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Membrane Initiated Effects of 1α,25-Dihydroxyvitamin D₃ in Prostate Cancer Cells: Effects on AP1 and CREB Mediated Transcription

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1. Introduction

The biologically active form of vitamin D₃, 1α,25-dihydroxyvitamin D₃ [1α,25(OH)₂D₃], is formed through a multistep process in the liver and the kidneys, initiated in the skin by solar UVB radiation. Vitamin D compounds are transported in the body by vitamin D binding protein (DBP) to either nuclear vitamin D receptors (nVDR) or putative membrane associated vitamin D receptors (mVDR) where it exerts its biological responses in target organs by nuclear- and membrane-initiated signaling pathways (Bouillon et al., 1995; Holmén et al., 2009). Finally, 1α,25(OH)₂D₃ becomes inactivated by 24-hydroxylase which transforms it into 1,24,25(OH)₃D₃, a substance with much lower affinity for VDR (Bouillon et al., 1995).

In the nuclear initiated signaling pathway, occupancy of the nuclear vitamin D receptor (nVDR) by 1α,25(OH)₂D₃ leads to modulation of gene transcription of hormone-sensitive genes (Krishnan & Feldman, 2010). In conformity with several other receptors of the nuclear steroid/thyroid superfamily, nVDR forms heterodimers with retinoid X receptor (RXR) (Sutton et al., 2003). Subsequent interaction with the vitamin D response element (VDRE) in the promoter sequence of target genes initiates induction or repression of transcription, hence generating a biological response (Haussler et al., 2011).

Vitamin D exerts multiple actions in the organism including the well-known regulation of calcium and phosphate homeostasis (Holick, 2006), but it also possesses anti-proliferative, pro-differential and pro-apoptotic actions in cancer cells as well as increasing the effect of a number of established anti-cancer drugs (Trump et al., 2010).

The association of vitamin D with human cancer is well described in adenocarcinoma of the prostate gland, i.e. prostate cancer (PCa). Clear correlation between vitamin D deficiency and risk factors for PCa, such as high age and darker pigmented skin, has been observed. Thus, the amount of vitamin D decline with age, and the elevated levels of melanin in African Americans partly inhibits sun initiated vitamin D synthesize (Hsing & Chokkaligam, 2006).
Furthermore, 1α,25(OH)₂D₃ has been proven to decrease the risk of PCa by controlling prostate cell proliferation (Holick, 2006).

In a previous paper, we have shown that 1α,25(OH)₂D₃ regulates prostate cell differentiation, apoptosis and proliferation via multiple pathways, which involves both nuclear and membrane receptors found in the JNK/SAPK (c-Jun N-terminal kinases/stress-activated protein kinase) pathway. (Hagberg et al., 2008; Larsson et al., 2008; Holmén et al., 2009; Karlsson et al., 2010).

The JNK/SAPK pathway may be induced by several different means, such as chemical and physical stress, UV-radiation and osmotic shock, as well as pro-inflammatory cytokines, and even G-coupled receptor signaling, (Matsukawa et al., 2004). Among the cytokines that triggers the JNK/SAPK pathway, TNF-α is predominant, and it is also known to regulate cellular events associated with cancer cell phenotype, such as apoptosis, cell proliferation and differentiation. Still, it has been shown that cancer cells are resistant to apoptosis induced by TNF-α (Chopra et al., 2004). This resistance seems to involve certain survival signals, one of which being the transcription factor, nuclear factor-kappa B (NF-kB). It is therefore thought that if NF-kB is inhibited, the cancer cells would become more sensitive to TNF-α induced apoptosis. Indeed, this seemed to be the case in TNF-α resistant leukemia cells that were treated with sulforaphane, a putative anti-cancer drug that showed a non-specific inhibition of the TNF-α induced NF-kB activation (Moon et al., 2009). In addition, there were indications that this inhibition of NF-kB lead to prolonged JNK/SAPK activation in the leukemia cells. Other studies indicate that activation of JNK also seems to regulate and can be regulated by NF-κB (Nachmias et al., 2004). A similar result to the sulforaphane inhibition of the TNF-α induced NF-kB has been reported for breast cancer cells treated with vitamin D. Thus, Michigan cancer foundation 7 (MCF-7) breast cancer cells, were found to become sensitized to apoptosis induced by TNF-α when treated with 1α,25(OH)₂D₃ (Golovko et al., 2005).

Transcription factor NF-kB can be regulated in concert with another transcription factor, activator protein 1 (AP1). AP1 is a complex consisting of homodimers and heterodimers of the jun and fos families and the activity of AP1 seems to be regulated by differential expression of the jun and fos families. The c-Jun components of AP1 can be regulated by the phosphorylating activity of active JNK (Dedieu and Lefebvre, 2006). Thus, in the two prostate carcinoma cell lines PC-3 and LNCaP, overexpression of the early growth response protein EGR-1, selectively increased the activity of both NF-kB and AP1 and the activation of these transcription factors appeared to be essential for the induction of proliferation and anchorage independence (Parra et al., 2011).

Another important mediator of cell proliferation, differentiation and apoptosis is the cyclic response element binding protein (CREB). CREB is part of the cAMP regulated pathway and is phosphorylated by protein kinase A (PKA). CREB does not have direct contact with the transcriptional machinery. Therefore it requires CREB binding protein (CBP) to achieve transcriptional activation. There are several steroid and thyroid hormones that act to bind CBP e.g. luteinating hormone, glucagon and adrenaline which all exert influence over cAMP and PKA. There are no direct evidences that vitamin D receptors are coupled to a protein complex which includes adenylate cyclase. However, several studies have demonstrated that 1α,25(OH)₂D₃ evoke rapid increases in PKA activity, intracellular cAMP concentrations which have been found to be associated with G-protein-coupled signaling as well as regulation of Ca²⁺ transport through Ca²⁺-channels (Massheimer et al., 1999; Schwartz et al., 2002; Dirks-Naylor & Lennon-Edwards, 2011).
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Knowing how the cell cycle arrest and the anti-proliferative effects are induced on a molecular level is important when developing successful therapeutic tools against cancer. 1α,25(OH)₂D₃ stands out as a potential anti-cancer drug, even with its severe side effect of hypercalcemia, and treating LNCaP cells with 1α,25(OH)₂D₃ results in an accumulation of cells in the G1 phase, growth arrest, and to some extent apoptosis. In order to get a better understanding of how this kind of action of 1α,25(OH)₂D₃ is regulated we have in this study made the following investigations:

First, monitor the response on JNK/SAPK complex dependent activation of AP1 to 1α,25(OH)₂D₃ and TNF-α in LNCaP prostate cancer cells. Secondly, decide whether 1α,25(OH)₂D₃ regulates TNF-α production and release by LNCaP cells and thus have an indirect effect via the TNF-α signaling pathway on cell growth, differentiation and apoptosis, and third, evaluate the PKA dependent activation of cyclic response element binding protein (CREB) in LNCaP cells treated with 1α,25(OH)₂D₃.

2. Materials and methods

2.1 Cell culture

LNCaP cells were cultured in Gibco RPMI 1640 media (Invitrogen, UK). The media contained FBS (10%), PEST (1%), L-glutamine (1%), HEPES and sodium pyruvate (1%). The cells were subcultured five days after the initial culture and seeded at a density of 20 000 cells/well onto a 96-well plate (Nunc, Thermo Fischer Scientific, US) or 50 000 cells/well at a 24 well plate (Nunc, Thermo Fisher Scientific). At the point of seeding to the plate, the growth media was substituted for Opti-MEM (Invitrogen, UK) with 5% FBS and 1% NEAA without phenol-red-free and without antibiotics to prepare for transfection. The cells were incubated at 37°C and 5% CO₂ for 48 hour prior 1α,25(OH)₂D₃ treatment.

2.2 Transient transfection and CREB reporter assay (cAMP/PKA)/AP1 reporter assay

SureFECT™ transfection reagent and Cignal™ CREB reporter/Cignal™ AP1 reporter kit was used according to the manufacturer’s protocol (SA BioSciences, USA) to monitor cAMP/PKA pathway activity and the activity of API-regulated transduction pathways. The CREB reporter is a viral vector based on the Cytomegalo virus (CMV) that has been rendered replication incompetent and robbed of all virulence factors. It consist of inducible firefly luciferase that response to CREB and constitutively expressed Renilla constructs. The reporter is designed to monitor cAMP/PKA pathway activity and together with a Dual Glo™ Luciferase Assay System (Promega, USA), it provides an easy approach to study the activity of this pathway. The API reporter contains a mixture of inducible API-responding firefly luciferase construct and a constitutively expressing Renilla luciferase construct. The luciferase construct codes for the firefly luciferase reporter gene which is under the control of a minimal cytomegalovirus (mCMV) promoter and tandem repeats of the TPA response element. The Renilla construct codes for the Renilla luciferase reporter gene, which acts under control of a CMV. It is used as a control for normalizing transfection efficiency and for monitoring cell viability.

Briefly, in both assays, the LNCaP cells were transfected using SureENTRY transfection reagent in a 96-well plate. The LNCaP cells were seeded at the time of transfection. The medium used for the transfection was Opti-MEM serum-free culture medium. Dilutions of
the AP1/CREB reporter and the positive and negative controls were prepared as well as dilutions for SureENTRY. The cells were then washed with PBS and trypsinised and then suspended in Opti-MEM serum-free cell culture medium. The cell pellet was the resuspended in Opti-MEM cell culture medium. A haemocytometer was used to determine cell density and 10 000 cells was then seeded into each well. The Cignal reporter, negative and positive control was added to the appropriate wells and SureENTRY was added to each well and incubated for 48 hours. The cells were then treated with 1\(\alpha\),25(OH)\(_2\)D\(_3\) and luminescence measurements were made for selected interval.

2.3 1\(\alpha\),25(OH)\(_2\)D\(_3\) and G-protein coupled PKA/CREB-dependent gene expression in LNCaP cells

Following incubation, cells transfected with the CREB reporter, were treated with 1\(\alpha\),25(OH)\(_2\)D\(_3\) in the concentrations 10\(^{-7}\) M with or without the G-protein inhibitor Guanosine 5’-[\(\beta\)-thio]diphosphate trilithium (GDP-\(\beta\)-S; 100 \(\mu\)M; Sigma-Aldrich). Ethanol (0.001%) was used as control.

2.4 Luminescence measurements

Luminescence levels were measured at four time points, 24, 48, 72 and 96h in a luminometer (FLUOstar Galaxy, BMG Labtech, Germany) using the Dual Glo\(^{TM}\) Luciferase Assay System (Promega, US). At the first interval, Dual Glo Reagent\(^{TM}\) was prepared by mixing Dual Glo Substrate\(^{TM}\) with Dual Glo Buffer\(^{TM}\) at a ratio of 1:1. Stop & Glo Reagent\(^{TM}\) was prepared by mixing Stop & Glo Substrate\(^{TM}\) with Stop & Glo Buffer\(^{TM}\), at a ratio of 1:100 at each time interval, before measuring activity. At each measuring interval, 75 \(\mu\)l of Dual Glo\(^{TM}\) was added to the examined wells to check inducible activity, according to manufacturer’s recommendations and were incubated at room temperature for 12-15 minutes. Following the Firefly luciferase reading, 75 \(\mu\)l of Stop & Glo Reagent\(^{TM}\) was added to check the non-inducible luciferase activity. As with Dual Glo Reagent\(^{TM}\), the wells were incubated for 12-15 minutes. The principle is that Firefly luciferase (Dual Glo Reagent\(^{TM}\)) is inducible, while Renilla luciferase (Stop & Glo Reagent\(^{TM}\)) is not. This provides a reference point to compare and normalize obtained data.

2.5 Effects of 1\(\alpha\),25(OH)\(_2\)D\(_3\) and TNF-\(\alpha\) on AP1-dependent gene expression in LNCaP cells

The cells were treated with 1\(\alpha\),25(OH)\(_2\)D\(_3\) (10\(^{-7}\) M) or TNF-\(\alpha\) (10\(^{-9}\) M) with or without 20 \(\mu\)M SP600125 (JNK/SAPK inhibitor (0.001% of ethanol was used as a control)). Luminescence measurements were taken after 24, 48, 72 and 96h. The experiments were repeated in triplicates.

2.6 Effects of 1\(\alpha\),25(OH)\(_2\)D\(_3\) on TNF-\(\alpha\) production in LNCaP cells

Each well of a 24 well plate was seeded with 50 000 LNCaP cells. The cells were then treated with 10\(^{-7}\) M 1\(\alpha\),25(OH)\(_2\)D\(_3\) and 20 \(\mu\)M of the JNK/SAPK inhibitor, SP600125. As a control, 0.001% of ethanol was used. TNF-\(\alpha\) production in the cell culture media was measured post 72 and 96 hours treatment using a commercial TNF-\(\alpha\) specific ELISAs according to the manufacturer’s instructions (Promega, USA). Each experiment was repeated three times.
2.7 Statistics

Two-way ANOVAs were performed using GraphPad Prism to evaluate the data from the assays. For the AP1 reporter assay, triplicates were used for all four time points and all treatments except for the ethanol controls, in which case duplicates were used for all time points. For the TNF-α specific ELISA four replicates were used for all the samples and duplicates for the standards, control and blank wells. The P value threshold was set to 0.05.

3. Results and discussion

Previous studies on prostate cancer cells have reported that 1α,25(OH)2D3 regulates proliferation and cell survival through membrane initiated signaling pathways (Hagberg et al., 2008; Larsson et al., 2008). Similar observations have been made in vitamin D responsive tissues, where membrane initiated signaling have been linked to PKA, PKC and MAPK signaling pathways (Schwartz et al., 2002; Dirks-Naylor and Lennon-Edwards, 2011). This study was aimed to further elucidate membrane initiated signaling by 1α,25(OH)2D3 in LNCaP prostate cancer cells by evaluating the effects of 1α,25(OH)2D3 on AP1 and CREB-dependent gene expression as well as testing the hypothesis that 1α,25(OH)2D3 might evoke membrane initiated effects through TNF-α and TNF-α initiated signaling pathways.

3.1 Effects of 1α,25(OH)2D3 on AP1-dependent gene expression

As shown in Figure 1, 1α,25(OH)2D3 increased AP1-dependent gene expression after treatment at 48 (p<0.05), 72 (p<0.001) and 96h (p<0.0001) compared to control treated LNCaP cells (0.001% ethanol). The effect was time-dependent and at 96h, a difference in AP1-dependent gene expression was observed between the cells treated with 1α,25(OH)2D3 and cells treated with 1α,25(OH)2D3 + SP600125 (JNK/SAPK inhibitor)(p<0.05). Thus, 1α,25(OH)2D3 increase AP1-dependent gene expression through the JNK/SAPK signaling pathway in LNCaP prostate cancer cells.

Fig. 1. Effects of 1α,25(OH)2D3 (10−7 M) on AP1-dependent gene expression.
AP1-dependent gene expression was determined in LNCaP prostate cancer cells by a AP1 reporter assay (SA Biosciences) by measuring Firefly luciferase activity relative to the Renilla luciferase activity after treatment with 1α,25(OH)₂D₃ (10⁻² M) with or without SP600125 (20 µM), using ethanol as control. LNCaP cell were treated for 24, 48, 72 or 96h before measuring the AP1 activity. Data were normalised and are expressed as % of control. The level of significance was set to p<0.05. Data are presented as mean ± SEM.

The data from this study supports previous observations in prostate cancer cells where 1α,25(OH)₂D₃ decrease cell proliferation (Larsson et al., 2008) and where at least a part of the decreased proliferation has been connected an increase in the phosphorylation of JNK/SAPK and c-jun (Larsson et al., 2008; Hagberg et al., 2008; Karlsson et al., 2010). Effects on MAPK signaling pathways by 1α,25(OH)₂D₃ is not limited to prostate cancer cells. In human myeloid leukemia HL-60 cells, Kim et al. (2007) 1α,25(OH)₂D₃ induced HL-60 cell differentiation through a pathway involved with PI3-K/FK/C/ERK/JNK and in human osteosarcoma SaOS-2 cells (Wu et al., 2007), 1α,25(OH)₂D₃ were reported to be involved in JNK/SAPK activation as well as ERK 1/2 MAPK signaling and that only sustained and not transient treatment with 1α,25(OH)₂D₃ induced AP1 activation.

### 3.2 Effects of TNF-α on AP1-dependent gene expression

TNF-α increased AP1-dependent gene expression after 72 (p<0.01) and 96h (p<0.05) compared to control treated LNCaP cells. The effect was not time-dependent and no differences in AP1-dependent gene expression was observed between the cells treated with TNF-α and cells treated with TNF-α + SP600125 (JNK/SAPK inhibitor) at any time-point. Thus, TNF-α increases AP1-dependent gene expression in LNCaP prostate cancer cells.

![Fig. 2. Effects of TNF-α (1 nM) on AP1-dependent gene expression.](image-url)
API1 activity. Data were normalised and are expressed as % of control. The level of significance was set to p<0.05. Data are presented as mean ± SEM.

The findings in the present study are in concert with reports from MIN6N8 pancreatic β-cells (Kim et al., 2005) and MCF7 breast cancer cells (Yin et al., 2009), where the JNK/SAPK signaling pathway was reported to increase API1 dependent gene expression. However, Yin et al. (2009) showed that the API1 transactivation activity had its peak after 3 hours but was still significantly elevated after 24h. In the present study, the response in an increased API1 activity came after 48 hours and persisted throughout the experiment. The difference in response reported in this study and by Yin et al. (2009) may be because of differences between breast cancer and prostate cancer cells but could also reflect that the concentration of TNF-α in experiments performed by Yin et al. (2009) could have been a limiting factor.

3.3 Effects of 1α,25(OH)2D3 on TNF-α production in LNCaP cells and TNF-α concentrations in culture media

The TNF-α specific ELISA showed that 1α,25(OH)2D3 did not affect the production of TNF-α in LNCaP cells and thus, does not have an effect on TNF-α signaling pathway by increased concentrations of the growth factor in the media. This suggest that TNF-α acts independently of 1α,25(OH)2D3 in activation of the JNK/SAPK signaling pathway. These results are consistent with the findings of Golovko et al. (2005), who reported that under physiological conditions, 1α,25(OH)2D3 does not affect the production of TNF-α, but that TNF-α mRNA expression was up-regulated by 1α,25(OH)2D3 as well as its analogue CB1093 in LNCaP and PC3 prostate cancer cells. Chopra et al. (2004) studied the role of TNF-α in regulation of growth and apoptosis in three different prostate cell lines: normal prostate epithelial cells, LNCaP cells and PC3 cells and could demonstrate that normal prostate epithelial cells and PC3 cells were resistant to growth arrest and apoptosis induced by TNF-α and LNCaP cells were highly sensitive to the growth factor. Thus, from the results in the present study as well as previous studies (Golovko et al., 2005; Chopra et al., 2004,) we conclude that 1α,25(OH)2D3 and TNF-α acts through independent pathways ending up in an up-regulation of API1-dependent gene expression.

3.4 Effects of 1α,25(OH)2D3 on CREB-dependent gene expression

1α,25(OH)2D3 increased CREB-dependent gene expression compared to control treated LNCaP cells (Figure 3). The effect was time- and G protein-dependent where treatment with 10^{-7} M 1α,25(OH)2D3 increased CREB-dependent gene expression compared to cells treated with 10^{-7} M 1α,25(OH)2D3 + GDP-β-s (G protein inhibitor) at 24 (p<0.05), 48h (p<0.0001) but were decreased compared to the G-protein inhibited cells at 72h (p<0.05). Thus, 1α,25(OH)2D3 increases CREB-dependent gene expression through a G protein-dependent PKA/CREB signaling pathway in LNCaP prostate cancer cells.

PKA/CREB-dependent gene expression was determined in LNCaP prostate cancer cells by a CREB reporter assay (SA Biosciences) by measuring Firefly luciferase activity relative to the Renilla luciferase activity after treatment with 1α,25(OH)2D3 (10^{-7} M) with or without GDP-β-S (100 µM), using ethanol as control. LNCaP cell were treated for 24, 48, 72 or 96h before measuring the CREB activity. Data were normalised and are expressed as % of control. The level of significance was set to p<0.05. Data are presented as mean ± SEM.
The fact that 1α,25(OH)₂D₃ both activate JNK/SAPK and PKA/CREB-dependent gene expression indicate that 1α,25(OH)₂D₃ exert effects through more than one pathway and mean that there might be more than one receptor that mediate the responses of this metabolite. Alternatively, the receptor could form different complexes that upon activation start unique signal cascades. An example of a similar observation is membrane initiated signaling in skeletal muscle, where six different signaling pathways have been described for 1α,25(OH)₂D₃ (Vasquez et al., 1996; Capiati et al., 2000; Dirks-Naylor and Lennon-Edwards, 2011). The point that there are two different vitamin D receptors (VDR, PDIα3) associated with the cell membrane (Holmen et al., 2009; Karlsson et al., 2010) and that PDIα3 has been suggested to form a trimer with at least three high affinity binding sites (Karlsson et al., 2010) make us to postulate that depending on the docking site of 1α,25(OH)₂D₃ to the receptor, the resulting change in three dimensional structure of the hormone-receptor complex, starts a subsequent signaling cascade. The response will thus be dependent on both time and space where both short-term and long-term effect will be important in regulating prostate cell biology.

3.5 Conclusions
In conclusion, our findings support previous reports and suggest that 1α,25(OH)₂D₃ regulate prostate cell biology via multiple pathways and targeting of specific pathways for 1α,25(OH)₂D₃ might provide more effective therapies compared to the vitamin D therapies currently clinically tested and may serve as a complementary treatment in patients with androgen independent prostate cancer.

3.6 Future directives
The nature of membrane initiated signaling as a response to 1α,25(OH)₂D₃ is not yet clarified. It has been debated over the last two decades and currently there are two major candidates to be a membrane associated receptor for 1α,25(OH)₂D₃ (VDR and PDIα3). Our laboratory have for several years studied membrane initiated signaling by 1α,25(OH)₂D₃, in silico and in vitro, to elucidate signaling pathways and its key components with the goal to
clarify the biological role of the pathways in regulating prostate cancer. Focus on future work will be to create specific antagonists and agonists (the pharmacopore approach) to the putative membrane associated receptors to clarify their functions in vitro and hopefully get molecules that have a high specificity to single receptor binding site.

4. References


