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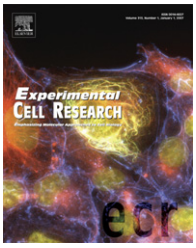

Research Article

1,25-Dihydroxyvitamin D_3 induces biphasic NF-κB responses during HL-60 leukemia cells differentiation through protein induction and PI3K/Akt-dependent phosphorylation/degradation of IκB

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ABSTRACT

1,25-Dihydroxyvitamin D_3 (VD_3) induces differentiation in a number of leukemia cell lines and under various conditions is able to either stimulate or inhibit nuclear factor kappa B (NF-κB) activity. Here we report a time-dependent biphasic regulation of NF-κB in VD_3-treated HL-60 leukemia cells. After VD_3 treatment there was an early ~4 h suppression and a late 8–72 h prolonged reactivation of NF-κB. The reactivation of NF-κB was concomitant with increased IKK activities, IKK-mediated IκBα phosphorylation, p65 phosphorylation at residues S276 and S536, p65 nuclear translocation and p65 recruitment to the NF-κB/vitamin D responsive element promoters. In parallel with NF-κB stimulation, there was an up-regulation of NF-κB controlled inflammatory and anti-apoptotic genes such as TNFα, IL-1β and Bcl-xL. VD_3-triggered reactivation of NF-κB was associated with PI3K/Akt phosphorylation. PI3K/Akt antagonists suppressed VD_3-stimulated IκBα phosphorylation as well as NF-κB-controlled gene expression. The early ~4 h VD_3-mediated NF-κB suppression coincided with a prolonged increase of IκBα protein which require *de novo* protein synthesis, lasted for as least 72 h and was insensitive to MAPK, IKK or PI3K/Akt inhibitors. Our data suggest a novel biphasic regulation of NF-κB in VD_3-treated leukemia cells and our results may have provided the first molecular explanation for the contradictory observations reported on VD_3-mediated immune-regulation.

Introduction

Nuclear factor kappa B (NF-κB) regulates the transcription of a wide array of genes involved in cancer development, inflammation, immune responses, apoptosis and cell proliferation. NF-κB encompasses a family of transcription factors including p65 (RelA), p105/p50, p100/p52, RelB and c-Rel. The classic form of NF-κB is the heterodimer p50/p65 that contains the

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Abbreviations: AML, acute myelogenous leukemia; CHX, cycloheximide; IKK, IκB kinases; IL-1β, interleukin 1beta; NF-κB, nuclear factor kappa B; TNF-α, tumor necrosis factor-α; VD_3, 1,25-dihydroxyvitamin D_3; VDR, vitamin D receptor; VDRE, vitamin D responsive element

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transcriptional activation domain and is sequestered in the cytoplasm as an inactive complex by the inhibitory protein IκB [1]. Acute immunological stimuli such as tumor necrosis factor-α (TNF-α), lipopolysaccharide or phorbol myristate acetate (PMA) lead to the activation of IκB kinase (IKK) which phosphorylates two key IκB serine residues, Ser32 and Ser36 [2]. Phosphorylated IκB would then undergo ubiquitination and proteolysis. The release of NF-κB from IκB complexes unmasks the nuclear localization signal and results in the translocation to the nucleus where it exerts its gene regulatory functions [2].

Constitutively increased NF-κB activity has been reported in human leukemia cells of both myelogenic [3,4] and lymphoblastic [5] origins. In these cells increased NF-κB results in anti-apoptotic activities, cell proliferation, induction of inflammatory gene expression and impediment of the therapeutic values of anti-cancer agents [6–8]. Constitutive NF-κB activation in leukemia cells is frequently caused by persistently activated IKK [4,5,9] or mutations in IκB [10,11].

1,25-Dihydroxyvitamin D_3 (VD_3), the biologically active form of vitamin D, may have anti-cancer and anti-inflammatory activities because of its anti-proliferative, immune modulatory and differentiation inducing actions [12,13]. VD_3 may induce terminal differentiation in human myeloid leukemia cell lines along the monocyte-macrophage lineage [13]. Paradoxical to VD_3's suggested anti-cancer activity, an essential element in the monocyte-macrophage maturation process is the activation of NF-κB [14–16] and the up-regulation NF-κB-controlled genes [17,18]. VD_3 activates NF-κB by stimulating IκBα phosphorylation/degradation in NB4 leukemia cells [19].

A number of mechanisms, including the activation of the PI3K-Akt pathway, may be involved in VD_3-stimulated NF-κB activation [20]. Protein kinase B/Akt is a mitogen-derived survival factor able to activate NF-κB through stimulating IKK [21]. Akt signaling is constitutively active in acute myelocytic leukemia cells [22] and may contribute to the increase in intrinsic NF-κB activity [23,24]. In HL-60 cells, Akt signaling may be stimulated by VD_3 [25] and blocking PI3K/Akt stimulation may result in apoptosis and G_1 arrest and the inhibition of monocytic differentiation [25–27].

Perplexingly under some conditions VD_3 has also been reported to produce inhibitory effects on NF-κB activity. VD_3 may increase the expression level of IκBα [28,29], decrease p50 and c-Rel levels [30], impair p65 nuclear translocation [31], decrease DNA binding of p65/p50 heterodimer [32] and diminish NF-κB binding activity to the IL-8 promoter [33]. In line with these VD_3 may also repress NF-κB gene products including the adhesion molecules ICAM-1 and VCAM-1 [34], anti-apoptotic protein A20 [35] and cytokines IL-1β, IL-6, IL-8 and TNF-α [36–40].

In the present study we demonstrated that in VD_3-treated HL-60 leukemia cells there was an early 4–8 h suppression and a delayed 16 to at least 72 h reactivation of NF-κB and an up-regulation of NF-κB controlled genes. The early suppression of NF-κB was associated with an increased IκBα level that required *de novo* protein synthesis and is independent from IKK, Akt and MAPK pathways. The late reactivation of NF-κB was associated with VD_3-stimulated phosphorylation of IκBα which required active Akt and IKK pathways.

Materials and methods

Chemicals and reagents

VD_3 was purchased from Alexis Biochemicals (San Diego, CA, USA) and was dissolved in ethanol as a 100 mM stock solution and stored at −20 °C. [γ-^{32}P] ATP was purchased from PerkinElmer Life Sciences (Hong Kong) Ltd. Anti-p-p65 (Ser^{536}), anti-p-p65 (Ser^{276}), anti-p-Akt (Thr^{308}), anti-p-IκBα (Ser^{32}), anti-IL-1β, anti-acetylated (lysine), anti-Akt, anti-p-PTEN and anti-PTEN antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Protein A/G plus-agarose, glutathione *S*-transferase (GST)-IκBα, anti-p65, anti-IκBα, anti-IKKα/β, anti-p-Akt (Ser^{473}), anti-lamin B and anti-β-tubulin were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). NF-κB consensus gel shift oligonucleotide was purchased from Promega (Madison, WI, USA). IKK, PI3K, Akt and MAPK inhibitors were from Calbiochem Ltd. (CA, USA).

Cell culture

The human promyelocytic leukemia HL-60 cell line was obtained from American Type Culture Collection (Manassas, VA, USA) and were grown in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, NY, USA) at 37 °C in humidified 5% CO_2 atmosphere. IKK, PI3K, Akt, MAPK and protein synthesis inhibitor were added to cells 2 h before the addition of VD_3. The final concentrations of ethanol or DMSO were below 0.2% and 0.1% (v/v) respectively, which did not affect cell growth and differentiation.

Cell surface CD11b antigen detection

The expression of cell surface marker CD11b was monitored by flow cytometry. Samples of 5×10^5 cells were washed twice with PBS, resuspended in 100 μl of PBS and then incubated for 45 min at 4 °C with 10 μl R-phycoerythrin-conjugated anti-human CD11b monoclonal antibodies (BD Pharmingen, USA). Cells were then washed twice, resuspended in 0.5 ml PBS, and analyzed on a Becton Dickson Flow Cytometer (BD Biosciences, USA) with CELL Quest software (Verity Software House, Inc., Topsham, ME).

Cell cycle analysis

Cells were collected, washed, suspended in cold PBS, fixed in 75% ethanol at −20 °C overnight, washed and resuspended in PBS with RNAase (0.1 mg/ml). Cellular DNA was stained with PI and cell samples were analyzed on Becton Dickson Flow Cytometer (BD Biosciences, USA) using CELL Quest software (Verity Software House, Inc., Topsham, ME).

Electrophoretic Mobility Shift Assay (EMSA)

EMSA and the nuclear extracts preparation were performed as described previously [41,42]. Briefly, 5 μg of nuclear protein was incubated with radiolabeled gel shift oligonucleotides for