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Differential expression of prohibitin is correlated with dual action of vitamin D as a proliferative and antiproliferative hormone in breast epithelial cells

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Abstract

Our previous microarray analysis showed that N-methyl-N-nitrosourea transformed MCF12F breast epithelial cells exhibited upregulation of several genes, including prohibitin, which was reversed by 1α -hydroxyvitamin D₅ (1α (OH)D₅) treatment. The *in silico* screening for putative transcription factor binding sites identified two VDR/RXR binding sites in the 1 kb promoter region of prohibitin. Other binding sites for EGR and GR which are also vitamin D target genes were identified in this region, indicating that prohibitin is a potential target gene for vitamin D. The combination of multiple binding sites also provides a basis for a possible dual regulation of prohibitin by vitamin D. Prohibitin upregulation by 1α (OH)D₅ treatment at both transcription and translation level was observed in vitamin D sensitive BT474 breast cancer cells, in which 1α (OH)D₅ significantly inhibited cell proliferation in normal culture condition. On the other hand, prohibitin down-regulation accompanied with vitamin D mediated maintenance of proliferation of breast epithelial cells was observed under stressed condition. These results demonstrated that vitamin D mediated antiproliferative activity in unstressed condition and growth maintaining activity under stressed condition involve differential expression of prohibitin.

Keywords

Prohibitin; Vitamin D; breast cancer cells; proliferation

Keywords

Prohibitin; Vitamin D receptor; breast cancer cells; CYP24 promoter

1. Introduction

We have previously shown that a relatively noncalcemic analog of vitamin D, 1α -Hydroxy-24-ethyl-cholecalciferol (1α (OH)D₅) is a promising chemopreventive agent in experimental mammary carcinogenesis models [1]. Using microarray analysis we identified several genes that are differentially regulated in carcinogen transformed human breast epithelial cells as compared to the untransformed cells (MCF12F) and the expression of a few genes including prohibitin, TCTP1 and thioredoxin was reversed with 1α (OH)D₅ treatment in the transformed

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cells [1]. Since prohibitin has been reported to have interactions with the cell cycle checkpoint molecules including E2F [2], p53 [3] and pRB [2] as well as exhibit tumor suppressive and anti-proliferative effects, we selected prohibitin to determine if it has a functional significance in vitamin D action. Prohibitin is a highly conserved protein and is localized into the inner membrane of the mitochondria, where it has a role as a mitochondrial chaperon protein in a complex with BAP37 in the maintenance of mitochondrial function [4]. Moreover, it is also reported to be localized in the nuclei of breast cancer cells and mediating hormone response in prostate cancer and ovary granulosa cells [2]. Recent reports indicate that prohibitin is upregulated in tumor cells as compared to normal cells [5]. Our attempts to characterize the role of prohibitin in VDR function led to identification of prohibitin as a target gene of vitamin D, which is dually-regulated by vitamin D and involved in vitamin D mediated cellular responses.

2. Materials and Methods

2.1. Cell culture

BT474, MCF-7, human breast cancer cells, were cultured in MEM containing 10% FBS as described previously [6]. MCF12F cells were cultured in DMEM/F12 containing 5% horse serum, supplemented with 20 ng/ml EGF (invitrogen), 10 µg/ml insulin (sigma), 100 ng/ml cholera toxin (sigma) and 0.5 µg/ml hydrocortisone. To induce stress in MCF12F cells, culture medium was replaced with MEM containing 2% FBS. The vitamin D analog, 1α(OH)D₃ was synthesized and dissolved as described previously [1]. 1α,25(OH)₂D₃ was purchased from Sigma.

2.2. Cell proliferation assay

Cell proliferation was evaluated using MTT assay [6] and BrdU (bromodeoxyuridine) incorporation assay. For BrdU incorporation assay, 10 µM BrdU (Calbiochem, La Jolla, CA) was added to the medium at 2 h before harvesting cells. Cells were trypsinized, washed with PBS and fixed in 1% paraformaldehyde in PBS for 15 min, followed by incubation in PBS containing 0.2% Tween-20 for 30 min at 37°C. Cells were then incubated with mouse monoclonal anti-BrdU antibody (Calbiochem) overnight at 4°C, then washed twice, incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. After 3 washes with PBS, cells were subjected to FACS analysis, a total of 20,000 events were measured per sample.

2.3. RNA interference

10⁵ cells per well were seeded in 6 well plates in culture media containing 5% FBS and incubated overnight. SiRNA against PHB (siPHB) and non-silencing siRNA (siCon, Qiagen, cat no. 1022076) were transfected using the RNAiCarrier kit (Epoch Biolabs, Sugar Land, TX). Cells were first incubated with siRNA complex (SiRNA at 80 nM, SiRNA:RNAi Carrier=1:6) for 6 h in Opti-MEM (invitrogen) containing 2% FBS, then FBS was increased to 5%. After overnight incubation, cells were incubated in fresh culture medium containing 5%FBS for 2 days in the presence or absence of 1α,25(OH)₂D₃, cells were then subjected to BrdU incorporation assay. Prohibitin knock-down was verified at the same time using western blot [6]. siPHB oligonucleotide duplex (SiPHB sense: 5'-CCCAGAAAUCACUG-UGAAAdTdT-3', antisense: 5'-UUUCACAGUGAUU-UCUGGG-dTdT-3') were designed to target the 3'UTR region at base pair 974–993 of sequence NM_002634 and synthesized by IDT Inc (Coralville, IA). Negative control siRNA (non-silencing RNA, sense: 5'-UUCUCCGAACGUGUCA-CGUdTdT-3', antisense: 5'-ACGUGACACGUUCGAGAAAdTdT-3') has no homology to known mammalian genes.

3. Results

3.1. Prohibitin upregulation by $1\alpha(\text{OH})\text{D}_5$ correlated to its inhibition of proliferation in BT474 cells

Our earlier studies have shown that prohibitin could be involved in cellular response to $1\alpha(\text{OH})\text{D}_5$ in breast epithelial cells [1]. To further address this issue, we analyzed the promoter region of prohibitin to examine if potential VDR/RXR binding sites exist. *In silico* analysis identified two potential DNA binding sites for VDR/RXR in the 1 kb promoter region and multiple EGR and GR binding sites (Fig 1A). Both EGR and GR are vitamin D target genes [7,8, unpublished data]. A time-course analysis of prohibitin mRNA expression in BT474 cells demonstrated that prohibitin mRNA level increased by ~2 fold after 8h treatment with $1\alpha(\text{OH})\text{D}_5$ (0.5 μM) (data not shown). Prohibitin protein upregulation by 24h incubation with $1\alpha(\text{OH})\text{D}_5$ was confirmed by western blot in the same cell line (Fig 1B). Cell proliferation assay demonstrated that $1\alpha(\text{OH})\text{D}_5$ inhibited cell proliferation by 50% after 4 day treatment in BT474 cells in normal culture condition (Fig 1C). These data identified prohibitin as a vitamin D target gene and that prohibitin upregulation by $1\alpha(\text{OH})\text{D}_5$ correlates to its inhibition of cell proliferation in BT474 cells.

3.2. Prohibitin downregulation by $1\alpha,25(\text{OH})_2\text{D}_3$ and RNAi in stressed MCF-7 cells was accompanied by increased BrdU incorporation

Given that prohibitin is a vitamin D target gene and can potentially contribute to the antiproliferative action of vitamin D, we investigated the effect of knock-down of prohibitin by RNAi on cell proliferation in MCF-7 cells. Transfection of siPHB for 24h, followed by 48 h incubation with regular culture medium containing 5% FBS decreased prohibitin protein by more than 70 % as evaluated by western blot analysis (Fig. 2C). BrdU incorporation assay using FACS analysis demonstrated a significant increase (> 3 folds) in BrdU-labeled cells after prohibitin knock-down (Fig. 2B) in comparison to non-silencing control siRNA (siCon) transfected cell (Fig 2A). Surprisingly, contrary to our expectation, $1\alpha,25(\text{OH})_2\text{D}_3$ treatment in siCon transfected MCF-7 cells significantly decreased prohibitin level (Fig 2C, lane 2) and increased cell proliferation (Fig 2A), which was comparable to the effects of prohibitin knock-down by siRNA.

3.3. Dual effects of $1\alpha,25(\text{OH})_2\text{D}_3$ in MCF12F cells under normal and stressed condition correlate to prohibitin regulation

Considering the results from siRNA transfected cells and the stress caused by transfection itself and transfection condition (low serum), we hypothesized that prohibitin could be a stress-associated protein, while vitamin D could play growth supportive role in the stressed cells by inhibiting prohibitin expression. We used MCF12F cells to further address this issue. Since MCF12F cells are generally grown in nutrient medium supplemented with growth factor and hormones. Replacement of the nutrient medium with regular medium containing low serum induces significant stress to the cells. As shown in Fig 3A, in normal culture condition, 4 day treatment with 100 nM $1\alpha,25(\text{OH})_2\text{D}_3$ significantly inhibited cell proliferation by ~40%; while in stressed culture condition, $1\alpha,25(\text{OH})_2\text{D}_3$ stimulated cell proliferation by ~2 fold. The dual effect of $1\alpha,25(\text{OH})_2\text{D}_3$ was also reflected in prohibitin regulation (Fig. 3B). In normal culture condition, 48 h treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ upregulated prohibitin (after normalized to β -actin), while in stressed condition, $1\alpha,25(\text{OH})_2\text{D}_3$ inhibited prohibitin expression. The dual regulation of prohibitin by $1\alpha,25(\text{OH})_2\text{D}_3$ in different culture condition correlated to its dual effects on cell proliferation as diagramed in Fig. 3C.

4. Discussion

In the literature vitamin D is often reported to inhibit both proliferation and apoptosis in cancer cells [9,10]. However the detailed signaling pathway for such dual effects has been poorly understood. In this study, we identified prohibitin as a vitamin D target gene, which is differentially expressed in cellular responses to vitamin D in normal and stressed conditions.

Prohibitin is a highly conserved protein and localizes to many cellular compartments and might have distinct but overlapping function in each of these [2]. Its role as a tumor-suppressor protein, however, is still controversial [2]. Our data show that prohibitin is dually-regulated by vitamin D in breast epithelial cells depending on the culture condition. *In silico* analysis identified two potential VDR binding sites and multiple putative EGR and GR binding sites in the promoter region of prohibitin. EGR and GR are also involved in cell stress [11,12]. The combination of these binding sites in promoter region provides a basis for dual regulation of prohibitin by vitamin D. Initially, the siRNA experiments were designed to evaluate the function of prohibitin in relation to cell proliferation and not stress. However the downregulation of prohibitin by $1\alpha,25(\text{OH})_2\text{D}_3$ in siCon transfected MCF-7 cells suggested that prohibitin may be a molecule associated with stress. Chemical reagents used for transfection are generally toxic, causing significant stress to cells. In addition, transfection is generally introduced in serum-starved medium, which also induces stress to cells. Our studies with MCF-12F cells using different culture condition confirmed this hypothesis and provided evidence for both up- and down-regulation of prohibitin by active vitamin D. Consistent with our finding, prohibitin was previously identified as a protein responding to mitochondrial stress [4]. In addition prohibitin was shown to actively translocate from nuclei to cytoplasm when cells were stressed with camptothecin, a strong inducer of apoptosis [3].

There is overwhelming evidence demonstrating protective function of vitamin D in addition to its antiproliferative effects. Zhang et al [9] previously showed a dual effect of $1,25(\text{OH})_2\text{D}_3$ in ovarian cancer cells. Pretreatment of these cells with $1\alpha,25(\text{OH})_2\text{D}_3$ decreased apoptosis induced by TRAIL and Fas ligand, however the persistent $1\alpha,25(\text{OH})_2\text{D}_3$ treatment induced apoptosis in ovarian cancer. Similarly, Riachy et al [10] reported that $1\alpha,25(\text{OH})_2\text{D}_3$ protects human pancreatic islets against cytokine-induced apoptosis via downregulation of the fas receptor. Considering that many genes involved in stress such as EGR, GR and prohibitin are vitamin D target genes, vitamin D seems to have protective anti-stress function. Cells can undergo transformation under stress; with vitamin D protection, the stress-associated transformation can be significantly inhibited. This supports the notion for vitamin D as a possible chemopreventive agent.

In summary, the results presented here provide evidence for the possible mechanism by which vitamin D can function both in maintaining cell proliferation while the cells are undergoing stress as well as an antiproliferative hormone in breast epithelial cells. We show here for the first time that prohibitin is both a vitamin D target gene and a stress-associated molecule and is responsible for vitamin D mediated cellular functions in breast epithelial cells.

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VDR/RXR -1000 TAGCCGGGTGTGGTGGTGCATGCCTGTAATCTCAGCTATT
 -960 CTGGGAG**GGTG**AGGCAGGAGAATCGCTTGAACCCGGGAGGCGGAGGTTGCGGTG.....
 -840 AACAAACAAAACAAAACAAGAAAATAAACACACAC**ACGC**ACACGGAGGT**GGGC**GGCGG.
 -540 AGTTTTTCAGCACAGAT**GGTT**CTCTCTGGGTCTGGTAATCAACGTCTCACAAC.....
 -420 CCTGCCGGTGATTTACACGACTTCCTCCAGAGTTCCACCT**GTCCT**CTTCATCAGCCAT
 -360 GAACCTGCCCCCTACTAATTTTTGCCACCAGCTGGACGCCACCTCCTCTTTCCC.....
 -60 TTCCACACCAATAGGAAGCGTGGATCTTGGAAAG**CGGGCGGGGC**AGAGGAGCTCATGCGC
 +1 **A**GTATGTGTGGTTGGGGAATTCATGTGGAG

Figure 1.

Prohibitin is a target gene of vitamin D. (A), *In silico* analysis of potential VDR/RXR binding sites in the promoter region of prohibitin gene. The core sequence (in bold face) of the putative transcription binding sites for VDR/RXR, EGR and GR with high identity to the authentic core and matrix sequences as identified by MatInspector V2.2 and Promo are underlined.

Nucleotides are numbered negatively to the left of the sequence with nucleotide + 1 corresponding to the transcription start site. (B) and (C) Upregulation of prohibitin by vitamin D correlates with its inhibition of cell proliferation in BT474 cells. (B), Western blot analysis of cell lysates from BT474 cells treated with 0.5 μM 1α(OH)D₅ for 24h. 1α(OH)D₅ treatment significantly increased prohibitin expression. β-actin was used as an internal control. (C), MTT assay of BT474 cell proliferation after 4 day treatment with 1α(OH)D₅ (0.5 μM) and 1,25 (OH)₂D₃ (100 nM). Results are expressed as mean ± SEM of three independent experiments with 8 wells per treatment in each experiment. *** p<0.001 compared to control.

Figure 2.

Downregulation of prohibitin by 1α,25(OH)₂D₃ or siRNA in stressed MCF-7 cells correlates with enhanced proliferative activity. (A), cells were transfected with siCon and incubated in the medium containing low serum to cause stress and then treated with 100 nM 1α,25(OH)₂D₃ for 48h. (B), transfection of siPHB served as a control for prohibitin downregulation. Both 1α,25(OH)₂D₃ and siPHB transfection downregulated prohibitin under the experimental condition, which was accompanied by increased BrdU incorporation in MCF-7 cells. (A), representative FACS analysis of BrdU incorporation in siCon transfected MCF-7 cells with ETOH (left) or 1α,25(OH)₂D₃ (right) treatment. (B), BrdU incorporation analysis of siPHB transfected MCF-7. (C), Western blot analysis of prohibitin expression corresponding to above treatments; lane 1 siCon transfection with ETOH treatment; lane 2, siCon transfection with 1α,25(OH)₂D₃ treatment; lane 3, siPHB transfection without treatment.

Figure 3.

Prohibitin downregulation by 1 α ,25(OH)₂D₃ in stressed MCF12F cells correlates to enhanced proliferative activity. (A), dual regulation of cell proliferation by 1 α ,25(OH)₂D₃ (100 nM) in normal culture condition (left) and stressed condition (low serum without supplements) (right). Cells were treated for 4 days and subjected to MTT assay. Results are expressed as mean $\pm$ SEM, *** p<0.001 compared to control. (B), prohibitin regulation by 1 α ,25(OH)₂D₃ in normal culture condition and stressed condition (2%FBS). MCF12F cells were treated for 48h and subjected to western blot analysis; lane 1 and 3, ETOH (control); lane 2 and 4, 1 α ,25(OH)₂D₃. (C), Schematic model of dual regulation of prohibitin by active vitamin D analog (VD) in relation to its proliferation-regulatory effects. Under stressed condition, active vitamin D plays a protective role by downregulating prohibitin and maintaining cell proliferation; while in normal culture condition, vitamin D upregulates or fine-tunes prohibitin which is involved in its antiproliferative effect.