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Journal of Nutritional Biochemistry 20 (2009) 629–637
Journal of Nutritional Biochemistry

# Mechanisms of nuclear vitamin D receptor resistance in Harvey-*ras*-transfected cells

Laura M. Taber[a], Lynn S. Adams[b], Dorothy Teegarden[a,*]

[a]*Department of Foods and Nutrition, Purdue University, West Lafayette, IN 47907, USA*
[b]*City of Hope, Duarte, CA 91010, USA*

Received 6 March 2008; received in revised form 28 May 2008; accepted 5 June 2008

**Abstract**

The hormone 1,25 dihydroxyvitamin D ($1,25(OH)_2D$) binds to the nuclear vitamin D receptor (nVDR), which heterodimerizes with retinoid X receptor α (RXRα), and this complex interacts with specific response elements [vitamin D response elements (VDREs)] to regulate gene transcription. Previous results show a significant reduction in $1,25(OH)_2D$-induced nVDR transcriptional activity in fibroblast (C3H10T1/2) cells transfected with the Harvey *ras* gene (*ras* cells) compared with parental cells. The purpose of this study was to investigate the mechanisms by which the H-*ras* gene interferes with nVDR transcriptional activity. Similar to the *ras* cells, transcriptional activity of the nVDR was reduced following induction of the H-*ras* gene for 9 days. The *ras* cells expressed similar protein levels of RXRα with the parent cells, and overexpression of the wild-type RXRα plasmid did not restore $1,25(OH)_2D$-mediated nVDR activity in *ras* cells. Inhibiting activation of extracellular signal-regulated kinase (ERK1/2) had no effect on nVDR activity in *ras* cells. Furthermore, the binding of nVDR to VDREs was reduced in $1,25(OH)_2D$-treated *ras* cells. In addition, neither treatment of *ras* cells with an inhibitor (ketoconazole) of the $1,25(OH)_2D$ degradative enzyme, 24-hydroxylase, nor the protein kinase C inhibitors, bisindoylmaleimide I and Gö 6976, had an effect on nVDR activity. In contrast, inhibition of phosphatidylinositol 3-kinase (PI3K) with LY294002 resulted in a 1.6-fold significant increase in the nVDR activity in the *ras* cells. Taken together, these results indicate that PI3K may, at least in part, mediate the suppression of the $1,25(OH)_2D$ regulation of nVDR transcriptional activity by the H-*ras* gene, leading to reduced ability to associate with response elements.


*Keywords:* 1,25-Dihydroxyvitamin D; Vitamin D; Vitamin D receptor; *ras*; Phosphoinositide 3-kinases

## 1. Introduction

Vitamin D is present in the diet and can also be synthesized endogenously from cholesterol in the skin through the action of sunlight or ultraviolet light. Epidemiologic studies have shown an inverse relationship between several types of cancer and moderate exposure to sunlight [1], which suggests a role for vitamin D as an anticancer agent. Current dietary recommendations for vitamin D are under debate [2], and it is critical to understand at which stages of cancer progression vitamin D may be effective to develop targeted dietary recommendations.

The active form of vitamin D, 1,25 dihydroxyvitamin D ($1,25(OH)_2D$), binds to the nuclear vitamin D receptor (nVDR) which heterodimerizes with the retinoid X receptor (RXR). The nVDR/RXR complex then translocates to the nucleus of target cells where it binds to vitamin D response elements (VDREs) on DNA. $1,25(OH)_2D$-Induced gene transcription has been shown to regulate cellular processes involved with carcinogenesis including differentiation, proliferation, apoptosis and angiogenesis in target cells [3–8].

The Ras protein is involved in an interrelated complex of signaling proteins, including Raf, Rac, Rho and PI3K [9]. The Ras protein, encoded by the Harvey-*ras* (H-*ras*) oncogene, has a single amino acid mutation at G12 that maintains it in a constitutively active conformation in cancer cells [9,10]. Constitutively active Ras leads to the increased activity of downstream protein kinase cascades, including extracellular regulated kinase 1/2 (ERK1/2), which can result in increased cellular proliferation and resistance to apoptosis [11]. The H-*ras* oncogene is present in many cancers including those in the cervix [12], salivary gland [13] and

* Corresponding author. Tel.: +1 765 494 8246; fax: +1 765 494 0906.
*E-mail address:* teegarden@purdue.edu (D. Teegarden).

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doi:10.1016/j.jnutbio.2008.06.008

thyroid [14]. In addition, both cellular [15] and animal models [16] have shown that transfection of mutated, constitutively active forms of Ras into untransformed cells results in an invasive and metastatic phenotype. Thus, elucidation of the mechanisms by which the activated Ras protein may alter regulation of cell signaling pathways associated with the action of $1,25(OH)_2D$ is critical to understanding how this hormone may be effective in the regulation of carcinogenesis.

Previous studies in our laboratory have shown that C3H10T1/2 cells stably transfected with the Harvey *ras* oncogene (*ras* cells) exhibit a significant reduction in $1,25(OH)_2D$-induced transactivation of the nVDR, compared with nontransfected control cells [17]. This observation is consistent with the results of Solomon et al. [18,19], who showed that transactivation of the VDR was reduced in *ras*-transformed human keratinocytes. These authors demonstrated that, in *ras*-transfected keratinocytes, VDR transcriptional activity was reduced through phosphorylation of RXRα by the constitutively activated ERK1/2 pathway [19]. In addition, Narayanan et al. [20] showed that the transcriptional activation of VDR by $1,25(OH)_2D$ is inhibited by the activation of ERK1/2 in cells in which RXRα is the partner of VDR. In contrast, in cells in which RXRβ or RXRγ is the VDR partner, the activation of ERK1/2 has been shown to stimulate VDR transcriptional activity [20].

The phosphoinositide 3-kinases (PI3Ks) are integral to the regulation of proliferation and survival of cancer cell lines. The p85 regulatory subunit of PI3K has two *src* homology-2 (SH2) domains that bind to phosphotyrosine residues of activated growth factor receptors or adaptor proteins. The receptor-associated p85 regulatory subunit binds to the p110 catalytic subunit of PI3K through an SH2 domain [21]. The p110 catalytic subunit phosphorylates phosphatidylinositol in the membrane at the D-3 position of the inositol ring. The 3-phosphoinositides that are generated include phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-triphosphate [22]. These phosphorylated inositides then function as docking sites for other second messenger signaling proteins containing pleckstrin-homology domains such as AKT, or proteins containing Fab1p, YOTB, Vac1p and early endosome antigen 1 (FYVE) domains [23]. The activation of these proteins regulates many processes including cell growth, cell-cycle progression, apoptosis and cytoskeletal changes [22,24]. The processes involving cytoskeletal modulation in cancer involve activation of the PI3K small GTPase effectors Rho, Rac and CDC 42 [22]. Furthermore, the interaction of PI3K with the Ras protein has been shown to induce PI3K activation [22,25].

The focus of the current study was to investigate the mechanisms of resistance to vitamin D-induced nVDR activation in the *ras* cell line. To achieve this goal, we investigated the level of nVDR binding to DNA in *ras* cells compared to C3H10T1/2 cells, possible degradation of $1,25(OH)_2D$ and the roles of RXRα, ERK1/2, PKC and PI3K in the modulation of $1,25(OH)_2D$-mediated nVDR transcriptional regulation in the *ras*-transfected cell line (Fig. 1).

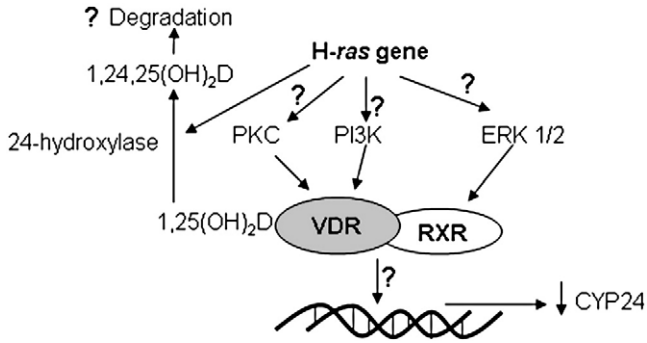


Fig. 1. Modulation of nVDR-mediated transcriptional regulation by H-*ras* oncogene. The presence of the H-*ras* oncogene reduces transcriptional activity of the nVDR. The H-*ras* oncogene leads to increased activity of PKC, PI3K and ERK1/2 which may subsequently regulate the activity of the nVDR or its heterodimer partner RXR. In addition, $1,25(OH)_2D$ is degraded through the activity of the 24-hydroxylase, which may reduce the nVDR activity by reducing cellular ligand concentration. The liganded nVDR with the RXR interacts with VDREs in the DNA to induce CYP24 expression, and the H-ras oncogene may impact the ability of this complex to interact with the VDRE.

## 2. Materials and methods

### 2.1. Reagents and chemicals

$1,25(OH)_2D$ was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, penicillin/streptomycin, lipofectamine and 6% Novex DNA Retardation Gels were obtained from Invitrogen (Carlsbad, CA, USA). Ketoconazole, bisindoylmaleimide I and Gö 6976 were purchased from Calbiochem (San Diego, CA, USA). Lilly 294002 (LY294002), PD98059, biotin antibodies, rabbit antibodies and Lumiglo reagents were purchased from Cell Signaling (Beverly, MA, USA). The RXRα antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tris-HCl Bio-Rad Ready Gels were purchased from Bio-Rad Laboratories (Hercules, CA, USA). The Nuclear Extract Kit and Nushift Human VDR Kit were obtained from Active Motif (Carlsbad, CA, USA). $T_4$ Polynucleotide Kinase and 10X Kinase buffer were from Promega Corporation (Madison, WI, USA). $^{32}$P-ATP was purchased from Amersham Biosciences (Piscataway, NJ, USA). Trans $^{35}$S was obtained from ICN Biochemicals (Irvine, CA, USA). Enhance solution was purchased from DuPont Corp. (Wilmington, DE, USA), and XAR film was from Kodak.

### 2.2. Cell model and cell culture

The C3H10T1/2 murine embryo fibroblast cell line (CCL-226) was purchased from ATCC (Rockville, MD, USA). The *rasneo*11A cell line was previously developed by

stable transfection of C3H10T1/2 cells with the Harvey *ras* oncogene (*ras* cells) [26]. This cell line is minimally transformed, as defined by the ability to grow in soft agar, but not to form tumors in nude mice [26–28]. Together, these cell lines comprise a model for multistage carcinogenesis in that the C3H10T1/2 cell line is untransformed and the *ras* cells are representative of the initiation stage of cancer. The pMT*rasneo*13 cell line is the C3H10T1/2 cell line containing a stably transfected Harvey *ras* oncogene under the control of an inducible truncated metallothionein promoter [26]. All cells were cultured in DMEM with 10% heat-inactivated fetal bovine serum, $1\times10^5$ U/L penicillin and 100 mg/L streptomycin, and grown at 37°C with 5% $CO_2$ and maintained in linear growth. Induction of the Harvey *ras* oncogene in the pMT*rasneo*13 cells was accomplished through the addition of zinc chloride ($ZnCl_2$) (50 μM) to the media. Ethanol was utilized as vehicle control for 1,25 $(OH)_2D$ at a concentration of <0.1% of total treatment.

### 2.3. Expression constructs

The cytochrome P450c24 (CYP24) luciferase reporter construct, which contains two vitamin D response elements [29], was a gift from J. Omdahl, and the thymidine kinase [pRL-TK-(*Renilla*)] luciferase internal control expression vector was purchased from Promega Corporation. The alanine 260 serine human RXRα mutant plasmid and RXRα wild-type plasmid were gifts from R. Kremer (McGill University, Montreal, Quebec, Canada) [19].

### 2.4. Determination of H-ras mRNA and protein content

pMT*rasneo*13 cells were labeled with Trans $^{35}S$ (specific activity >1000 Ci/mmol) for 6 h and treated with 50 μM $ZnCl_2$ for 9, 24 or 48 h to induce H-*ras* expression. Total mRNA was isolated and H-*ras* was detected via Northern blot analysis as previously described [30]. To determine the relative amount of total Ras protein, the radiolabeled Ras protein was immunoprecipitated from the cell lysate using the Y13-259 antibody. Proteins were then separated by SDS-PAGE and the gel was treated with Enhance solution and dried, and the bands were visualized using XAR film.

### 2.5. Immunologic detection of proteins

Following treatment with either $1,25(OH)_2D$ (100 nM) or ethanol vehicle control (<0.1% of total treatment) for 24 h, cells were rinsed with cold CMF-PBS and harvested by scraping on ice into cold lysis buffer (25 mmol/L HEPES, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.1% Triton), 1% protease inhibitor cocktail [containing 104 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride, 0.08 mmol/L aprotinin, 2 mmol/L leupeptin, 4 mmol/L bestatin, 1.5 mmol/L pepstatin A and 1.4 mmol/L E-64] and 1% phosphatase inhibitor cocktail (containing sodium vanadate, sodium molybdate, sodium tartrate and imidazole). Cells were placed on ice for 10 min, sonicated for 20 s and centrifuged at 18,000×*g* for 10 min, and the supernatant collected for analysis. Protein concentration was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) and 12 μg of protein was loaded onto a 12% Tris-HCl gel. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with either RXRα antibody (1:1000) or β-actin antibody (1:1000). Bands were visualized via chemiluminescence using HRP-conjugated secondary antibodies and quantified using Biorad Quantity One software in conjunction with the Biorad Fluor S Multi Imager system.

### 2.6. Reporter gene assay

Cells at approximately 70% confluence were transiently transfected with 0.1 to 0.5 μg pRL-TK-Renilla, 1.7 to 2 μg CYP-24, 2 μg wtRXRα or 2 μg RXR-Ala260 using the Lipofectamine Plus system (Invitrogen) according to manufacturer's instructions. Twenty-four hours post-transfection, cells were treated with 100 nM $1,25(OH)_2D$ or vehicle for 24 h. Luciferase activity was assayed via the Dual Luciferase Assay (Promega) on a Turner TD-20/20 (Turner Designs) luminometer, and transfection efficiency was normalized by pRL-TK-*Renilla* luciferase activity. Data are expressed as relative luciferase units with a ratio of treated cells to vehicle controls (mean±S.E.M., $n\geq3$).

### 2.7. Electromobility shift assay

Nuclear extracts (14–16 μg/μl) from C3H10T1/2 and *ras* cells treated with 100 nM $1,25(OH)_2D$ or vehicle were analyzed according to manufacturer's instructions using the Nushift Human VDR Kit. The contents of each reaction were separated using a 6% Novex DNA Retardation Gel. The gels were dried on a gel air dryer and the bands were visualized employing a Perkin Elmer Cyclone Storage Phosphor System (Downers Grove, IL, USA).

### 2.8. Inhibitor studies

To determine the involvement of signaling pathways in the reduced transcriptional activation of the nVDR in the *ras* cells, cells were pretreated using inhibitors to MEK1 (50 μM PD98059), PKC (2 μM bisindoylmaleimide I or 10 nM Gö 6976) and PI3K (50 μM LY294002). To inhibit 24-hydroxylase, cells were co-treated with 0.1 μg/ml ketoconazole and 100 nM $1,25(OH)_2D$ or vehicle control (DMSO <0.1% of total volume) for 18 h. Following pretreatment with inhibitors (PD98059 for 1 h, bisindoylmaleimide I, Gö 6976 and LY294001 for 6 h), cells were harvested and assayed for CYP24 luciferase activity as described above. Results are expressed as relative luciferase units (sample/vehicle). Values are means of two experiments±S.E.M. ($n$=6).

### 2.9. Statistical analysis

Data were analyzed by either Student's $t$ test or one-way ANOVA followed by Dunnett's Multiple Range test ($\alpha$=0.05) using Prism GraphPad 4 software.

## 3. Results

To determine whether the reduced activation of nVDR transcriptional activity in the *rasneo*11A cells is due to the constitutive activation of H-*ras* in this cell line, or if rapid activation of *ras* would also have a suppressive effect, an inducible Harvey-*ras* C3H10T1/2 clonal cell line was developed using a truncated metallothionein promoter (pMT*rasneo*13). In this cell model, Harvey-*ras* mRNA cannot be detected using a Northern blot prior to zinc chloride treatment. Following the addition of 50 μM zinc chloride, Harvey-*ras* mRNA was expressed within 24 h (Fig. 2A). Harvey-*ras* protein expression increased fivefold by 24 h (Fig. 2B). In addition, the transformed phenotype was apparent within 2 days (Fig. 2C). These data show that the Harvey-*ras* gene is expressed and functional in the pMT*rasneo*13 cells following zinc chloride treatment.

Following induction of the *ras* gene with 50 μM zinc chloride for 9 days, we observed a significant decrease in nVDR transcriptional activity in 1,25(OH)$_2$D-treated cells compared with control cells as assessed by reporter assay using the CYP-24 promoter luciferase gene construct ($P$<.001) (Fig. 2D). There was no significant difference in nVDR transcriptional activity in 1,25(OH)$_2$D-treated cells at earlier times (1, 2, 4 and 7 days). These results suggest that the differential transcriptional activity of the nVDR is not unique to the stably Harvey-*ras* transfected *rasneo*11A cells and does not occur rapidly after induction of the *ras* oncogene.

Our laboratory has previously shown that the expression level of the nVDR is not different between the C3H10T1/2 and *rasneo*11A cells [17]. Therefore, we hypothesized that differential expression of the heterodimer partner of nVDR (RXRα) between the cell lines may be responsible for the altered nVDR transcriptional activity. However, results of Western blot analyses indicated no significant difference in the levels of RXRα between C3H10T1/2 and *rasneo*11A cells (Fig. 3A and B). We also tested the role of RXRα and ERK1/2 activity in the reduced nVDR transcriptional activity in the *rasneo*11A cells. Results showed that

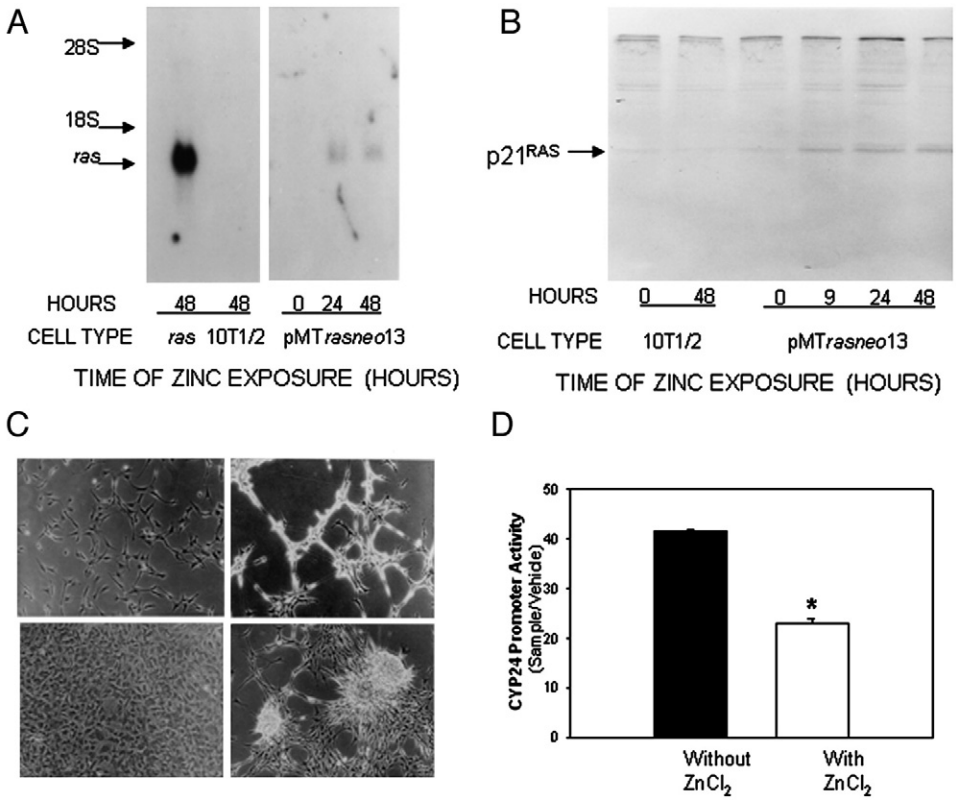


Fig. 2. Induction of Harvey-*ras* gene in pMT*rasneo* 13 cells following zinc chloride treatment. (A) A representative Northern blot showing Harvey-*ras* mRNA in pMT*rasneo*13 cells after 24 and 48 h of exposure to 50 μM zinc chloride. The left two lanes show Harvey-*ras* mRNA levels from *rasneo*11A cells (*ras*) and C3H10T1/2 cells (10T1/2) which serve as controls. (B) Results of immunoprecipitation of radiolabeled methionine-labeled cells showing increased p21$^{RAS}$ protein in pMT*rasneo*13 cells following treatment with 50 μM zinc chloride for indicated times. The left two lanes contain proteins isolated from C3H10T1/2 cells (10T1/2) following zinc chloride treatment for 0 and 48 h. (C) Phenotype of pMT*rasneo*13 cells without (upper left panel) and with (upper right panel) addition of zinc chloride to the media for 3 weeks. Phenotype of pMT*rasneo*13 cells at high density without (lower left panel) and with (lower right panel) addition of zinc chloride to the media for 3 weeks. (D) nVDR transcriptional activity in pMT*rasneo*13 cells. pMT*rasneo*13 cells were pretreated with 50 μM zinc chloride for 7 days, transiently co-transfected with the CYP24 luciferase and *Renilla* luciferase control plasmids for 24 h, and treated with 100 nM 1,25(OH)$_2$D or 0.1% ethanol vehicle control for 24 h. CYP24 luciferase activity was assayed and normalized for transfection efficiency via *Renilla* luciferase activity. Results are expressed as relative luciferase units (sample/vehicle). Values are means±S.E.M. ($n$=3); asterisk indicates $P$<.001 between groups.

transfection of *rasneo*11A cells with the mutant RXRα plasmid (A260S), the proposed site of ERK1/2 phosphorylation and subsequent inhibition of heterodimer formation, or overexpression of the wild-type RXRα plasmid, did not change nVDR transcriptional activity in the *rasneo*11A cells (Fig. 3C). To further test whether constitutive activation of ERK1/2 in the *rasneo*11A cells interferes with the transcriptional activity of the nVDR–RXR complex (potentially by phosphorylation of RXRα) [19], the MAPKK/MEK inhibitor PD98059 was utilized. Results of this study showed that inhibition of ERK1/2 did not increase nVDR transcriptional activity in the *rasneo*11A cells (Fig. 3D). These results suggest that ERK1/2 activity does not interfere with the differential nVDR transcriptional activity in this cell model.

To determine whether there is a differential level of nVDR binding to nuclear DNA in the *ras*-transfected cells compared to control cells, we performed the electrophoretic mobility shift assay in both cell lines. Results showed that

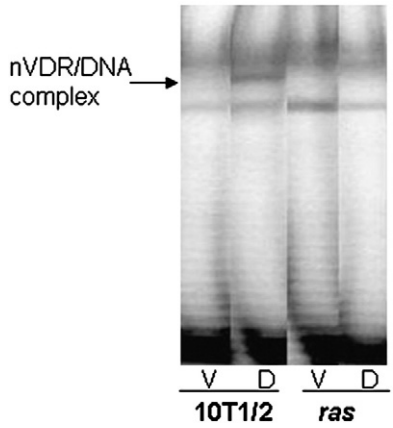


Fig. 4. Electromobility shift assay in 1,25(OH)$_2$D-treated cells. A nVDR/RXR oligo probe was labeled with $^{32}$P and incubated with 100 nM 1,25(OH)$_2$D (1,25D) or ethanol vehicle (V) control-treated nuclear extracts from C3H10T1/2 and *rasneo*11A cells. The nVDR/DNA complex is indicated. Lanes are electronically arranged for consistency in presentation.

the nVDR binds the nuclear DNA in the C3H10T1/2 cells treated with 1,25(OH)$_2$D, but not in the *rasneo*11A cells (Fig. 4), suggesting that reduced binding of the nVDR to the VDRE in the *rasneo*11A cells may lead to inhibition of nVDR transcriptional activity.

24-Hydroxylase is the enzyme responsible for the conversion of 1,25(OH)$_2$D to the inactive metabolites 1,25(OH)$_2$D$_3$-26,23-lactone and calcitroic acid [31,32]. To ascertain whether conversion of 1,25(OH)$_2$D to its inactive metabolites is responsible for the decreased activation of

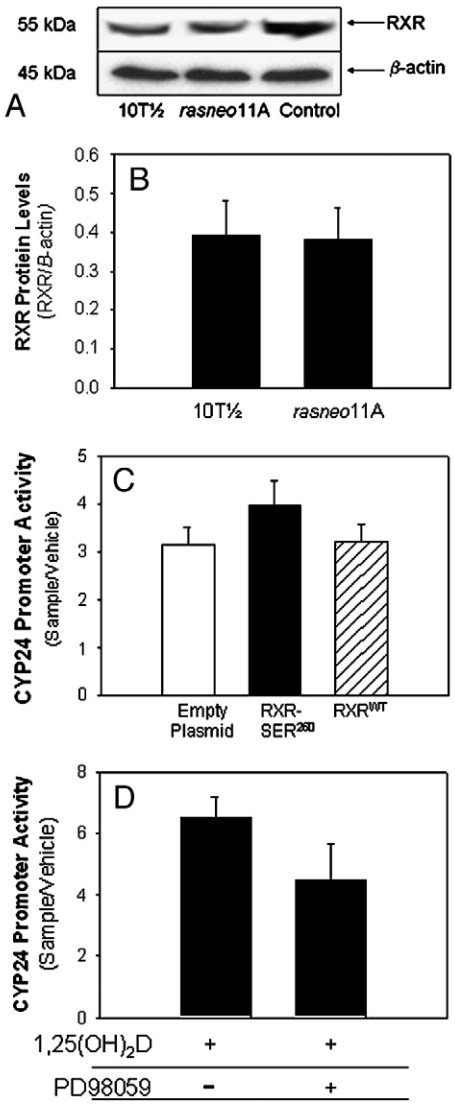


Fig. 3. Role of RXRα in nVDR transcriptional activity in stably Harvey-*ras* transfected cells. (A) RXRα protein expression in C3H10T1/2 and *rasneo*11A cells. C3H10T1/2 and *rasneo*11A cells were treated with ethanol vehicle or 100 nM 1,25(OH)$_2$D for 24 h and probed with RXRα antibody. The upper panel is a representative autoradiograph of RXRα and the lower panel is the actin control of a Western blot of three separate cell preparations with RXRα C3H10T1/2 (10T1/2), *rasneo*11A and control protein cell lysates. (B) Histogram representative of the quantification of RXRα protein expression by Western blot analysis (RXRα/actin control). Results are expressed as relative optical density. Values are means±S.E.M. ($n$=3). (C) nVDR transcriptional activity in *rasneo*11A cells following transient transfection with empty plasmid, RXRα (A260S) mutant plasmid (RXR-SER$^{260}$) or overexpression of wild-type RXRα (RXR$^{WT}$). *Rasneo*11A cells were transiently co-transfected with the CYP24 luciferase plasmid and *Renilla* luciferase control plasmids for 24 h. Each of the three groups was treated with 0.1% ethanol vehicle control or 100 nM 1,25(OH)$_2$D for 24 h. CYP24 luciferase activity was assayed and normalized for transfection efficiency via *Renilla* luciferase activity. Results are expressed as relative luciferase units (sample/vehicle). Values are means±S.E.M. ($n$=3). There was no significant effect between groups as determined by one-way ANOVA. (D) Effect of MAPKK/MEK inhibition of nVDR transcriptional activity. *Rasneo*11A cells were transiently co-transfected with the CYP24 luciferase and *Renilla* luciferase control plasmids for 24 h, pre-treated with 50 μM PD98059 for 1 h and co-treated with 0.1% DMSO and 0.1% ethanol vehicle control or 100 nM 1,25(OH)$_2$D for 24 h. CYP24 luciferase activity was assayed and normalized for transfection efficiency via *Renilla* luciferase activity. Results are expressed as relative luciferase units (sample/vehicle). Values are means±S.E.M. ($n$=3); there was o significant effect between groups.

1,25(OH)$_2$D -mediated transcriptional activation in the *ras* cells, we utilized ketoconazole, an inhibitor of 24-hydroxylase. Treatment with ketoconazole did not cause a significant difference in the 1,25(OH)$_2$D-induced nVDR activity of C3H10T1/2 [20.36±1.54 with 1,25(OH)$_2$D vs. 18.61±0.82 with 1,25(OH)$_2$D and ketoconazole] or *rasneo*11A cells [4.04±0.2 with 1,25(OH)$_2$D vs. 4.01±0.20 with 1,25(OH)$_2$D and ketoconazole]. This indicates that the differential 1,25(OH)$_2$D activation of the nVDR in these cell lines is not mediated through conversion of 1,25(OH)$_2$D to an inactive metabolite.

In addition, protein kinase C (PKC) is known to be rapidly activated by 1,25(OH)$_2$D [33] and to regulate nVDR activity. Therefore, the role of PKC in 1,25(OH)$_2$D-regulated nVDR activity was assessed. Inhibition of PKC with bisindoylmaleimide I in combination with treatment by 1,25(OH)$_2$D resulted in a significant decrease in nVDR transcriptional activity in both C3H10T1/2 ($P$=.02) and *rasneo*11A cells ($P$<05) (Fig. 5). Treatment of C3H10T1/2 cells with the PKC inhibitor Gö 6976 in combination with 1,25(OH)$_2$D also resulted in a significant decrease in nVDR transcriptional activity ($P$=.03) (Fig. 5). These results suggest that PKC is required for the activity of the nVDR in both cell lines; however, its activation is not responsible for the differential transcriptional activity of the nVDR in C3H10T1/2 and *rasneo*11A cells.

PI3K has been shown to interact directly with the nVDR in human myeloid leukemia (THP-1) cells [34], and AKT, a downstream effector of PI3K, is activated by 1,25(OH)$_2$D in C3H10T1/2 cells [7] and HL60 cells [35]. Therefore, the role of PI3K in the differential transcriptional activity of the nVDR in C3H10T1/2 and *rasneo*11A cells was investigated. Treatment of *rasneo*11A cells with the PI3K inhibitor LY294002 in combination with 1,25(OH)$_2$D showed a significant 1.6-fold increase in the nVDR transcriptional activity compared to cells treated with 1,25(OH)$_2$D alone ($P$=.0003), whereas in C3H10T1/2 cells, there was no significant change in the transcriptional activity of the nVDR (Fig. 6). These data suggest that the reduced nVDR transcriptional activity in *rasneo*11A cells may be mediated by the PI3K signaling pathway.

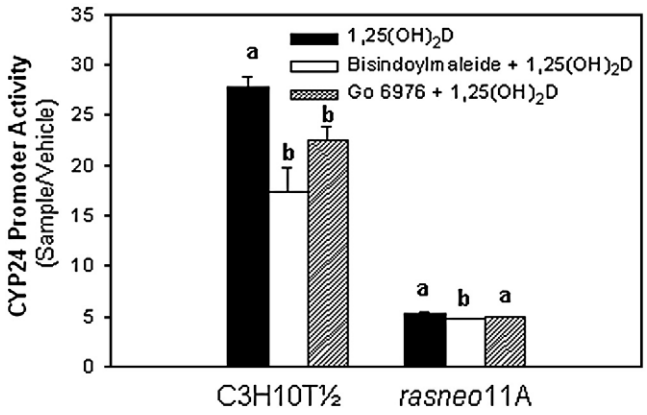


Fig. 5. Effect of PKC in C3H10T1/2 and *rasneo*11A cells on nVDR transcriptional activity. C3H10T1/2 and *rasneo*11A cells were transiently co-transfected with the CYP24 luciferase and *Renilla* luciferase control plasmids for 24 h, pretreated with 2 μM bisindoylmaleimide I or 10 nM Gö 6976 for 6 h and treated with 0.1% DMSO and 0.1% ethanol vehicle control or 100 nM 1,25(OH)$_2$D for 18 h. CYP24 luciferase activity was assayed and normalized for transfection efficiency via *Renilla* luciferase activity. Results are expressed as relative luciferase units (sample/vehicle). Values are means of one representative experiment for each cell type± S.E.M. ($n$=3). Bars with different letters indicate significant differences in treatment within cell type ($P$<05).

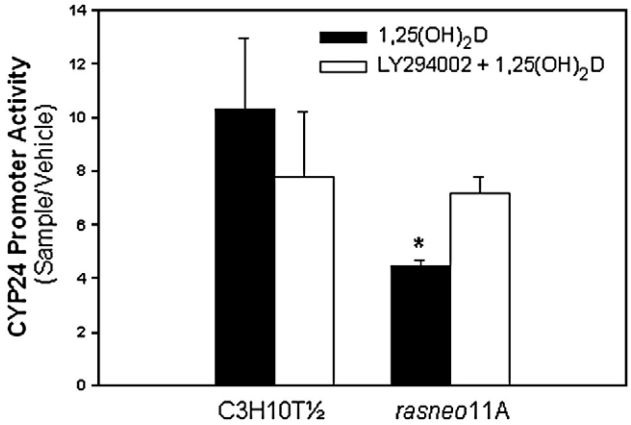


Fig. 6. Role of PI3K in 1,25(OH)$_2$D-induced nVDR transcriptional activity. Inhibition of PI3K with LY294002. C3H10T1/2 and *rasneo*11A cells were transiently co-transfected with the CYP24 luciferase and *Renilla* luciferase control plasmids for 24 h, pretreated with 50 μM LY294002 for 6 h and treated with 0.1% DMSO and 0.1% ethanol vehicle control or 100 nM 1,25(OH)$_2$D for 18 h. CYP24 luciferase activity was assayed and normalized for transfection efficiency via *Renilla* luciferase activity. Results are expressed as relative luciferase units (sample/vehicle). Values are means±S.E.M. For C3H10T1/2 cells, two experiments ($n$=5). For *rasneo*11A cells, three experiments ($n$=8); asterisk indicates significant differences between treatments within group ($P$=.0003).

## 4. Discussion

The current studies investigated the mechanisms underlying the resistance of the transcriptional activity of the nVDR, as assessed by a CYP-24 luciferase reporter gene, in 1,25(OH)$_2$D-treated *ras*-transfected mouse fibroblast cells compared with the parent C3H10T1/2 cell line. Together with our previous results [17], the current study indicates that neither differential expression of the nVDR nor RXRα protein is responsible for the difference in the transcriptional activity of the nVDR in *ras*-transformed compared to parent C3H10T1/2 cells. Our studies also suggest that neither degradation of 1,25(OH)$_2$D nor activation of PKC or ERK1/2 is involved in the reduced nVDR transcriptional activity in the H-*ras* transfected C3H10T1/2 fibroblast cells. However, we show that the *ras* cells exhibit reduced nVDR/RXR complex binding to VDRE and this may be modulated by the PI3K signaling pathway.

Decreased transcriptional activity of the nVDR in *ras*-transfected human keratinocytes (HPK1A cells) has also

been reported by Solomon et al. [18,19] and in *ras*-transfected murine mammary (HC11) cells by Rozenchan et al. [36]. Although Rozenchan et al. [36] reported that nVDR mRNA expression is lower in *ras*-transformed cells than in the parent HC11 cells, our laboratory previously reported that the expression of the nVDR protein is the same in *ras*-transfected and the parent C3H10T1/2 cells [17]. This indicates that the mechanism of interference of the transactivation of the nVDR in *ras*-transfected cells may vary between cell lines.

In the present study, gel shift analysis indicated interference with the binding of the nVDR to its DNA response element (VDRE) in nuclear extracts from the *ras-neo*11A cell line in the presence of $1,25(OH)_2D$, while the nVDR complex does bind the VDRE in the parental C3H10T1/2 nuclear extracts. Therefore, we hypothesized that an upstream signaling pathway may interfere with the binding of the nVDR to the VDRE, subsequently reducing its activity in the *rasneo*11A cell line. Due to reports that ERK1/2 interferes with nVDR–RXRα interaction in the HPK1A cell line [19], we investigated the role of the ERK1/2 signaling pathway in the regulation of nVDR transcriptional activity in the *ras*-transfected C2H10T1/2 cells. Our studies showed that inhibition of MAPKK/MEK, the upstream activator of ERK1/2, with PD98059, did not change the transcriptional activity of the nVDR in the *ras*-transfected cells. In addition, inhibition of the phosphorylation of RXRα by ERK1/2, using a nonphosphorylatable RXRα mutant plasmid in the *ras* cells, did not change the transcriptional activity of the nVDR either. Our results are in contrast to the results of Solomon et al. [19] who showed that RXRα is phosphorylated through the ERK kinase cascade in *ras*-transformed human keratinocytes, and that this phosphorylation results in the inhibition of vitamin D signaling. In their study, inhibition of MAPKK/MEK with PD98059 restored the activation of the VDR by $1,25(OH)_2D$ [19], and the addition of a nonphosphorylatable RXRα mutant plasmid restored activation of the nVDR in *ras*-transfected keratinocytes. Thus, cell line-specific differences in the regulation of nVDR transcriptional activity occur. This cell line-specific variation may be explained by a report by Narayanan et al. [20], which showed that the transactivation of the VDR varied according to the specific isoform of RXR to which it was bound.

In our previous studies, we demonstrated that H-*ras* transfection of C3H10T1/2 cells results in reduced nVDR transcriptional activation. To determine whether this effect was specific to this cell line, an inducible *ras* gene was transfected into the C3H10T1/2 cell line (pMT*rasneo*13 cells). Our results showed that earlier than 9 days post-induction, there was no change in CYP-24 reporter gene activity. However, after 9 days of induction of the *ras* gene, a significant reduction in CYP-24 transcriptional activity was observed, suggesting that long-term H-*ras* gene expression is necessary to reduce nVDR transcriptional activity in this cell model.

The enzyme 24-hydroxylase is known to convert $1,25(OH)_2D$ to the inactive metabolites $1,25(OH)_2D_3$-26,23-lactone and calcitroic acid [31,32]. Previous experiments revealed that ketoconazole, an inhibitor of 24-hydroxylase [37], increased the growth-inhibitory activity of $1,25(OH)_2D$ in human prostate cancer cells, and that induction of 24-hydroxylase by $1,25(OH)_2D$ or the vitamin D analog, EB1089, was partially blocked [38]. In the current study, inhibition of 24-hydroxylase with ketoconazole in C3H10T1/2 cells and *rasneo*11A cells had no effect on nVDR transcriptional activity. Thus, we conclude that the differential activation of the nVDR is unlikely due to conversion of $1,25(OH)_2D$ to an inactive metabolite.

We further explored the role of the PKC and PI3K signaling pathways in the interference of H-*ras* expression on $1,25(OH)_2D$-induced nVDR transcriptional activity in the C3H10T1/2 cells. Our results showed that PKC activity is not responsible for the differential activation of the nVDR in the C3H10T1/2 and *ras*-transfected cells. Alternatively, inhibition of PI3K in *ras* cells significantly increased the $1,25(OH)_2D$-induced transcriptional activity of the nVDR. These results suggest that PI3K plays a role in the Harvey-*ras*-mediated reduction in $1,25(OH)_2D$-induced nVDR transcriptional activity. Humeniuk-Polaczek and Marcinkowska [39] observed an impairment in the nuclear localization of the nVDR in THP-1 leukemia cells that were resistant to $1,25(OH)_2D$-induced differentiation. Based on reports that the VDR interacts with PI3K in THP-1 cells [34], the authors hypothesized that PI3K may "trap" the nVDR in the cytosol [39]. In a later study, Gocek et al. [40] reported that the cytosolic portion of VDR is found near the F-actin cytoskeleton next to the plasma membrane in THP1 cells. Taken together with the current study, these results suggest that the nVDR transcriptional activity may be partially inhibited by PI3K. Although our studies as well as previous studies support a role for PI3K in regulation of the nVDR, the effect of PI3K on the nVDR transcriptional activity may not be direct, as inhibition of PI3K will impact other signaling pathways. Further investigations are necessary to understand the mechanism underlying the role of PI3K in inhibition of nVDR transcriptional regulation via the *ras* oncogene.

Vitamin D status is inversely associated with risk of several cancers; however, the basis for this relationship has not been clarified. Currently, the dietary recommendations for vitamin D are under debate [2], and an understanding of the response of cells at various stages of carcinogenesis will contribute to the development of targeted recommendations. In addition, vitamin D is also available through exposure to ultraviolet light. Due to the low availability of vitamin D in the diet, if the preventive capability of vitamin D is established, it may be important to develop appropriate recommendations for exposure to the sun as excessive exposure to ultraviolet light is a major risk factor for skin cancer. The current studies in the murine fibroblast model of multi-stage carcinogenesis provide valuable insight into the

effects of constitutive H-*ras* activation, a common mutation in many cancers, on $1,25(OH)_2D$-induced gene transcription during cancer progression. Our studies suggest that the cell signaling lipid kinase PI3K partially mediates the differential nVDR transcriptional activity in the *rasneo*11A cell line compared with the C3H10T1/2 parental cell line. A clear understanding of how the nVDR is affected by oncogenes commonly mutated in cancer such as H-*ras* is important to the development of strategies to prevent and/or treat cancer via dietary components such as $1,25(OH)_2D$.

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