1,25-dihydroxyvitamin D regulation of Vascular Endothelial Growth Factor (VEGF), Prolyl Hydroxylase (PHD), and Hypoxia-Inducible Factor-1 (HIF-1) in mammary epithelial cells with ERB2 expression

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Harpenau, Erin, "1,25-dihydroxyvitamin D regulation of Vascular Endothelial Growth Factor (VEGF), Prolyl Hydroxylase (PHD), and Hypoxia-Inducible Factor-1 (HIF-1) in mammary epithelial cells with ERB2 expression" (2009). CFS Honors Program Undergraduate Theses. Paper 5.
1,25-DIHYDROXYVITAMIN D REGULATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF), PROLYL HYDROXYLASE (PHD), AND HYPOXIA-INDUCIBLE FACTOR-1 (HIF-1) IN MAMMARY EPITHELIAL CELLS WITH ERB2 EXPRESSION

by

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A Thesis Submitted in Partial Fulfillment
Of the Requirements for a Degree with Honors
(Dietetics/Nutrition, Fitness, & Health)

The College of Consumer and Family Sciences
Purdue University
May 2009
West Lafayette, Indiana

Approved by:

Advisor: Dorothy Teegarden
Abstract

Breast cancer is one of the most prevalent forms of cancer among American women. Vitamin D has been shown to reduce the risk of developing breast cancer, in part by preventing the formation of new blood vessels (termed angiogenesis) within tumors. Vascular Endothelial Growth Factor (VEGF), a potent angiogenic factor, has been shown to be regulated by the active form of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)₂D). It has been proposed that 1,25(OH)₂D does not directly decrease the expression of VEGF, but rather acts indirectly through Hypoxia Inducible Factor-1α (HIF-1α), a direct transcriptional regulator of VEGF. 1,25(OH)₂D may impact HIF-1α in one of two ways: gene transcription or stability of the protein, which is degraded through the actions of Prolyl Hydroxylase (PHD). The purpose of this study was to determine the impact of 1,25(OH)₂D on VEGF, HIF-1α, and PHD mRNA expression in MCF10A breast epithelial cells which are untransformed (LXSN) or which contain the ErbB2 oncogene. ErbB2 is an oncogene that is commonly overexpressed and associated with an increased aggressiveness in breast cancer. The MCF10A are a model of multistage carcinogenesis, models that are important to use when studying cancer prevention. The hypothesis of this study is that 1,25(OH)₂D decreases the gene expression of VEGF and HIF-1α, and increases the gene expression of PHD in untransformed breast epithelial cells and breast epithelial cells transfected with ErbB2.

Treatment of LXSN cells with increasing concentrations of 1,25(OH)₂D (1 nM, 10 nM, and 100 nM) for 24 hours resulted in increasing concentrations of VEGF mRNA and decreasing concentration of PHD mRNA levels compared to the vehicle control. However, these differences were not significant (p>0.05) except at a high dose of 1,25(OH)₂D (100 nM). Increasing doses of 1,25(OH)₂D did not significantly alter HIF-1α mRNA abundance (p>0.05) compared to the vehicle control. Treatment of the ErbB2-containing cells with the same increasing concentrations of 1,25(OH)₂D showed no significant difference in the concentration of VEGF or HIF-1α mRNA (p>0.05) compared to the vehicle control. The PHD mRNA expression decreased with increasing concentrations of 1,25(OH)₂D, however this was only significant (p>0.05) at the high dose of 1,25(OH)₂D (100 nM). These results suggest that in untransformed MCF10A breast epithelial cells, 1,25(OH)₂D increases the expression of VEGF mRNA at high doses, but not through an increased expression of HIF-1α mRNA. In the MCF10A breast epithelial cells transfected with ErbB2, 1,25(OH)₂D had no significant impact on the expression of VEGF or HIF-1α mRNA.
Purpose

The purpose of this study was to determine the impact of 1,25(OH)₂D on VEGF, HIF-1α, and PHD mRNA expression in untransformed MCF10A (LXSN) and oncogene (ErbB2) transfected MCF10A breast epithelial cells.

Literature & Rationale

Breast cancer is a form of cancer that is quite prevalent among American women. The CDC reports that 186,467 women were diagnosed with breast cancer and 41,116 died from the disease in 2005(16). Breast cancer, like all forms of cancer, progresses through a series of steps that is termed multi-stage carcinogenesis (15). Tumor development, or carcinogenesis, can be divided into 4 steps: tumor initiation, tumor promotion, malignant conversion, and tumor progression (13). Tumor initiation is the result of the accumulation of irreversible genetic damage and is commonly the result of the activation of an oncogene. An oncogene is a normally functioning gene in a cell that has been altered by mutation and acts to contribute to the growth of a tumor (4). The next step in carcinogenesis is an accumulation of initiated cells and is termed tumor promotion. Once there are a large number of initiated cells, they become susceptible to a malignant conversion. This means that the initiated cells are altered to express a malignant phenotype, which usually causes the uncontrolled growth that is the hallmark characteristic of cancer cells. The dominant step of carcinogenesis is tumor progression. This is the step where the cell displays uncontrollable growth and the development of tumors. Metastasis is also observed in this stage of cancer development. Metastasis is the process of the spreading of cancer from its initial site to other areas of the body (3). Breast cancer is particularly fatal because of the increased likelihood of metastasis (9). One gene that is an important regulator of metastatic potential and is responsible for the rapid growth found in the mammary epithelium tissues that is associated with the presence of this form of cancer is the ErbB2 oncogene (14,9). An over expression of this oncogene is found in approximately 30% of breast cancer patients (9).

The rapid, uncontrollable growth of tumor cells requires in part, an increase in angiogenesis. Angiogenesis is the process of developing new blood vessels and is a major determinant in the growth of a tumor (12). In order for tumor cells to grow and thrive, they must have a ready supply of oxygen and nutrients, which are supplied by the bloodstream. The development of new blood vessels to tumor cells is critical for the growth of the tumor. The process of angiogenesis has four stages: (1) degrading the basement membrane of existing vasculature, (2) proliferation and migration of endothelial cells, (4) attachment of preexisting endothelial cells to new channels, and (10) formation of new basement membrane and maturation of the new vasculature (12).
In addition to the other effects of ErbB2, the protein product of this oncogene is linked to an increased production of Vascular Endothelial Growth Factor (VEGF), a potent stimulator of angiogenesis (10). The action of VEGF is primarily targeted towards the endothelial cell of the vasculature. VEGF acts by binding to a receptor (VEGFR2) and activating a signaling cascade. This cascade then acts to increase the permeability, proliferation, and migration of vascular endothelial cells (6). Since the proliferation and migration of vascular endothelial cells are major events in angiogenesis, a compound with the ability to inhibit the production of VEGF may hinder the rapid growth of tumors and prevent the development of metastatic cancers.

Recently, the link between vitamin D and a decreased risk of cancer has received much attention (1). Vitamin D is available in two main forms: in the diet or from a biological conversion that occurs in the skin. Dietary sources of vitamin D include natural and fortified foods. The most typical source of natural vitamin D is the fatty fish, such as salmon. Some of the fortified sources of vitamin D include: milk, orange juice and some cereals (7). The vitamin D that is produced in reaction to the absorption of sunlight represents the major source of vitamin D in most people. When sunlight is absorbed by the 7-dehydrocholesterol in an individual’s skin, it is converted to previtamin D₃. Previtamin D₃ is quickly converted into a biologically significant isomer, vitamin D₃. Vitamin D₃ is then released into the bloodstream where it binds to the vitamin D-binding protein (7).

Once the vitamin D₃ that is produced in the skin and the vitamin D that is consumed in the diet are present in the bloodstream they are taken up by the liver where they are metabolized to 25-hydroxyvitamin D [25OHD]. The vitamin D metabolite 25OHD is the circulating form of vitamin D that is used to assess an individual’s vitamin D status. However, this is not the biologically active form of vitamin D and another hydroxylation is required to produce the biologically active form 1,25-dihydroxyvitaminD [1,25(OH)₂D] (10). 25(OH)D was traditionally thought to be converted to 1,25(OH)₂D via the kidneys and functioned to regulate serum calcium levels, blood pressure, and insulin secretion (7). However, it was discovered that extrarenal tissues, such as colon, prostate, breast, and immune cells, also express the enzyme 25-hydroxyvitamin D-1α-hydroxylase (1-OHase) which is necessary in the conversion of 25(OH)D to 1,25(OH)₂D. 1, 25(OH)₂D which is produced in these extrarenal cells may act to inhibit cell proliferation and cell differentiation and act to induce apoptosis (5). It is this ability of 1,25(OH)₂D in specific tissues to arrest cell growth and differentiation that is drawing much attention in the role of cancer prevention.
There have been several studies performed testing the link between vitamin D and cancer. In 1941, epidemiological evidence was used to support an observation that individuals living in northern states in the United States were more likely to die of cancer than individuals living in the southern states (5). This led others to consider the link between sunlight exposure, vitamin D status, and cancer risk. John, et al., analyzed data from The National Health and Nutrition Examination Survey (NHANES) I Epidemiologic Follow-up Study to look at the potential protective role of vitamin D in breast cancer risk (8). They looked at the relationship of sunlight exposure and dietary intake of vitamin D to the development of breast cancer. They found that women who lived in areas with adequate sunlight exposure and dietary intake of vitamin D were associated with a 25-65% reduction in breast cancer risk. It can be inferred from this data that adequate intake of vitamin D and a corresponding adequate vitamin D status provides breast cells with adequate substrate [25OHD] for conversion into the active form 1,25(OH)₂D, which in turn may act to inhibit tumor progression.

Another study that was conducted to study the link between vitamin D status and tumor progression was performed by Tangpricha et al (8). Tangpricha et al. studied the progression of a cancer cell line in mice that were vitamin D deficient or vitamin D sufficient. They placed the cancer cells in the two mouse treatment groups and observed the growth of the tumor. The mice that were vitamin D deficient had tumors that grew much more rapidly than the tumors in the vitamin D sufficient mice. This observation suggests that vitamin D status is an important factor in the reduction of tumor growth and slowing cancer progression.

In 2002, one study looked at the local production of 1,25(OH)₂D and its role in inhibiting cancer growth (5). They studied a prostate cancer cell line which either had 1-OHase or lacked 1-OHase activity. It was found that the cells without 1-OHase were not able to use 25OHD but if they were treated with 1,25(OH)₂D there was a marked decrease in cellular growth. The cells that had active 1-OHase present also displayed a marked decrease in cellular growth. This data also supports the theory that increased concentrations of 1,25(OH)₂D in cancer cells is related to an inhibition of cellular growth.

The exact mechanism of the role of vitamin D in the inhibition of cancer progression is not known. However, a main role for vitamin D in cancer prevention appears to be the inhibition of angiogenesis (1). Since VEGF is such a potent stimulator of angiogenesis, the link between the regulation of VEGF by 1,25(OH)₂D and the progression of cancer is vital. It is proposed that the action of the active vitamin D metabolite 1,25(OH)₂D acts to decrease the expression of VEGF (1) in two ways: by decreasing the expression of HIF-1α mRNA or increasing the expression of Prolyl Hydroxylase (PHD) mRNA(Figure 1).
Figure 1 - Impact of increased concentrations of 1,25(OH)₂D in cell models

The active vitamin D compound, 1,25(OH)₂D has been shown to decrease the concentration of Hypoxia Induced Factor1α (HIF-1α) mRNA. This was shown by Ben-Shoshan et al, who demonstrated that treating prostate and colon cancer cells with increasing concentrations of 1,25(OH)₂D reduced the levels of HIF-1α protein produced in a dose-dependent manner. They also demonstrated in this study that this regulation of HIF-1α occurs during translation, decreasing the concentration of HIF-1α mRNA. While HIF-1α does not directly influence the process of angiogenesis, it has a role in activating angiogenic stimulators such as VEGF. HIF-1α heterodimerizes with HIF-1β to form the HIF-1-transcription factor (10). This compound stimulates angiogenesis by binding to the promoter region of the VEGF gene and up-regulating VEGF mRNA expression (10). This increase in VEGF mRNA expression leads to an increase in VEGF protein and a corresponding increase in angiogenesis.

The other action of 1,25(OH)₂D may be to increase the expression of PHD mRNA. Increasing the expression of PHD mRNA leads to a corresponding increase in concentration of the PHD protein, which results in an increased degradation of the HIF-1α protein (2). PHD contributes to the regulation of HIF-1α by binding to HIF-1α under normoxia conditions. The resulting complex is targeted for degradation via a complex ubiquitin-proteasome pathway (8). Although there is evidence that 1,25(OH)₂D both up and down-regulates the VEGF protein, dependent on the status of the cell(1), the mechanism by which 1,25(OH)₂D mediates these changes has not been explored. This study sought to try and close this gap in the knowledge of the how vitamin D acts to inhibit the progression of cancer.
**Study Design & Methods**

*Cell Type*

The MCF10A human epithelial breast cells were used in this experiment. The MCF10A cells are derived from normal, untransformed tissues and maintained in Mammary Epithelial Growth Medium (MEGM). The two cell models used include: MCF10A which expresses the ErbB2 oncogene and the untransformed MCF10A cell containing LXSN, the empty plasmid for the ErbB2 oncogene.

*Cell Treatments*

The LXSN and the ErbB2 cell models were plated in 6-well-plates and treated with varying concentrations of 1,25(OH)2D. The four experimental groups included: an ethanol vehicle, 1 nM 1,25(OH)2D, 10 nM 1,25(OH)2D, and 100 nM 1,25(OH)2D. The cells were harvested 24 hours following the treatment.

*Methods*

After harvest, total RNA was extracted and quantified. Extraction of the RNA allowed the expression of HIF-1α, VEGF, and PHD mRNA to be assessed with real time polymerase chain reaction (PCR). Primers for HIF-1α, VEGF, and PHD have been designed and optimized for the MCF10A cell line.

- **Human VEGF primer:** CAAGATCCGCACACGTGTA
  TCACATCTGCAAGTACCTCG
- **Human HIF-1 primer:** TGCTGAAGACACAGAAGCAAA
  AAAGCGCAAGTCCTCAAAGC
- **Human GAPDH primer:** TCACCCTTCCAGGAGCG
  CTGCTTCACCACCTTCTTGA
- **Prolyl Hydroxylase primer:** CTTTGACCGGTTGCTCATTT
  TAGGCGGCTGTGATACAGGT

The controls included no temperate controls and no RT, to ensure the lack of genomic DNA contamination. The SYBR green system was used for visualization. The normalization gene was 18S and data is expressed as ΔΔ Ct method for determination of fold change over control samples. Standards were composed of aliquots of PCR products in 10-fold serial dilutions ranging from 10⁰ to 10¹ molecules. Standards were amplified in triplicate and used to calculate a regression of threshold cycle on the molecule copy number to determine a log value of starting abundance for each cDNA aliquot, amplified in duplicate, based on individual threshold cycle. The abundance of mRNA was analyzed, quantified, and statistically evaluated.
Statistical Analysis

The statistical significance was assessed by an analysis of variance (ANOVA). A statistical significance is defined as p<0.05. A trend towards statistical significance is defined as being between 0.005-0.100.

Results

Impact of 1,25(OH)2D on VEGF mRNA expression in Untransformed Breast Epithelial Cells

In the untransformed breast epithelial cells, the LXSN cell line, increasing concentrations (1 nM, 10 nM, 100 nM) of 1,25(OH)2D did not significantly impact the expression of VEGF mRNA at a physiological level of 10 nM (Figure 2). However, at the higher concentration (100 nM) an increase was observed, although this increase was not significant (p>0.05). There was also a decrease observed at the low concentration (1 nM) which had a p-value with a trend toward significant (0.05 < p < 0.10).

Impact of 1,25(OH)2D on HIF-1α mRNA expression in Untransformed Breast Epithelial Cells

In the untransformed breast epithelial cells, the LXSN cell line, increasing concentrations (1 nM, 10 nM, 100 nM) of 1,25(OH)2D did not significantly impact the expression of HIF-1α mRNA within a physiological range of 10 nM or less (Figure 3). However, at the higher concentration (100 nM) an increase was observed, but was not significant (p>0.05). There was a significant difference (p > 0.05) between the 1 nM and 100 nM treatments, but not between either of these treatments and the vehicle.

Impact of 1,25(OH)2D on PHD mRNA expression in Untransformed Breast Epithelial Cells

In the untransformed breast epithelial cells, the LXSN cell line, increasing concentrations (1 nM, 10 nM, 100 nM) of 1,25(OH)2D did not significantly impact the expression of PHD mRNA at a physiological level of 10 nM (Figure 4). However, at high concentrations (100 nM) there was a decrease in the amount of PHD mRNA, although this decrease was not significant (p > 0.05). There was a significant difference (p > 0.05) between the 1 nM and 100 nM treatments, but not between either of these treatments and the vehicle.
Figure 2 - The Regulation of VEGF mRNA Expression by 1,25(OH)_2D. Untransformed (LXSN) cells were treated with vehicle or increasing concentrations of 1,25(OH)_2D, and following harvest the expression of VEGF mRNA assessed by PCR. Bars with different letters are significantly different (p-value < 0.05) as assessed by ANOVA.

Figure 3 - The Regulation of HIF-1α mRNA Expression by 1,25(OH)_2D. Untransformed (LXSN) cells were treated with vehicle or increasing concentrations of 1,25(OH)_2D, and following harvest the expression of VEGF mRNA assessed by PCR. Bars with different letters are significantly different (p-value < 0.05) as assessed by ANOVA.
Impact of 1,25(OH)₂D on VEGF mRNA expression in Breast Epithelial Cells Transfected with ErbB2

In the breast epithelial cells transfected with the ErbB2 oncogene, increasing concentrations (1 nM, 10 nM, 100 nM) of 1,25(OH)₂D did not significantly impact the expression of VEGF mRNA (Figure 5).

Impact of 1,25(OH)₂D on HIF-1α mRNA expression in Breast Epithelial Cells Transfected with ErbB2

In the breast epithelial cells transfected with the ErbB2 oncogene, increasing concentrations (1 nM, 10 nM, 100 nM) of 1,25(OH)₂D did not significantly impact the expression of HIF-1α mRNA (Figure 6).

Impact of 1,25(OH)₂D on PHD mRNA expression in Breast Epithelial Cells Transfected with ErbB2

In the breast epithelial cells transfected with the ErbB2 oncogene, increasing concentrations (1 nM, 10 nM, 100 nM) of 1,25(OH)₂D did not significantly impact the expression of PHD mRNA at physiological level of 10 nM (Figure 7). However, at high concentrations (100 nM), 1,25(OH)₂D acted to decrease the expression of PHD mRNA.
Figure 5 – The Regulation of VEGF mRNA Expression by 1,25(OH)₂D. Epithelial cells transfected with the ErbB2 oncogene were treated with vehicle or increasing concentrations of 1,25(OH)₂D, and following harvest the expression of VEGF mRNA assessed by PCR. Bars with different letters are significantly different (p-value < 0.05) as assessed by ANOVA.

Figure 6 – The Regulation of HIF-1α mRNA Expression by 1,25(OH)₂D. Epithelial cells transfected with the ErbB2 oncogene were treated with vehicle or increasing concentrations of 1,25(OH)₂D, and following harvest the expression of VEGF mRNA assessed by PCR. Bars with different letters are significantly different (p-value < 0.05) as assessed by ANOVA.
Figure 7 - The Regulation of PHD mRNA Expression by 1,25(OH)\(_2\)D. Epithelial cells transfected with the ErbB2 oncogene were treated with vehicle or increasing concentrations of 1,25(OH)\(_2\)D, and following harvest the expression of VEGF mRNA assessed by PCR. Bars with different letters are significantly different (p-value < 0.05) as assessed by ANOVA.

Discussion

In this study we treated two cell lines, untransformed breast epithelial cells and breast epithelial cells transfected with the ErbB2 oncogene, with varying concentrations (1 nM, 10 nM, & 100 nM) of 1,25(OH)\(_2\)D for 24 hours. We then isolated and quantified the RNA from these cells and performed a PCR test to determine the amount of mRNA that was present in the cells. We looked at the amount of VEGF, HIF-1\(\alpha\), and PHD present in these cells.

There were two main questions that were considered with this study. First, was to determine what the effect of 1,25(OH)\(_2\)D was on the gene expression of VEGF, HIF-1\(\alpha\), and PHD in untransformed breast epithelial. Upon statistical analysis of the data, we observed trends. With respect to VEGF, it appeared that as the concentration of 1,25(OH)\(_2\)D increased, the concentration of VEGF mRNA increased in the untransformed cells, although the results were not statistically significant (Figure 2). The expression of HIF-1\(\alpha\) mRNA did change with increasing concentrations of 1,25(OH)\(_2\)D, except at a high concentration of 100 nM, although this increase was not significant (Figure 3). The PHD mRNA decreased, though not significantly, with increasing concentrations of 1,25(OH)\(_2\)D (Figure 4). We expected that the VEGF and HIF-1\(\alpha\) mRNA would increase together since HIF-1\(\alpha\) is a regulator of VEGF. However, this might be explained by alterations in protein level. In our laboratory, other students determined the effect of treatment of untransformed cells and cells transfected with the ErbB2 oncogene.
with the same varying concentrations of 1,25(OH)2D (1 nM, 10 nM, 100 nM) on protein expression of HIF-1α. They showed that in the untransformed cells, increasing concentrations of 1,25(OH)2D corresponded with an increasing expression of HIF-1α protein. This suggests that VEGF might be regulated by HIF-1α at a protein level rather than at a transcriptional level. This hypothesis is supported by the decrease in PHD mRNA expression. With less PHD being expressed, there might be less PHD protein to degrade the HIF-1α protein and more available HIF-1α protein available to alter VEGF transcription.

The other main question that was considered in this study was the effect of the ErbB2 oncogene on the 1,25(OH)2D-induced gene expression of VEGF. The VEGF mRNA expression in the ErbB2 cells appeared to decrease with increasing concentrations of 1,25(OH)2D (Figure 5). However, this trend was not significant (p > 0.05). There did not appear to be a significant difference in the gene expression of HIF-1α in the ErbB2 transfected cells (Figure 6). The expression of PHD was not significantly different except at high concentrations (100 nM) of 1,25(OH)2D (Figure 7). The protein expression of HIF-1α determined in our laboratory indicated that protein expression of HIF-1α in ErbB2 cells was not significantly different from the vehicle. While the trends in VEGF expression are not significant, they are similar to the decreases in VEGF mRNA expression with treatment of 1,25(OH)2D that are found in the literature (1). It may be that with a larger sample size and subsequent reduction in error, a statistically significant result would be achieved.

**Conclusion**

In untransformed MCF10A breast epithelial cells, 1,25(OH)2D increases the expression of VEGF mRNA but not through an increased expression of HIF-1α. We also observed that in MCF10A breast epithelial cells transfected with the ErbB2 oncogene 1,25(OH)2D did not significantly impact the expression of VEGF or HIF-1α expression.
References:


