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Vitamin D and prostate cancer: The role of membrane initiated signaling pathways in prostate cancer progression☆

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ABSTRACT

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) has been demonstrated to mediate both genomic and non-genomic responses in prostate cancer (CaP) cells. Here, we give an overview of membrane initiated $1,25(OH)_2D_3$ signaling in prostate cancer cell progression. The presence of PDIA3 was investigated and homologous modeling of the putative PDIA3 receptor complex was conducted. Furthermore, the cellular distribution of nVDR was analyzed. We could show that both nVDR and PDIA3 are expressed in the prostate cancer cell lines investigated. The homologous modeling of PDIA3 showed that the receptor complex exists in a trimer formation, which suggests for allosteric activity. Our findings support previous reports and suggest that $1,25(OH)_2D_3$ is an important therapeutic agent in inhibiting prostate cancer progression. Furthermore, our data show that $1,25(OH)_2D_3$ regulate prostate cell biology via multiple pathways and targeting specific pathways for $1,25(OH)_2D_3$ might provide more effective therapies compared to the vitamin D therapies currently clinically tested.

1. Introduction

Prostate cancer (CaP) is one of the most common cancer types in men, and etiological factors such as age, ethnical and genetic background, diet and hormones are believed to be involved in both development and progression of the disease [1].

Androgens have been shown to be involved in the regulation of prostate growth and proliferation [2], but not all CaP patients have responded to inhibition of androgens [3]. Androgen independent prostate cancer (AIPC) is a very aggressive and metastatic form of CaP and does not correspond to any of today's therapies. However, the present assumption is that steroid hormones can activate mutated androgen receptors in prostate cancer cells and therefore affect cancer cell growth [4].

During the last 20 years, an increasing number of reports have demonstrated that 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) regulates CaP cell biology, through regulation of transcription and activation of key components governing cell growth, differentiation and apoptosis via the cytosolic/nuclear vitamin D receptor VDR [5,6]. Recently, studies suggest a newly discovered protein, membrane-associated rapid response steroid specific for $1,25(OH)_2D_3$ (called 1,25-MARRS or the correct gene and protein name protein disulfide isomerase family A, member 3 (PDIA3)), as a potential membrane receptor that binds $1,25(OH)_2D_3$ [7–9]. This protein has been hypothesized to function as a membrane-associated scaffold molecule, when bound to $1,25(OH)_2D_3$, the ligand–receptor complex mediates several rapid signal transductions [10]. However, other studies have demonstrated a localization of nVDR at the plasma membrane, and suggest that nVDR may be the mediator of the rapid membrane-associated effects [11–15].

Rapid effects of vitamin D include opening of ion channels, changed enzyme activities, or activation of signaling cascades, and was initially referred to as non-genomic response [9,12,13,16]. However, rapid increases in intracellular calcium concentrations and activation of second messengers, such as PKC, PKA, and MAP kinases, have effects on gene expression, and constitute novel long-term regulatory pathways for vitamin D. The origin of membrane initiated signaling caused by metabolites of the vitamin D endocrine system is debated and several putative receptors have been suggested (Annexin II, PDIA3, Catalase, and nVDR) [13,19–23].

The vitamin D receptor-mediated regulation of cell and organ physiology is complex. To fully understand the dynamics involved in this process, the kinetic and dynamic parameters integrated within bioinformatical and mathematical models need to be characterized [24]. By modeling ligand–receptor interactions (*in silico* docking), two distinct, but overlapping binding sites for the nVDR have been suggested [23]. To explain the initiations of the differ-

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ent signaling pathways, the potential differences in stabilities of cis- and trans-locked 1,25$(OH)_2D_3$ in binding to their respective receptor pocket should be studied.

In this paper, we present an overview of the ongoing vitamin D research at our lab with emphasis on membrane initiated vitamin D signaling in prostate cancer. The aim of the present work was also to investigate the presence and partially characterize the previously identified membrane receptor, PDIA3, in prostate cancer cells. Thus, expression on transcriptional and translational levels of PDIA3 was examined in LNCaP, PNT-2 and PC-3 cell lines and homologous modeling of the PDIA3 receptor complex was performed.

2. Materials and methods

2.1. In vitro cell culture conditions

LNCaP cancer cells (clone FGC Ecacc Cat. 89110211 Sigma–Aldrich), PC-3 cancer cells (ECACC Cat. 90112714 Sigma–Aldrich) and PNT-2 normal cells (ECACC Cat. 95012613 Sigma–Aldrich) were cultured in RPMI 1640 (Cat. 31870-025 Gibco Invitrogen) supplemented with 10% FBS and antibiotics at 37 °C in an atmosphere of 95% humidity and 5% CO_2.

2.2. RNA extraction

Total RNA were automatically extracted from the harvested cells of the different cell lines with a KingFisher mL Instrument (Thermo Electron Corporation, USA) according to the manufacturer's protocol (MagAttract Tissue Mini M48 Kit, Qiagen). RNA was quantified using a NanoDrop (NanoDrop technologies, USA).

2.3. Reverse transcription PCR

cDNA was generated from the total RNA using reverse transcription PCR kit from Qiagen (10× reaction buffer, 5 mM dNTP mix, 10 units/μl RNAse inhibitor, 4 units Omniscript reverse transcriptase), oligo(dT)-18 primers. In each reaction, 100 ng of total RNA was used. The reaction was performed during 60 min in 37 °C. Primer sets specific for *PDIA3* and *GADPH* were designed using the Primer3 program, the Center for Genome Research at the Whitehead Institute (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The primers were constructed to amplify across two or more different exons, thus ruling out DNA amplification (*PDIA3* exons 3–5, forward primer TTGATTGCACTGCCAACACT, reverse primer AGTTGCTGGCTGCTTTTAGG (expected product size 285 bp), *GADPH* forward primer CCACCCATGGCAAAATTCCATG, reverse primer TCTAGACGGAGGTCAGGTCCACC (expected product size 550 bp)). The PCR conditions was as follow: 10× PCR buffer, 5 mM dNTP mix, 25 mM magnesium, 5 U/μl Taq polymerase, 10 μM *PDIA3* primers and 3 μM *GADPH* primers using the following program: 1 min at 95 °C, 1 min at 59 °C and 30 s at 72 °C, this cycle was repeated 28 times.

2.4. Western blot analysis

LNCaP cells were harvested in a lysis buffer and an equal amount of cellular proteins was subjected to 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were subsequently transferred to PVDF membranes (Biorad) and the blots were probed with a primary anti-PDIA3 antibody (dilution 1:500) (Atlantis Antibodies, Sweden) followed by incubations by incubations with dilution 1:500 of alkaline phosphatase conjugated anti-rabbit IgG (Sigma). The bands of PDIA3 were visualized using the nitro blue tetra zolium/5 bromo-4cloro-3-indolyl-phosphatase reagent (BCIP) (Sigma).

2.5. Immunocytochemistry

The cells were fixated in −20 °C methanol for 3 s and subsequently preheated at 60 °C for 12–14 s. To block non-specific sites, the cells were incubated in 1% bovine serum albumin (BSA) diluted with PBS for 1 h at room temperature (RT). After four washes with PBS the cells were incubated with primary antibodies (VDR [1:50] Santa Cruz Biotechnology) for 1 h at RT. The cells were then washed four times with PBS and incubated with the secondary antibodies (Chicken anti-rabbit [1:300] Santa Cruz Biotechnology) for 45 min at RT. After four washes with PBS the cells were mounted in VectaShield, 14 μl/chamber (Vector Labs, USA). Leica microscope with excitations wavelength of 520–600 nm was used for analysis of the slides.

2.6. Homologous modeling of PDIA3

The PDIA3 protein (e.g. PDB id 3F8U) [25] consists of two structural domains; the N-terminus domain (SER25-SER367) and the C-terminus domain (ASN360-GLU493). To get a possible idea of the complex of PDIA3, we used a modeling procedure available in Modeller (9v5) [26]. The N-terminal domain as base for the ligand interaction of the complex was used, since a part of the complex formation of PDIA3 consists of a trimer of N-terminus domains interacting to form a complex, PDB id 2H8L [27]. To incorporate the remaining part of the N-terminus and the C-terminus domain, a model of the PDIA3 protein, PDB id 3F8U, was used. Several models were generated and the best model was selected based on stereochemistry PROCHECK [28] and minimized error according to Modeller's restraint profile.

3. Results and discussion

The expression analysis showed that PDIA3 was expressed in all three prostate cancer cell lines at both transcriptional and translational level (Fig. 1). The immunocytochemistry analysis revealed that nVDR is localized in the nucleus, and with a high abundance in the cytosol, where a part of the receptor population is present in a close vicinity to the plasma membrane (Fig. 2). These results are in concert with earlier findings [7–9,12,13,23] that have suggested PDIA3 and nVDR as membrane-associated receptors for 1,25$(OH)_2D_3$. We also performed homologous modeling of the complex of PDIA3, which predicted that PDIA3 exists as a trimer (Fig. 3). Ligand (1,25$(OH)_2D_3$)-receptor (PDIA3) modeling studies by our group have demonstrated that the receptor exhibit several putative ligand binding sites (Larsson et al., manuscript in preparation). Earlier investigations on 1,25$(OH)_2D_3$ binding to membrane fractions of intestinal epithelial cells [29–31], chondrocytes [32] and osteoblasts [32] have demonstrated positive cooperativity, which support the results from the homologous modeling of PDIA3. We have previously shown that 1,25$(OH)_2D_3$ regulates apoptosis, proliferation and invasiveness of CaP cells by more than one signaling pathway [33,34]. The metabolites act via the classic nVDR and a previously unknown membrane initiated signaling pathway, where the receptor protein has been described to be PDIA3. Thus, this novel membrane-associated signaling pathway is a potential therapeutic target for prostate cancer. New data from our lab (Larsson et al., manuscript in preparation) demonstrate that 1,25$(OH)_2D_3$ is more effective evoking apoptosis after inhibition of the SAPK/JNK pathway, suggesting a negative interaction by the PDIA3 signaling pathway on nVDR mediated apoptosis and propose that pro-apoptotic effects are mediated through the nVDR.

In conclusion, our findings support previous reports and suggests that 1,25$(OH)_2D_3$ is an important therapeutic agent in

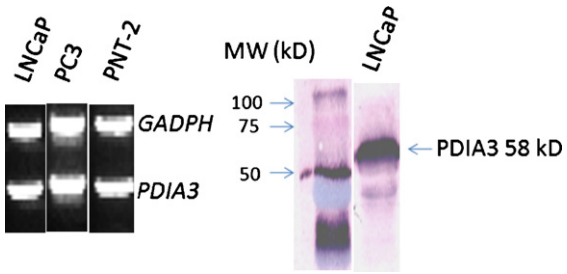

Fig. 1. Expression analysis using semi-quantitative RT-PCR and Western blot showed that PDIA3 is expressed in the LNCaP-, PC3 prostate cancer and PNT-2 cell lines. cDNA was generated from the total RNA using reverse transcription PCR kit from Qiagen. In each reaction, 100 ng of total RNA was used. The reaction was performed during 60 min in 37 °C. Primer sets specific for *PDIA3* and *GADPH* were designed using the Primer3 program, the Center for Genome Research at the Whitehead Institute (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The primers were constructed to amplify across two or more different exons, thus ruling out DNA amplification (*PDIA3* exon 3-5, forward primer TTGATTGCACTGCCAACACT, reverse primer AGTTGCTGGCTGCTTTTAGG (expected product size 285 bp), *GADPH* forward primer CCACCCATGGCAAAATTC-CATG, reverse primer TCTAGACGGAGGTCAGGTCCACC (expected product size 550 bp). The PCR conditions was as follow: 10× PCR buffer, 5 mM dNTP mix, 25 mM magnesium, 5 U/μl Taq polymerase, 10 μM *PDIA3* primers and 3 μM *GADPH* primers) using the following program: 1 min at 95 °C, 1 min at 59 °C and 30 s at 72 °C, this cycle was repeated 28 times. For Western analysis, LNCaP cells were harvested in a lysis buffer (RIPA) and an equal amount of cellular proteins was subjected to 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were subsequently transferred to PVDF membranes (Biorad) and the blots were probed with a primary anti-PDIA3 antibody (dilution 1:500) (Atlantis Antibodies, Sweden) followed by incubations by incubations with dilution 1:500 of alkaline phosphatase conjugated anti-rabbit IgG (Sigma). The bands of PDIA3 were visualized using the nitro blue tetra zolium/5 bromo-4cloro-3-indolyl-phosphatase reagent (BCIP) (Sigma).

decreasing prostate cancer progression. Furthermore, our data show that $1,25(OH)_2D_3$ regulate prostate cell biology via multiple pathways and targeting specific pathways for $1,25(OH)_2D_3$ might provide more effective therapies compared to the vitamin D therapies currently clinically tested and may serve as a complementary treatment in patients with AIPC.

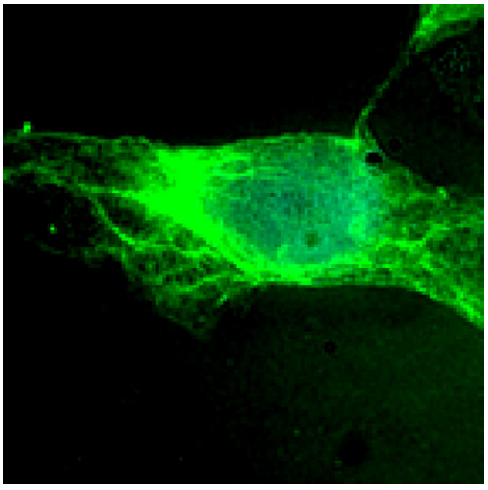

Fig. 2. Immunocytochemistry analysis of the classical vitamin D receptor (nVDR) in LNCaP prostate cancer cells. LNCaP prostate cells were seeded on Chamber Slides (Nunc, DK) at low density (20,000 cells/chamber) in 1 ml of serum depleted culture media for 48 h. In order to visualize and study the localization of nVDR, LNCaP cells were incubated with primary antibodies (VDR [1:50] Santa Cruz Biotechnology) for 1 h followed by incubation with secondary antibodies (Chicken anti-rabbit [1:300] Santa Cruz Biotechnology) for 45 min. The cells on slides were mounted in VectaShield, 14 μl/chamber (Vector Labs, USA). Leica microscope with excitations wavelength of 520–600 nm was used for analysis of the slides.

Fig. 3. A suggested model of the putative PDIA3 receptor complex. To get a possible idea of the complex of PDIA3, we used a modeling procedure available in Modeller (9v5). The N-terminal domain as base for the ligand interaction of the complex was used, since a part of the complex formation of PDIA3 consists of a trimer of N-terminus domains interacting to form a complex, PDB id 2H8L. To incorporate the remaining part of the N-terminus and the C-terminus domain, a model of the PDIA3 protein, PDB id 3F8U, was used. Several models were generated and the best model was selected based on stereochemistry PROCHECK and minimized error according to Modeller's restraint profile. The results from the homologous modeling of PDIA3 revealed a trimer complex (blue: chain A; green: chain B; red: chain C).

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