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Activation of Rapid Signaling Pathways Does Not Contribute to 1α,25-Dihydroxyvitamin D_3-Induced Growth Inhibition of Mouse Prostate Epithelial Progenitor Cells

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ABSTRACT

The active form of vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2$D) inhibits the growth of prostate epithelial cells, however the underlying mechanisms have not been clearly delineated. In the current study, the impact of 1,25$(OH)_2$D on the rapid activation of extracellular-regulated kinase (ERK) 1/2 and protein kinase C α (PKCα), and the role of these pathways in growth inhibition was examined in immortalized mouse prostate epithelial cells, MPEC3, that exhibit stem/progenitor cell characteristics. 1,25$(OH)_2$D treatment suppressed the growth of MPEC3 in a dose and time dependent manner (e.g., 21% reduction at three days with 100 nM 1,25$(OH)_2$D treatment). However, ERK1/2 activity was not altered by 100 nM 1,25$(OH)_2$D treatment for time points from 1 min to 1 h in either serum-containing or serum-free medium. Similarly, PKCα activation (translocation onto the plasma membrane) was not regulated by short-term treatment of 100 nM 1,25$(OH)_2$D. In conclusion, 1,25$(OH)_2$D did not mediate rapid activation of ERK1/2 or PKCα in MPEC3 and therefore the growth inhibitory effect of 1,25$(OH)_2$D is independent of rapid activation of these signaling pathways in this cell type. J. Cell. Biochem. 107: 1031–1036, 2009.

KEY WORDS: PROSTATE; VITAMIN D; 1,25-DIHYDROXYVITAMIN D; MAPK; PROTEIN KINASE C; GROWTH INHIBITION

The active hormonal form of vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2$D), has been proposed to mediate protection from prostate cancer [Schwartz and Hulka, 1990; Skowronski et al., 1993]. The mechanism of 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2$D) cellular actions involves both traditional, genomic signaling pathway through the nuclear vitamin D receptor (nVDR) and rapid (within minutes or seconds) activation of kinases. The nVDR plays a critical role in mediating ligand-induced transcriptional regulation of a variety of genes that are important for cell proliferation, differentiation, and apoptosis. In addition, several kinases have been shown to be rapidly regulated by 1,25$(OH)_2$D, including extracellular-regulated kinase (ERK) 1/2 [Beno et al., 1995; Marcinkowska et al., 1997; Song et al., 1998; Chen et al., 1999; Morelli et al., 2001; Buitrago et al., 2001a,b; Galbiati et al., 2002; Schwartz et al., 2002; Johansen et al., 2003; Capiati et al., 2004; Pardo and de Boland, 2004] and protein kinase C α (PKCα) [Balogh et al., 2000; Capiati et al., 2000; Rivera-Bermudez et al., 2002; Boyan et al., 2003; Schwartz et al., 2003; Wali et al., 2003], which may contribute to the regulation of genes that are not direct targets of nVDR [Farach-Carson and Davis, 2003]. However, the mechanisms mediating growth inhibition by vitamin D that lead to prostate cancer prevention, as well as therapy by vitamin D analogues, are not known definitively. One hypothesis is that these effects are not mediated by transcriptional activation requiring the VDR but by the rapid, membrane-initiated actions of 1,25$(OH)_2$D.

The regulation of ERK1/2 by 1,25$(OH)_2$D has been shown in a variety of cell types. ERK1/2 is a member of the mitogen-activated protein kinases (MAPK). Upon upstream activation by extracellular stimuli, such as growth factors, ERK1/2 phosphorylates a variety of downstream proteins including transcription factors, and therefore modulates their activity and corresponding gene transcription. Rapid activation of ERK1/2 by 1,25$(OH)_2$D has been observed in muscle cells [Buitrago et al., 2001b; Morelli et al., 2001], enterocytes [Pardo and de Boland, 2004], costochondral chondrocytes [Schwartz et al., 2002], colon carcinoma [Chen et al., 1999], insulinoma cells [Galbiati et al., 2002], hepatic cells [Beno et al., 1995], acute promyelocytic leukemia cells [Marcinkowska et al., 1997; Song et al., 1998], and keratinocytes [Marcinkowska et al., 1997; Johansen et al., 2003].

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Another rapid signal previously reported to be induced by $1,25(OH)_2D$ is PKCα [Balogh et al., 2000; Capiati et al., 2000; Rivera-Bermudez et al., 2002; Boyan et al., 2003; Schwartz et al., 2003; Wali et al., 2003]. PKCα is classified as a classical PKCs (cPKC), and a downstream target of extracellular stimulated receptors, including G-protein coupled receptors (GPCRs) [Breitkreutz et al., 2007]. Activated GPCR induces phospholipase C (PLC) activity to hydrolyze 4,5-bisphosphate (PIP2) and consequently produce inositol 1,4,5-trisphosphate (IP3) into cytosol and diacylglycerol (DAG) on the plasma membrane [Breitkreutz et al., 2007]. IP3 triggers calcium release through the IP3 receptor located on endoplasmic reticulum. The interaction of both DAG and calcium with PKCα brings PKCα to the plasma membrane and activates its kinase activity [Breitkreutz et al., 2007]. Activated PKCα phosphorylates proteins on serine/threonine residues and thereby regulating their activity [Breitkreutz et al., 2007]. Rapid PKC activation by $1,25(OH)_2D$ has been shown in myoblasts and myotubes [Capiati et al., 2000], duodenal mucosae [Balogh et al., 2000], chondrocytes [Boyan et al., 2003; Schwartz et al., 2003], osteoblasts [Wali et al., 2003], osteosarcoma cell line [Rivera-Bermudez et al., 2002].

Induction of cell cycle arrest by $1,25(OH)_2D$ has been observed in both normal and transformed prostate epithelial cells [Miller et al., 1992; Skowronski et al., 1993, 1995; Peehl et al., 1994; Blutt et al., 1997; Zhuang and Burnstein, 1998; Sprenger et al., 2001; Rao et al., 2002]. However, the activation of ERK1/2 and PKCα by $1,25(OH)_2D$, and the role of these pathways in $1,25(OH)_2D$-mediated growth inhibition have not been investigated in prostate progenitor/stem cells. The cancer stem cell theory proposes the origin of prostate cancer is from a small prostate stem cell population [Bonkhoff, 1996]. These cells have the self-renewal and multilineage differentiation properties of normal stem cells. They are responsible for tumor reoccurrence because of their resistance to radiotherapy and androgen-deprivation therapy [Maitland et al., 2006]. Therefore it is critical to study the impact of potential chemopreventive agents in untransformed progenitor cells. The purpose of these studies is to investigate whether $1,25(OH)_2D$ rapidly activates ERK1/2 and PCKα and whether these rapid signaling pathways play a role in $1,25(OH)_2D$-mediated growth inhibition in normal prostate progenitor cells.

MATERIALS AND METHODS

MATERIALS

Unless otherwise noted, all chemicals were obtained from Sigma (St. Louis, MO). Bicinchoninic acid (BCA) assay was obtained from Pierce (Rockford, IL), $1,25(OH)_2D$ was from Biomol (Plymouth Meeting, PA) and Lipofectamine 2000 from Invitrogen (Carlsbad, CA). The vector expressing a fusion of PKCα and enhanced green fluorescent protein (PKCα-EGFP) and the GFP monoclonal antibody were from Clonetech (Mountain View, CA). Phorbol-12-myristate-13-acetate (TPA) and phosphospecific ERK1/2 (Thr202/Tyr204) or total ERK1/2 antibody were from Cell Signaling (Danvers, MA).

CELL CULTURE

The normal mouse prostate epithelial progenitor cells (MPEC)-3 was a gift from Dr. Scott Cramer (Wake Forest University, Winston-Salem). The isolation, culture condition, and properties of MPEC3 were described previously [Barclay and Cramer, 2005; Barclay et al., 2008]. Medium was changed every 48 h. Medium in experiments which use serum-free medium contains BSA, gentamycin, and trace elements in DMEM/F12 (50:50). The progenitor origin of MPEC3 cells were verified by their expression of prostate stem cell markers, Sca 1 and CD49f, as well as their ability of multilineage differentiation into organized prostatic ductal structures [Barclay et al., 2008]. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

CELL PROLIFERATION ASSAY

Relative cell number was assessed using the MTT assay according to the manufacturers instructions. Results were quantified spectrophotometrically using PowerWaveX Microplate Spectrophotometer (BioTek Instruments, Winooski, VT). Results were from three separate experiments with at least 6 wells per experiment for each treatment condition.

CELL TREATMENT FOR ERK1/2 ACTIVITY

Two conditions were used to determine whether $1,25(OH)_2D$ modifies ERK1/2 activity: (1) serum-containing medium and (2) serum-free medium. Cells were treated by adding $1,25(OH)_2D$ (final concentration 100 nM) or vehicle directly to the medium to avoid activation of the pathways due to addition of medium. An untreated control which was not manipulated until harvest was included in all experiments. At the end of the treatment period, cells were rinsed twice with CMF-PBS buffer (136.7 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 pH 7.4) and collected into lysis buffer containing 25 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1% Triton, protease inhibitor cocktail, and phosphatase inhibitor cocktail. The lysate was centrifuged at 12,000*g* for 10 min and the supernatant used for immunoblot for phosphorylated and total ERK1/2.

CELL TREATMENT FOR PKCα ACTIVATION

PKCα activation was determined by its association with plasma membrane. MPEC3 were transfected with the PKCα-EGFP construct using Lipofectamine 2000 according to the manufacturer's instructions. Twenty-four hours after transfection, cells were rinsed and given serum-free medium (described above) and treated with 100 nM $1,25(OH)_2D$ or 1 μM PKCα agonist, TPA, for the indicated times. Following the incubation, cells were rinsed and harvested into hypotonic HES buffer (20 mM HEPES, 1 mM EDTA, and 250 mM Sucrose, pH 7.4) containing protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and phosphatase inhibitor cocktail (Sigma). Cell lysate was prepared using a glass tissue homogenizer. The cell membrane fraction was separated from cytosolic fraction by ultracentrifugation (Beckman OptimaTM TL Ultracentrifuge) of the homogenate at 100,000*g* for 1 h at 4 °C. The pellet (membrane fraction) was re-suspended in 20 μl of HES buffer which included 1% of Triton X-100. Samples were frozen at −20°C for immunobloting for PKCα-EGFP.

IMMUNOBLOT

Total cellular protein concentration from the collected samples was assessed with the BCA assay. Samples were prepared and separated

by SDS–PAGE (10% Tris–HCl). The membranes were incubated with blocking buffer followed by incubation with first antibody: 1:500 GFP monoclonal antibody for PKCα-EGFP, 1:1,500 phosphospecific ERK1/2 (Thr202/Tyr204) or 1:2,000 total ERK1/2 antibody. Immunoreactive bands were visualized with horseradish peroxidase-conjugated anti-rabbit antibody and ECL Advance visualization solution (Amersham Biosciences, Piscataway, NJ). Immunoreacting bands were quantified with Fluor-STM Multi-Imager (Bio-Rad, Hercules, CA). ERK1/2 activity was calculated by first correcting the density of immunoreacting bands with the mean of no treatment control in the same blot, and results expressed as the ratio of phosphorylated ERK1/2 over total ERK1/2.

STATISTICAL ANALYSIS

All experiments were completed at least twice with a final n of at least four for each treatment. Student's *t*-tests where used to test difference between two groups. Differences were considered significant when $P \leq 0.05$.

RESULTS

The impact of $1,25(OH)_2D$ on the growth of MPEC3 was examined. $1,25(OH)_2D$ suppressed MPEC3 cell growth in a time- and dose-dependent manner. A significant inhibitory effect was observed as early as 24 h after 1 nM $1,25(OH)_2D$ treatment (4% reduction) and a maximum 21% reduction in cell number was observed at 72 h after 100 nM $1,25(OH)_2D$ treatment (Fig. 1).

The effect of $1,25(OH)_2D$ on the rapid activation of ERK1/2 was assessed in MPEC3. A non-significant 50% increase in pERK1/2 level was seen after 15 min of $1,25(OH)_2D$ treatment when cells were cultured in serum-containing medium (Fig. 2A) but no trend towards activation in cells cultured in serum-free medium (Fig. 2B).

The influence of $1,25(OH)_2D$ on PKCα activity was assessed in MPEC3. Although the PKCα agonist, TPA, stimulated a sixfold increase in membrane associated PKCα in MPEC-3 cells after 15 min. $1,25(OH)_2D$ treatment had not impact on PKCα activation at any time point examined (Fig. 3).

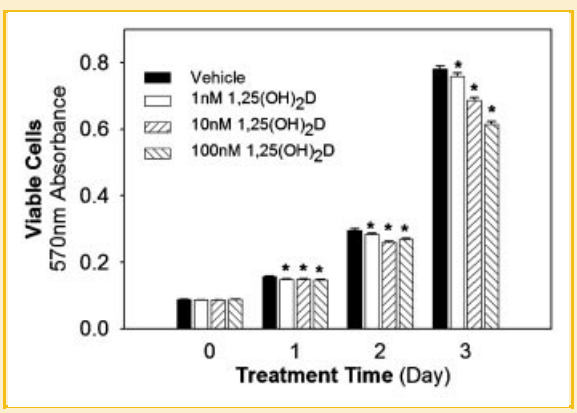

Fig. 1. $1,25(OH)_2D$ regulation of MPEC3 growth. MPEC3 were treated with $1,25(OH)_2D$ (D) or vehicle (V) with indicated doses and times. MTT cell growth assay was performed to assess cell viability. Values represent the means of n = six samples ± SE. * Indicates statistically significant difference compared to vehicle control at $P < 0.05$.

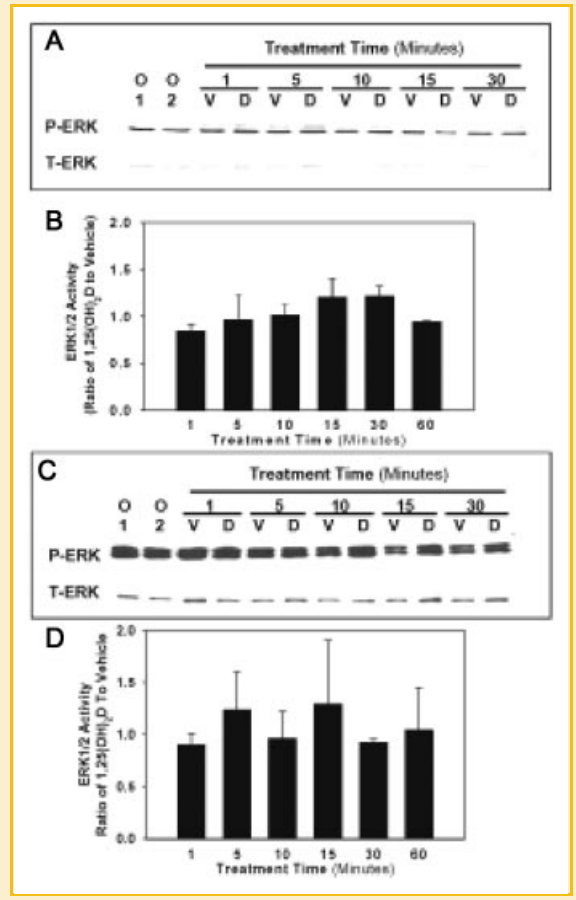

Fig. 2. $1,25(OH)_2D$ regulation of ERK1/2 activity in MPEC3. ERK1/2 activity was assessed with phosphospecific and total antibodies. Cells were treated with 100 nM $1,25(OH)_2D$ (D) or vehicle (V) for indicated time points. $1,25(OH)_2D$ was added in serum-containing medium (A,B) or serum-free medium (C,D). An untreated control (O), which was not manipulated until harvest, was loaded in duplicate on each gel. A representative immunoblot of ERK1/2 activity in serum-containing medium (A) and serum-free medium (C) are shown. The quantification of the blots in serum-containing are shown in B and serum-free medium in D. ERK1/2 activity was calculated from the density of bands of phospho-ERK1/2 divided by total-ERK1/2, both of which were corrected against no treatment control (O). Data are expressed as a ratio of ERK1/2 activity of treated samples to vehicle controls. Values represent mean ± SE, and n = four. There were no significant differences between $1,25(OH)_2D$ and vehicle treated ERK1/2 activity.

DISCUSSION

Our data show that $1,25(OH)_2D$ causes significant growth arrest in a proliferating prostate progenitor cell line but it does so without the need to activate either ERK1/2 or PKCα signaling. Therefore, $1,25(OH)_2D$-induced growth arrest does not depend on rapid activation of these signaling pathways in these cells.

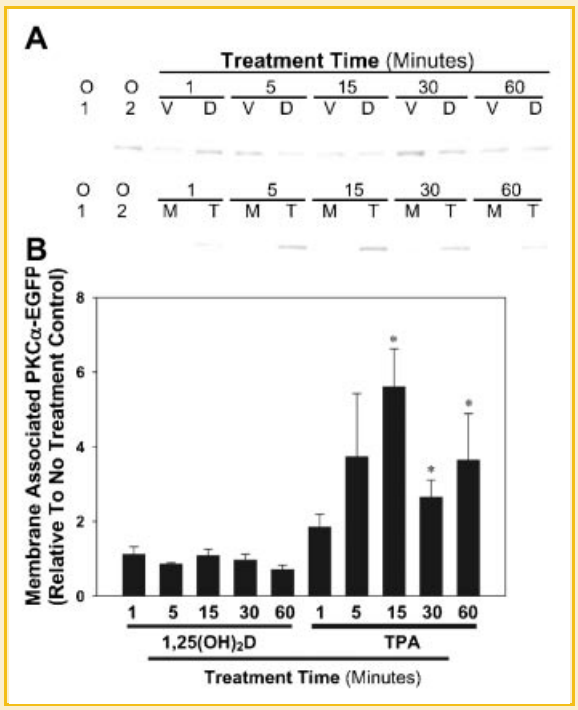

Fig. 3. $1,25(OH)_2D$ regulation of PKCα activity in MPEC3. MPEC3 were transiently transfected with EGFP-PKCα vector, followed by treatment with vehicle (V), $1,25(OH)_2D$ (D) (100 nM), TPA (T) (1 μM, positive control), or DMSO (M) (vehicle for TPA) for time points indicated. Cell membranes were isolated immediately after harvest and used for immunoblot with anti-GFP antibody. A: A representative immunoblot showing membrane associated PKCα at indicated time points after $1,25(OH)_2D$ or TPA treatment. The quantification of the blots is shown in B. Data are expressed as ratio of treated samples to vehicle controls. Values represent the means of n = four for each treatment group ± SE. * Indicates significant ($P \leq 0.05$) difference between TPA and vehicle (M) treatment. There were no significant differences in PKCα activity between $1,25(OH)_2D$ and vehicle treated cells.

ERK1/2 has been shown to be rapidly activated by $1,25(OH)_2D$ in a variety of cell types, for example, primary cultures of myocytes [Beno et al., 1995; Buitrago et al., 2001a,b], chondrocytes [Beno et al., 1995; Schwartz et al., 2002], osteosarcoma cells [Wu et al., 2007], hepatic ito cells [Beno et al., 1995], VDR+ and VDR− breast cancer cells [Cordes et al., 2006], and promyelocytic NB4 leukemia cell line [Song et al., 1998] in either serum containing or serum free medium. With only one exception, where ERK1/2 activity was rapidly suppressed by $1,25(OH)_2D$ treatment in the MCF7 breast cancer cell line [Capiati et al., 2004], the majority of published research has shown $1,25(OH)_2D$ treatment causes a rapid increase in either or both of ERK isoforms. ERK1/2 activation by has been linked with $1,25(OH)_2D$-mediated alteration of cell behavior, such as cell growth and differentiation [Marcinkowska et al., 1997; Chen et al., 1999; Galbiati et al., 2002; Capiati et al., 2004]. For example, rapid (3 min) and transient activation of ERK1/2 is evident in human colonic carcinoma cell line, CaCo-2 cells, treated with $1,25(OH)_2D$ (100 nM) in serum-containing medium [Chen et al., 1999]. Consistent with a role for ERK1/2 in $1,25(OH)_2D$-mediated alterations in cell behavior, a specific inhibitor of the upstream kinase of ERK1/2 (PD 098059) completely blocked $1,25(OH)_2D$ induced activation of activator protein-1 (AP-1), which induces the differentiation of CaCo-2 [Chen et al., 1999]. Contrary to previous reports our data do not support a link between ERK1/2 activation and $1,25(OH)_2D$ mediated growth arrest in the prostate epithelial progenitor cell.

PKCα is another kinase that has been shown to be activated rapidly by $1,25(OH)_2D$ treatment in a variety of cell types [Balogh et al., 2000; Capiati et al., 2000; Rivera-Bermudez et al., 2002; Boyan et al., 2003; Schwartz et al., 2003; Wali et al., 2003]. In contrast to these reports showing rapid activation of PKCα by $1,25(OH)_2D$, our study did not show this effect in prostate epithelial progenitor cells. These indicates that $1,25(OH)_2D$-mediated activation of PKCα is not required for growth suppression in normal prostate epithelial progenitor cells.

It is not clear why $1,25(OH)_2D$ treatment does not stimulate ERK or PKC signaling in the prostate epithelial cell progenitor. Several possible explanations exist. First, there may be cell type specific differences in the expression of MARRS, an alternate membrane associated $1,25(OH)_2D$ receptor that has been shown to mediate rapid responses of $1,25(OH)_2D$ [Khanal and Nemere, 2007]. In addition, while Huhtakangas et al. (2004) found that the traditional VDR may mediate membrane signaling pathways by associating with caveolae [Huhtakangas et al., 2004], not all tissues have high caveolae-associated VDR levels (e.g., kidney = liver > heart ⋙ lung = duodenum). It is not clear whether VDR exists in the caveolae of prostate epithelial progenitor cells. Future studies will need to be conducted to determine whether alterations in the cellular status or location of the traditional VDR or the MARRS protein exist in prostate epithelial progenitor cells.

Lack of rapid activation of ERK1/2 and PKCα indirectly strengthens the importance of the traditional, genomic signaling pathway of $1,25(OH)_2D$ as a mediator of growth arrest. Activation of VDR by $1,25(OH)_2D$ leads to the induction of transcription. A number of genes which may mediate the anti-proliferative activity of $1,25(OH)_2D$ have been shown, including insulin-like growth factor binding protein (IGFBP)-3 [Stewart and Weigel, 2005], cell cycle cyclin-dependent kinase inhibitory protein (CDKIs), p21 [Liu et al., 1996; Zhuang and Burnstein, 1998; Rao et al., 2004; Saramaki et al., 2006]. Based on our results, we believe that future studies should focus on identifying direct VDR target genes that are crucial for vitamin D-mediated growth arrest.

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