-fold) after exposure

to hypoxia in all cells and significantly was inhibited by $1,25(OH)_2D_3$ under normoxia as well as under hypoxia with various extents (between 40–60%) among the different cell lines (Fig. 2). These results show that $1,25(OH)_2D_3$ inhibits VEGF secretion in cancer cells under normoxic and hypoxic conditions.

$1,25(OH)_2D_3$ Inhibits HIF-1α Protein Expression and HIF-1 Transcriptional Activity

Because our results showed that $1,25(OH)_2D_3$ inhibited cancer cell proliferation and VEGF secretion even under hypoxia, we sought to determine whether $1,25(OH)_2D_3$ affects the HIF-1 pathway. Prostate (LNCaP, PC-3, and CL-1) and colon (SW-480) cancer cells were treated with

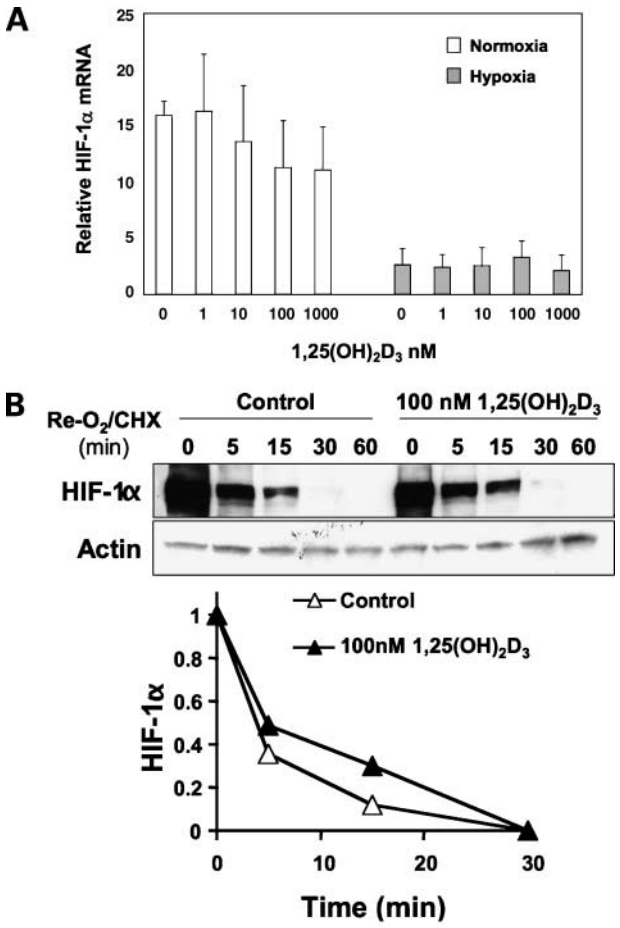

Figure 5. $1,25(OH)_2D_3$ does not affect the transcription or degradation of HIF-1α. **A,** total RNA was isolated from CL-1 cells treated with $1,25(OH)_2D_3$ under normoxic and hypoxic conditions for 24 h and analyzed by quantitative real-time PCR using primers specific to HIF-1α. The results were normalized to Niemann-Pick expression. *Columns,* mean (n = 2); *bars,* SD. **B,** CL-1 cells were treated with 100 nmol/L $1,25(OH)_2D_3$ under hypoxic conditions for 24 h and subsequently exposed to room air in the presence of 10 μg/mL cycloheximide (*CHX*) for the indicated time (min). *Top,* whole-cell extracts were prepared and resolved by SDS-PAGE, and Western blotting was done with antibodies against HIF-1α and actin; *bottom,* quantification of the HIF-1α signal by densitometry following normalization to actin levels. HIF-1α levels from untreated and treated cells at time zero are arbitrarily given the value of 1.

increasing concentration of $1,25(OH)_2D_3$ under hypoxic conditions (Fig. 3). $1,25(OH)_2D_3$ reduced the levels of HIF-1α protein in a dose-dependent manner (Fig. 3A) between 60% to 90% among the various cells (Fig. 3B).

We next studied the effects of $1,25(OH)_2D_3$ on HIF-1 transcriptional activity using a reporter gene assay (Fig. 4A and B). CL-1 and LNCaP cells were transiently transfected with a construct containing the luciferase gene under the control of the HREs from the *VEGF* promoter (13). Exposure to hypoxia induced HIF-1 activation by >50-fold compared with normoxia in CL-1 cells, whereas $1,25(OH)_2D_3$ treatment caused a significant inhibition of HIF transcriptional activity under both normoxia (Fig. 4A, *top*) and hypoxia (Fig. 4A, *bottom*). In LNCaP cells, luciferase activity was dramatically induced under hypoxia compared with no detectable activity under normoxic conditions and this activity was significantly inhibited by increasing doses of $1,25(OH)_2D_3$ (Fig. 4B). Similar effects of significant 40% to 60% inhibition on HIF transcriptional activity were also obtained from the noncancerous HEK 293 cells (data not shown). As an internal control, the cells were simultaneously cotransfected with both *Renilla* Tk-luciferase and the firefly HRE-luciferase plasmids and subjected to dual-luciferase assay. There were no changes in *Renilla* Tk-luciferase activity either under normoxia, hypoxia, or after $1,25(OH)_2D_3$ treatment (data not shown).

To further investigate the effects of $1,25(OH)_2D_3$ on HIF-1 transcriptional activity, we measured the transcript levels of HIF target genes, *VEGF, ET-1*, and *Glut-1*. Total RNA was prepared from untreated and treated cells with $1,25(OH)_2D_3$ and analyzed by quantitative real-time PCR. The results showed that the mRNA expression of all genes was significantly induced under hypoxia (Fig. 4C). After $1,25(OH)_2D_3$ treatment, there was ≥50% significant inhibition of the expression of all genes tested especially under hypoxia (Fig. 4C). Taken together, our results show that $1,25(OH)_2D_3$ inhibits HIF-1α protein expression as well as HIF-1–mediated transcriptional activation of VEGF and other HIF target genes.

$1,25(OH)_2D_3$ Affects HIF-1α Protein Expression on the Translational Level

To clarify the mechanism by which $1,25(OH)_2D_3$ involves HIF-1, we examined the effects of $1,25(OH)_2D_3$ on HIF-1α transcriptional and posttranscriptional levels. Analysis of RNA prepared from untreated and treated CL-1 cells with increasing doses of $1,25(OH)_2D_3$ under normoxia or hypoxia revealed that HIF-1α mRNA expression was not significantly ($P > 0.05$) affected by any concentration of $1,25(OH)_2D_3$ (Fig. 5A). HIF-1α mRNA levels were relatively decreased under hypoxia compared with normoxia (Fig. 5A).

We next examined the effects of $1,25(OH)_2D_3$ on HIF-1α protein stability. HIF-1α was induced by exposing the cells to hypoxia and subsequent exposure to room air (reoxygenation) in the presence of the protein translation inhibitor, cycloheximide (Fig. 5B, *top*). Reoxygenation causes a rapid HIF-1α degradation and, in the presence of cycloheximide, the new protein synthesis is inhibited;

thus, HIF-1α levels predominantly reflect the degradation process of the protein. CL-1 cells were treated with either 0.1% ethanol or 100 nmol/L 1,25(OH)$_2$D$_3$ under hypoxia and then they were exposed to room air in the presence of cycloheximide for various times and analyzed by Western blotting for HIF-1α protein levels. Within 5 min of exposure to O_2 in the presence of cycloheximide, HIF-1α protein levels from untreated and treated cells were decreased by 50% (Fig. 5B, *bottom*). Although the levels of the HIF-1α signal were reduced by 40% after 1,25(OH)$_2$D$_3$ treatment (different signals at the zero time point), the degradation rate of HIF-1α protein was almost the same under both conditions. Taken together, these results indicate that 1,25(OH)$_2$D$_3$ does not affect HIF-1α either on the transcriptional or on the posttranslational levels. It is most likely that 1,25(OH)$_2$D$_3$ affects HIF-1α new protein synthesis.

1,25(OH)$_2$D$_3$ Does Not Inhibit VEGF Expression in HIF-1α Knockout HCT116 Cells

Our results showed that 1,25(OH)$_2$D$_3$ treatment inhibits both the HIF-1α and the VEGF protein levels. To determine whether the decrease in VEGF levels by 1,25(OH)$_2$D$_3$ was mediated directly by the HIF-1 pathway, we used HIF-1α knockout colon cancer cells (HCT116$^{HIF\text{-}1\alpha-/-}$). These cells do not exhibit any transcriptional activity of HIF as measured by reporter gene assay, which indicates that, although they may express HIF-2α, it is transcriptionally inactive (data not shown). We measured the proliferation and VEGF secretion of these cells before and after treatment with 1,25(OH)$_2$D$_3$. As a control, we used the parental HCT116 cells, which express HIF-1α protein and exhibit induction of HIF-1 transcriptional activity under hypoxia unlike HCT116$^{HIF\text{-}1\alpha-/-}$ cells (data not shown). 1,25(OH)$_2$D$_3$ inhibited the proliferation of both HCT116 and HCT116$^{HIF\text{-}1\alpha-/-}$ cells under normoxic and hypoxic conditions (Fig. 6A and B). Of note, HCT116 cells were more sensitive to 1,25(OH)$_2$D$_3$ under hypoxic than normoxic conditions similar to the observed results from SW-480 colon cancer cells (Fig. 1B).

HCT116$^{HIF\text{-}1\alpha-/-}$ cells expressed basal levels of VEGF protein similar to those of HCT116 cells (Fig. 6C and D). Whereas VEGF was significantly induced under hypoxia in HCT116 cells (Fig. 6C), its levels were not affected by hypoxia in HCT116$^{HIF\text{-}1\alpha-/-}$ cells (Fig. 6D). Moreover, 1,25(OH)$_2$D$_3$ inhibited the basal levels of VEGF and the hypoxia-induced VEGF in HCT116 cells (Fig. 6C), whereas 1,25(OH)$_2$D$_3$ did not affect VEGF protein levels under either normoxia or hypoxia in HCT116$^{HIF\text{-}1\alpha-/-}$ cells (Fig. 6D). These findings showed that the inhibition of VEGF levels by 1,25(OH)$_2$D$_3$ in this cancer cell model is dependent on the presence of HIF-1α.

Discussion

Inhibition of angiogenesis is one of the mechanisms that contribute to the antitumoral activity of 1,25(OH)$_2$D$_3$. 1,25(OH)$_2$D$_3$ inhibits the proliferation of tumor-derived endothelial cells *in vitro* (15) and also inhibits angiogenesis *in vivo* (6, 16). In the current study, we show that a significant part of the antiangiogenic effects of 1,25(OH)$_2$D$_3$ are mediated through the HIF pathway.

1,25(OH)$_2$D$_3$ reduces the expression of HIF-1α protein in various human cancer cells, leading to attenuation of HIF-1 transcriptional activity (Figs. 3 and 4). It seems that 1,25(OH)$_2$D$_3$ affects HIF-1α protein translationally rather than transcriptionally or posttranslationally (Fig. 5), similar to growth factors (8) and androgens (17).

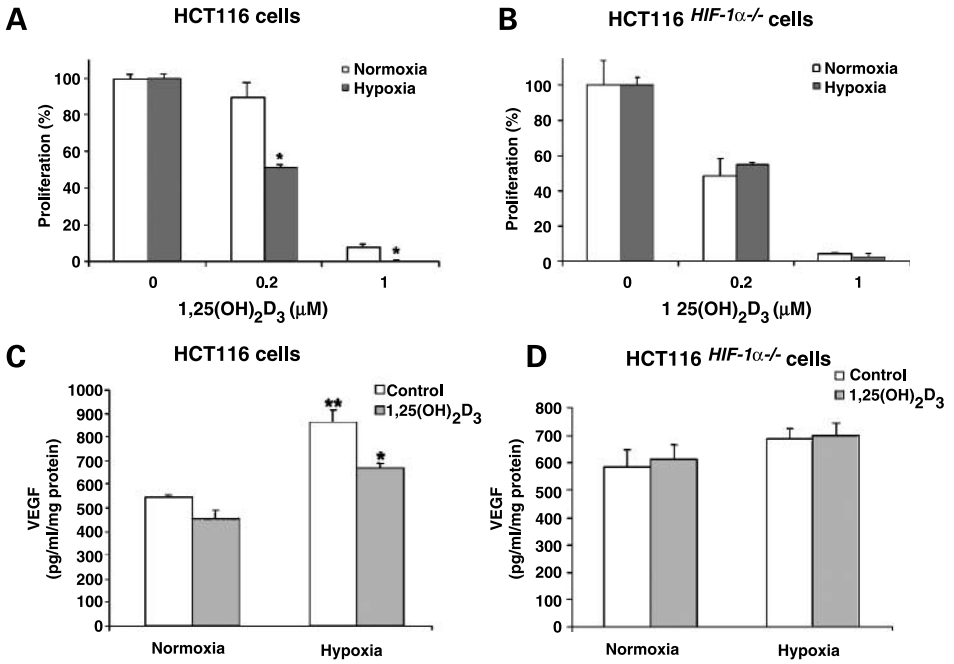

Figure 6. 1,25(OH)$_2$D$_3$ does not affect VEGF expression in HCT116$^{HIF\text{-}1\alpha-/-}$ colon cancer cells. HCT116 (**A**) and HCT116$^{HIF\text{-}1\alpha-/-}$ (**B**) cells were treated with 1,25(OH)$_2$D$_3$ under normoxic and hypoxic conditions. After 4 d, the cells were processed for 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt proliferation assay. Proliferation was expressed as decrease in percentage of the initial absorbance that was measured in untreated cells (100%) under normoxia or hypoxia. *Columns,* mean (n = 3); *bars,* SD. *, $P < 0.01$ between hypoxia and normoxia values of the same concentration. Conditioned media from HCT116 (**C**) and HCT116$^{HIF\text{-}1\alpha-/-}$ (**D**) cells treated with 0.1% ethanol (control) or 1 μmol/L 1,25(OH)$_2$D$_3$ under normoxic and hypoxic conditions for 24 h were analyzed for VEGF levels expressed as picogram per milliliter per total amount of protein in each well. *Columns,* mean (n = 2); *bars,* SD. *, $P < 0.05$ between the indicated concentrations and hypoxia control; **, $P < 0.01$ between hypoxia and normoxia control.

The effects of $1,25(OH)_2D_3$ on the HIF/VEGF pathway were confirmed when we used the HIF-1α knockout colon cancer cells ($HCT116^{HIF\text{-}1\alpha-/-}$). $1,25(OH)_2D_3$ failed to suppress VEGF expression levels under either normoxia or hypoxia in $HCT116^{HIF\text{-}1\alpha-/-}$ cells (Fig. 6). These results further emphasize the importance of the HIF pathway in $1,25(OH)_2D_3$ inhibition of angiogenesis. It should be pointed out that $1,25(OH)_2D_3$ concentrations used were above the physiologic range (0.1–1 μmol/L), which are usually applied for *in vitro* studies. It is therefore warranted to confirm the effects of $1,25(OH)_2D_3$ on HIF *in vivo* using physiologic concentrations.

Interestingly, $1,25(OH)_2D_3$ inhibited the proliferation of cancer cells also under hypoxia (Figs. 1 and 6). The results may indicate on the potency of $1,25(OH)_2D_3$ to inhibit tumor growth under hypoxia, which exist in most solid tumors (18).

HIF-1 has become an acceptable target for cancer therapeutics over the past few years, with studies on cancer patients showing a positive role of HIF in tumor progression (19). HIF-1α is clearly overexpressed in the majority of human cancers and is associated with patient mortality and poor response to treatment (19). Thus, inhibiting the HIF pathway, whether selectively or non-selectively, would be useful for potential therapeutic implications. Our *in vitro* studies support the clinical observations that when $1,25(OH)_2D_3$ was administrated in combination with docetaxel to treat androgen-independent prostate cancer patients, it significantly enhanced the effects of docetaxel (20). This further supports the rationale to use multiple targeted strategies, including $1,25(OH)_2D_3$ analogues, in cancer therapy.

In this study, we found that HIF-1 is downstream to the vitamin D receptor. Interestingly, Banach-Petrosky et al. (21) very recently have shown that the development of high-grade prostate intraepithelial neoplasia can be prevented in *Nkx3.1;Pten* mutant mice by the early administration of $1,25(OH)_2D_3$. In this model, AKT is constitutively activated (22). Because HIF-1α is downstream of AKT, it might be possible that a part of the preventive activity of $1,25(OH)_2D_3$ in this model is attributed to HIF-1 inhibition. HIF-1 activation is an early event in prostate and other cancers (23, 24). It therefore would be more warranted to use $1,25(OH)_2D_3$ and its low-calcemic analogues in cancer chemoprevention and treatment.

To the best of our knowledge, this is the first documentation that a significant part of the antiangiogenic effects of $1,25(OH)_2D_3$ in hypoxic cancer cells are mediated via the HIF-1 pathway. Our results further support the rationale to use vitamin D as an antineoplastic agent. In addition, we believe that newly developed low-calcemic analogues of vitamin D could be initially tested in HRE-based high-throughput screening assays to evaluate their antiangiogenic potencies.

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