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MAPK inhibition by 1α,25(OH)$_2$–Vitamin D$_3$ in breast cancer cells. Evidence on the participation of the VDR and Src☆

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Abstract

1α,25-Dihydroxyvitamin D$_3$ [1α,25(OH)$_2$D$_3$], the hormonally active form of Vitamin D$_3$, has been shown to be a potent negative growth regulator of breast cancer cells both in vitro and in vivo. 1α,25(OH)$_2$D$_3$ acts through two different mechanisms. In addition to regulating gene transcription via its specific intracellular receptor (Vitamin D receptor, VDR), 1α,25(OH)$_2$D$_3$ induces, rapid, non-transcriptional responses involving activation of transmembrane signal transduction pathways. The mechanisms that mediate the antiproliferative effects of 1α,25(OH)$_2$D$_3$ in breast cancer cells are not fully understood. Particularly, there is no information about the early non-genomic signal transduction effectors modulated by the hormone. The present study shows that 1α,25(OH)$_2$D$_3$ rapidly inhibits serum induced activation of ERK-1 and ERK-2 MAP kinases. The non-receptor tyrosine kinase Src is involved in the pathway leading to activation of ERK 1/2 by serum. Furthermore, 1α,25(OH)$_2$D$_3$ increases the tyrosine-phosphorylated state of Src as well as it inhibits its kinase activity and induces the association of the VDR with Src. These data suggest that 1α,25(OH)$_2$D$_3$ inhibits MAPK by inactivating Src tyrosine kinase through a so far unknown mechanism that seems to be mediated by the VDR.
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Keywords: 1α,25(OH)$_2$D$_3$; Breast cancer cells; MAP kinase; Src; VDR

1. Introduction

1α,25-dihydroxyvitamin D$_3$ [1α,25(OH)$_2$D$_3$], the active form of Vitamin D$_3$, acts as a negative growth regulator of a wide array of cancer cells, including breast cancer cells [1]. 1α,25(OH)$_2$D$_3$ acts through two different mechanisms [2]. In addition to regulating gene transcription via its specific intracellular receptor, VDR [3], 1α,25(OH)$_2$D$_3$ induces rapid, non-transcriptional responses involving activation of transmembrane signal transduction pathways [4]. The mechanisms that mediate the antiproliferative effects of 1α,25(OH)$_2$D$_3$ in breast cancer cells are not fully understood. Particularly, there are no data concerning early non-genomic signal transduction effectors modulated by 1α,25(OH)$_2$D$_3$.

The ERK 1/2 (p42/p44 MAPK) members of the MAP kinases are involved in transduction of externally derived signals regulating cell growth [5], therefore, an unregulated activation of this cascade can result in oncogenesis [6]. It is well known that mitogenic factors contained in serum stimulate the ERK 1/2 pathway and thus promote proliferation [7]. A mechanism by which 1α,25(OH)$_2$D$_3$ may inhibit breast cancer cell growth could be through a blockade of the mitogenic effects of serum. Src, a non-receptor tyrosine kinase which may also be activated by serum, can trigger the Ras/Raf/MAPK cascades through the recruitment of the Shc–Grb2–Sos complex [8]. The activation of signal transduction pathways by 1α,25(OH)$_2$D$_3$ suggests the existence of a receptor that mediates this rapid action. It remains unclear whether the VDR, which mediates the genomic actions also triggers the signaling cascades or, alternatively, there is another 1α,25(OH)$_2$D$_3$-binding protein different from the VDR responsible for the non-genomic effects. Therefore, it was considered of interest to determine if the VDR is involved in the non-genomic effects of 1α,25(OH)$_2$D$_3$ in breast cancer cells.

Specifically, the aim of the present study was to characterize the rapid effects of 1α,25(OH)$_2$D$_3$ on MAP kinase activity in MCF-7 breast cancer cells and to investigate the

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involvement of Src and the VDR in the modulation of this key mitogenic signaling pathway.

2. Materials and methods

2.1. Chemicals

$1\alpha,25(OH)_2D_3$ was from Hoffmann-La Roche (Basel, Switzerland). Protein A- or G-Sepharose and Immobilon P membranes were from Sigma (St. Louis, MO, USA). RPMI-1640 medium was from Hyclone (Milan, Italy). Chemiluminescence reagents and $[\gamma^{32}P]$-ATP were from New England Nuclear (Chicago, IL, USA).

2.2. Cell culture

MCF-7 cells were from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in RPMI-1640 media plus 10% fetal bovine serum (FBS), penicillin and streptomycin. For the analysis of $1\alpha,25(OH)_2D_3$ actions, serum was removed and cells were incubated for 24 h in RPMI-1640 medium without phenol red plus 0.1% bovine seroalbumin.

2.3. Western blot analysis

Proteins were subjected to SDS–PAGE and transferred to Immobilon P membranes. Membrane probing with antibodies was performed according to antibodies manufacturers' instructions. Bands were visualized by chemiluminescence detection. In some cases, membranes were incubated in stripping buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 50 mM 2-mercaptoethanol) for 30 min at 55 °C and blotted again with a different antibody.

2.4. Immunoprecipitation

Cells were homogenized in immunoprecipitation buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1mM EDTA, 0.5% Nonidet P40, 1% Triton X-100, 1 mM PMSF, 8 μg/ml aprotinin, 6 μ/ml leupeptin, 1 mM Na_3VO_4, 1 mM NaF) and after clarifying by centrifugation, lysates were incubated with the corresponding antibodies (overnight at 4 °C), followed by precipitation with protein A-sepharose or protein G-sepharose (2 h at 4 °C). After washing, immunocomplexes were subjected to kinase activity assays or immunoblot analysis. Controls were performed using protein A- or G-sepharose alone in each experiment.

2.5. MAP kinase activity

After immunoprecipiation with anti-MAP kinase antibody, the immune complexes were incubated at 30 °C for 30 min in 35 μl of buffer containing 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM sodium orthovanadate, 0.5 mg/mL myelin basic protein as substrate, 40 μM ATP, and $[\gamma^{32}P]$ATP (2 μCi/assay). The reaction mix was spotted onto Whatman P-81 papers, washed with 75 mM H_3PO_4, and the radioactivity was measured.

2.6. Src kinase activity

After immunoprecipitation with anti-Src antibody, the immune complexes were incubated at 30 °C for 15 min in 35 μl of buffer containing 50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, 1 mM sodium orthovanadate, 0.15 mg/mL enolase as substrate for Src kinase, 40 μM ATP, and $[\gamma^{32}P]$ATP (5 μCi/assay). The reaction was stopped with sample buffer containing SDS. The samples were subjected to electrophoresis, transferred to PVDF membranes as described and imaged by autoradiography.

2.7. Co-immunoprecipitation

Co-immunoprecipitation was performed as described for *immunoprecipitation*, but cells were homogenized under native conditions to preserve protein–protein associations in 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 3 mM KCl, 0.5 mM EDTA, 1% Tween 20, containing protease inhibitors (1 mM PMSF, 6 μg/ml leupeptin, 8 μg/ml aprotinin) and phosphatase inhibitors (1 mM sodium fluoride, 1 mM sodium orthovanadate).

3. Results

To establish whether $1\alpha,25(OH)_2D_3$ inhibits ERK 1/2 MAP kinase, MCF-7 cells were treated with 20% FBS or 20% FBS plus different doses of $1\alpha,25(OH)_2D_3$ (1-100 nM) for 3 min. The hormone decreased the amounts of the phosphorylated (and thereby active) form of ERK1 and ERK2 MAP kinases, without affecting the total quantity of the enzymes, in a dose-dependent manner (Fig. 1). These results were confirmed determining MAP kinase activity. The hormone (1 nM) decreased serum-induced MAPK activity after 3 min treatment ($-46.15 \pm 3.79\%$; $P < 0.05$, $n = 3$). Thus, the hormone rapidly opposes serum-induced activation of MAP kinase, using a concentration range at which $1\alpha,25(OH)_2D_3$ has been shown to cause growth inhibition of breast cancer cells both in vitro and in vivo [1]. To our knowledge, this are the first data demonstrating that the hormone antagonizes the mitogenic action of serum, or the growth factors therein, on the MAPK cascade of tumorigenic breast cells.

As Src is an upstream positive modulator of ERK 1/2 [8], the participation of Src in serum-induced MAPK activation was investigated. Pretreatment of MCF-7 cells with Src inhibitors PP1 or PP2 decreased serum-induced MAPK activation (Fig. 2), confirming this hypothesis.

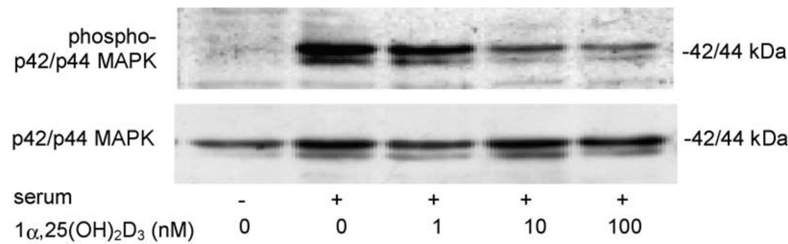

Fig. 1. $1\alpha,25(OH)_2D_3$ inhibits serum-induced MAPK activation in MCF-7 cells. MCF-7 cells were treated with serum (20% FBS) plus vehicle (isopropanol <0.1%) or serum plus different doses of $1\alpha,25(OH)_2D_3$ (1-100 nM) for 3 min. Controls were treated with vehicle alone. Cells were homogenized and subjected to Western blot analysis using anti-(phospho)-active p42/p44 MAPK antibody followed by reblotting with anti-p42/p44MAPK antibody (Promega, Madison, WI).

Then, experiments were carried out to determine whether $1\alpha,25(OH)_2D_3$ inhibits Src activity. Phosphorylation of Tyr-527 residue of Src negatively modulates its activity [9]. Once activated, autophosphorylation in Tyr-416 is required to achieve maximal stimulation [10]. Cells were treated with 20% FBS or 20% FBS plus $1\alpha,25(OH)_2D_3$ (10 nM) for 3 min. As illustrated in Fig. 3A, the hormone increased tyrosine phosphorylation of Src as evidenced by immunoprecipitation of lysates with anti-Src antibody followed by blotting with anti P-tyrosine and vice versa (the latter not shown). To investigate whether this increase in Src tyrosine phosphorylation state correlates with a decrease in its activity, Src activity was assayed after immunoprecipitation of Src. As expected, two bands were observed, one corresponding to P-enolase and another corresponding to phosphorylated Src. $1\alpha,25(OH)_2D_3$ decreased Src kinase activity (Fig. 3B). MCF-7 cells exhibit elevated basal Src activity [11]; this may be related to high levels of phosphorylation at Tyr-416. This could account for the fact that the stimulatory effects of serum on Src phosphorylation and activity are not very pronounced. The $1\alpha,25(OH)_2D_3$-induced increase in tyrosine phosphorylation may be ascribed to Tyr-527 which should prevail over that of Tyr-416 explaining thereby the decrease of Src activity. As Src activity is lowered by the hormone, a lesser extent of autophosphorylation of Tyr-416 may be the reason of the decreased phosphorylation of Src in the enzymatic assays. Taken together, these results suggest that the hormone exerts its inhibitory effect on MAPK, at least in part, at the level of Src.

The exact way by which $1\alpha,25(OH)_2D_3$ modulates the MAP kinase pathway in MCF-7 cells remains unknown. It is possible that the VDR could be involved by interacting with Src. The human VDR sequence contains a putative tyrosine phosphorylation site corresponding to aminoacids 141–147 (KTYDPTY), which could be recognized by the SH2 domain of Src. In order to evaluate this possibility, VDR-Src association in response to $1\alpha,25(OH)_2D_3$ was investigated. Cells were treated with 20% FBS or 20% FBS plus $1\alpha,25(OH)_2D_3$ (10 nM) for 3 min. Cell lysates were immunoprecipitated with anti-Src antibody under native conditions, followed by Western blot analysis of VDR, and vice versa. As illustrated in Fig. 4, $1\alpha,25(OH)_2D_3$ increased the

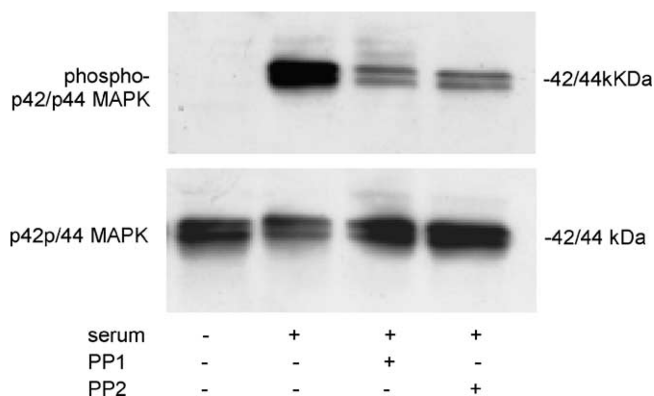

Fig. 2. Serum-induced MAPK activation is blocked by the Src inhibitors PP1 and PP2. MCF-7 cells were pretreated with PP1 or PP2 (10 μM) for 30 min before incubation with serum (20% FBS) for 3 min. Controls were treated with vehicle alone (DMSO <0.1%). Cells were homogenized and subjected to Western blot analysis using anti-(phospho)-active p42/44 MAPK antibody followed by reblotting with anti-p42/p44 MAPK.

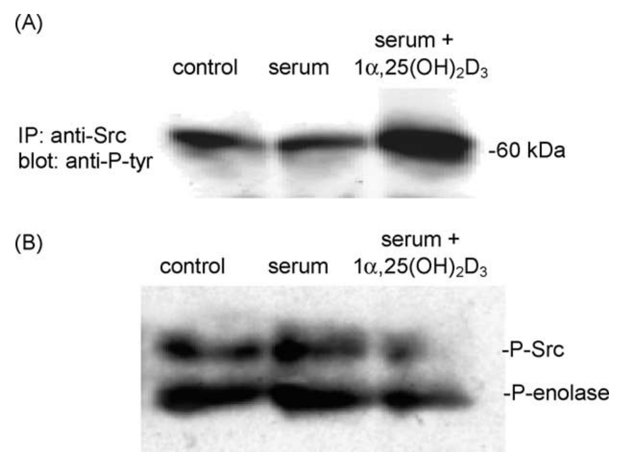

Fig. 3. $1\alpha,25(OH)_2D_3$ increases tyrosine-phosphorylated state of Src and inhibits its activity. (A) MCF-7 cells were treated with serum (20% FBS) plus vehicle (isopropanol <0.1%) or serum plus $1\alpha,25(OH)_2D_3$ (10 nM) for 3 min. Controls were treated with vehicle alone. Cell lysates were obtained, Src was immunoprecipitated with anti-Src antibody (Santa Cruz Biotechnology, Santa Cruz, CA) under dissociating conditions, resolved by SDS–PAGE and then immunoblotted with anti-phosphotyrosine antibody (anti-P-tyr, Promega). (B) Alternatively, Src tyrosine kinase activity was determined as described in Section 2.

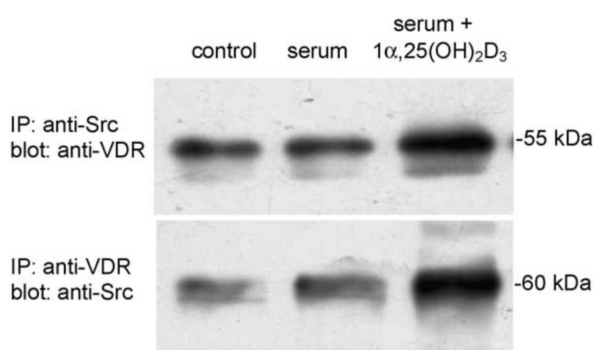

Fig. 4. $1\alpha,25(OH)_2D_3$ induces association of VDR with Src. MCF-7 cells were treated with serum (20% FBS) plus vehicle (isopropanol <0.1%) or serum plus $1\alpha,25(OH)_2D_3$ (10 nM) for 3 min. Controls were treated with vehicle alone. Cell lysates were obtained, Src was immunoprecipitated with anti-Src antibody under native conditions, resolved by SDS–PAGE and then immunoblotted with anti-VDR (Affinity Bioreagents, Golden, CO). Immunoprecipitation with anti-VDR antibody followed by blotting with anti-Src was also performed.

association between Src and VDR, suggesting the involvement of the Vitamin D receptor in the inhibition of Src activity. However, the significance of this interaction on the inhibition of Src by the hormone should be further investigated.

In conclusion, the results obtained in the present work indicate that $1\alpha,25(OH)_2D_3$ inhibits the MAPK cascade in breast cancer cells by inactivating Src tyrosine kinase through a so far unknown mechanism that seems to be mediated by the VDR.

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