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NFκB pathway is down-regulated by 1α,25(OH)$_2$-vitamin D$_3$ in endothelial cells transformed by Kaposi sarcoma-associated herpes virus G protein coupled receptor

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ABSTRACT

We have previously demonstrated that 1α,25 dihydroxy-vitamin D$_3$ (1α,25(OH)$_2$D$_3$) has antiproliferative effects on the growth of endothelial cells transformed by the viral G protein-coupled receptor associated to Kaposi sarcoma (vGPCR). In this work, we have investigated whether 1α,25(OH)$_2$D$_3$ exerts its growth inhibitory effects by inhibiting the Nuclear Factor κ B (NFκB) pathway which is highly activated by vGPCR. Cell proliferation studies demonstrated that 1α,25(OH)$_2$D$_3$, similarly to bortezomib, a proteosome inhibitor that suppresses the activation of NFκB, reduced the proliferation of endothelial cells transformed by vGPCR (SVEC-vGPCR). The activity of NFκB in these cells decreased by 70% upon 1α,25(OH)$_2$D$_3$ treatment. Furthermore, time and dose response studies showed that the hormone significantly decreased NFκB and increased IκBα mRNA and protein levels in SVEC-vGPCR cells, whereas in SVEC only IκBα increased significantly. Moreover, NFκB translocation to the nucleus was inhibited and occurred by a mechanism independent of NFκB association with vitamin D$_3$ receptor (VDR). 1α,25(OH)$_2$D$_3$-induced increase in IκBα required *de novo* protein synthesis, and was independent of MAPK and PI3K/Akt pathways. Altogether, these results suggest that down-regulation of the NFκB pathway is part of the mechanism involved in the antiproliferative effects of 1α,25(OH)$_2$D$_3$ on endothelial cells transformed by vGPCR.

1. Introduction

Kaposi Sarcoma (KS) is a highly vascular tumor, and the tumor cells display features of activated endothelial cells, including high expression of several phenotypic markers as well as various specific tyrosine kinases [1]. The Kaposi Sarcoma-associated herpes virus G protein-coupled receptor (KSHV-GPCR) is a key molecule in the pathogenesis of Kaposi Sarcoma. Recently, it has been determined that Nuclear Factor κ B (NFκB) gene expression was increased in KS and demonstrated that vGPCR potently activates the NFκB pathway [2]. It has also been found that constitutive NFκB is not sufficient for transformation of endothelial cells, but is required for direct induction of neoplasia by vGPCR, being an important therapeutic target for the treatment of Kaposi Sarcoma [2].

NFκB regulates the transcription of a wide array of genes involved in cancer development, inflammation, immune responses, apoptosis and cell proliferation. NFκB encompasses a family of transcription factors including p65 (RelA), p105/p50, p100/p52, RelB and c-Rel. The classic form of NFκB is the heterodimer p50/p65 that contains the transcriptional activation domain and is sequestered in the cytoplasm as an inactive complex by the inhibitory protein IκB [3]. Acute immunological stimuli such as tumor necrosis factor-α (TNF-α), lipopolysaccharide or phorbol myristate acetate lead to the activation of IκB kinase (IKK) which phosphorylates two key IκB serine residues, Ser32 and Ser36 [4]. Phosphorylation of IκB by IKK initiates the ubiquitylation and degradation of IκB by the proteasome, leading to nuclear translocation and activation of NFκB. Many proinflammatory cytokines and chemokines, such as IL-1, IL-6, IL-8, IL-12, and TNF-α, are targets of NFκB regulation [4,5]. Deregulation of NFκB and IκB phosphorylations are a hallmark of chronic inflammatory diseases and cancer, and newly designed drugs targeting these constitutively activated signaling pathways represent promising therapeutic tools [6]. 1α,25dihydroxyvitamin D$_3$ (1α,25(OH)$_2$D$_3$, calcitriol), a steroid hormone, plays an essential role in the regulation of calcium homeostasis, cell proliferation and differentiation, and in the immune system [7,8]. More recently, its ability to induce apoptosis, inhibit angiogenesis, tumor invasion and metastasis, and to modulate cytokine production has been demonstrated [9]. Most of the activity of

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$1\alpha,25(OH)_2D_3$ is mediated by the vitamin D receptor (VDR), a member of the nuclear receptor superfamily [10]. As a ligand-activated transcription factor, VDR heterodimerizes with retinoid X receptor once activated by $1\alpha,25(OH)_2D_3$ and binds to the vitamin D response element in the target gene promoter to regulate gene transcription. The VDR has also been shown to physically interact with other regulatory proteins such as Smad3 [11], β-catenin [12], NFκB p65 [13], and cyclin D_3 [14], but the physiological relevance of these protein–protein interactions remains to be determined. The ability of $1\alpha,25(OH)_2D_3$ to decrease NFκB DNA binding activity upon IL-1, IL-8 and TNF-α exposure has been demonstrated in macrophages, leukemia and breast cancer cells [15–17]. $1\alpha,25(OH)_2D_3$ has also been shown to decrease the DNA binding capacity of NFκB in human fibroblasts [18]. Moreover, in fibroblasts lacking VDR the activity of NFκB increased due to a reduction in IκBα levels and in NFκB-VDR interaction [19]. It has also been reported that $1\alpha,25(OH)_2D_3$ directly regulates the NFκB pathway by decreasing NFκB translocation to the nucleus and increasing IκBα protein expression in macrophages [20]. We have previously demonstrated that $1\alpha,25(OH)_2D_3$ has antiproliferative effects on the growth of endothelial cells transformed by KSHV-GPCR [21]. In the present work, we demonstrated that $1\alpha,25(OH)_2D_3$ exerts its inhibitory effects by down regulation of the NFκB pathway. $1\alpha,25(OH)_2D_3$ decreased NFκB activity by interfering with its translocation to the nucleus. Moreover NFκB mRNA and protein levels were decreased whereas IkBα mRNA and protein levels were increased. In addition, inhibition of the NFκB pathway occurs independently of the association of VDR with NFκB.

2. Experimental

2.1. Chemicals and reagents

$1\alpha,25(OH)_2D_3$ and Immobilon P (polyvinylidene difluoride; PVDF) membranes were from Sigma–Aldrich (St. Louis, MO, USA). The CellTiter 96 AQueous One Solution Cell Proliferation Assay was from Promega (Madison, WI, USA). The Transcription Factor kit for NFκB p65 was from Thermo scientific (Pierce Biotechnology, Rockford, Il, USA). Bortezomib was from Selleck (Nuclilab.nl, Rotterdam, NL). The antibodies used were: mouse monoclonal anti-VDR (Affinity Bioreagents, Golden, CO, USA), rabbit polyclonal anti-NFκB and mouse monoclonal IκBα from Cell Signaling (Cell Signaling Technology, Migliore Laclaustra, Buenos Aires, AR), anti-actin and rabbit polyclonal anti-VDR, mouse monoclonal anti-NFκB as well as anti-rabbit, anti-mouse and anti-rat horseradish peroxidase–conjugated secondary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse Alexa 467 and anti-rabbit Alexa 488 were from Invitrogen (Buenos Aires, AR). Protein A/G agarose and anti-lamin B were from Santa Cruz Biotechnology. PCR primers and fluorogenic probes for mouse RelA (NFκB), IκBα, and β-actin were purchased from Eurogentec (Seraing, Belgium). For most applications, $1\alpha,25(OH)_2D_3$ was used at 10 nmol/l because this concentration consistently shows antiproliferative effects in multiple assays in a variety of tumor cell types.

2.2. Cell lines and transfections

SV-40 immortalized murine endothelial cells stably expressing vGPCR full-length (SVEC-vGPCR) or empty vector pCEFL (SVEC) as a control was used. Stable overexpression of vGPCR promotes tumor formation when these cells are injected into immuno-suppressed mice and induces angiogenic lesions similar to those developed in Kaposi Sarcoma [21,22]. Transfected cells were selected with 500 μg/ml G418 (Cellgro, Manassas, VA, USA). Stable SVEC-vGPCR endothelial cells targeted with small hairpin RNA against mouse vitamin D receptor (shVDR) or control shRNA were obtained by transduction of lentiviral particles [21]. The stable cell lines were selected with 2 μg/ml puromycin (Invivogen, San Diego, CA, USA) and the medium was freshly changed every other day. VDR knock-down was monitored by Western blot analysis.

2.3. Proliferation assays

SVEC-vGPCR cells were seeded in 96-well plates, at 2500 cells per well. After overnight growth, the cells were starved for 24 h and then treated with $1\alpha,25(OH)_2D_3$ (10 nM) or vehicle (0.01%, ethanol), in the presence or absence of Bortezomib (0.5 nM) in triplicate in DMEM-2% fetal bovine serum (FBS) for 48 h. CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay containing the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) was used to determine cell proliferation according to the manufacturer's instructions. Absorbance (OD) was measured at 490 nm.

2.4. Nuclear extracts

Cells were collected and transferred to a centrifuge tube in cold PBS and centrifuged at 250 g for 5 min. The supernatant was discarded and the cell pellet was resuspended in Buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.1% Triton X-100 and protease inhibitor cocktail) and incubated for 15 min on ice followed by 5 min centrifugation at 250 g. The cell pellet was resuspended in two volumes of ice cold Buffer A and disrupted using a syringe with a small gauge needle (5 strokes) and centrifuged at 8000*g* for 20 min. The supernatant containing the cytosolic fraction was transferred to a fresh tube and stored at −70 °C. The nuclear fraction of the cell lysate (pellet) was resuspended in 2/3 of the original cell pellet volume in ice cold Buffer B (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 1 mM DTT, 1.0% NP 40, 25% (v/v) glycerol and protease inhibitor cocktail). The nuclei were disrupted using a syringe (with a No. 27 gauge needle) and agitated at 4 °C for 30–60 min. Finally, the nuclear suspensions were centrifuged at 16,000 for 5 min at 4 °C. The supernatant (nuclear extract) was transferred to a fresh tube and used to perform the NFκB activity assay.

2.5. NFκB activity assay

The Transcription Factor kit for NFκB p65 from Thermo scientific (Pierce Biotechnology, Rockford, Il, USA) was used to measure the activity of Nuclear Factor κ B (NFκB) from nuclear extracts following manufacture's protocol. Chemiluminescence reaction was captured with a microplate reader Synergy HT Biotek (Biotek Instruments, Inc., Winooski, VT, USA).

2.6. SDS–PAGE and Western blot analysis

Whole-cell lysates from cultures treated with $1\alpha,25(OH)_2D_3$ as indicated for each experiment and their protein content determined by the Bradford procedure [23] were resolved by SDS–PAGE and transferred to PVDF membranes. Western blot analyses were performed as reported before [23]. Antibodies used include monoclonal anti-VDR (1:1500), rabbit anti-NFκB/p65 (1:2000), mouse anti-NFκB/p65 (1:500), anti-IκBα (1:3000) and anti-actin (1:7500), combined with anti-rabbit (1:10000), anti-mouse (1:5000) or anti-rat (1:5000) horseradish peroxidase–conjugated secondary antibodies.

2.7. Quantitative real-time PCR

Total RNA for quantitative reverse chain polymerase reaction (qRT-PCR) analysis was isolated by High Pure RNA Isolation Kit

(Roche). 0.5–1 μg of RNA was reverse transcribed using the Superscript II Reverse transcriptase (Invitrogen, Belgium) and qRT-PCR reactions were performed on the resulting cDNA (2 μl of cDNA; dilution 1/10) in a 7500 Fast Real Time PCR system (Applied Biosystems). Specific primers were used to detect NFκB and IκBα levels and β actin to normalize gene expression. Sequences of forward primers (Fw), reverse primers (Rv), and detection probes (Tp) were as follows: VDR: AGGAGAGCACCCTTGGGCT (Fw), ACACACT CCACAGATCCGAGG (Rv), CCAGCACCTCCCTGCCTGACCC (Tp). RelA: CTGTCCTCTCACATCCGATTTTT (Fw), CGGTTTACTCGGCAGATCTTG (Rv); IκBα: TGGCCAGTGTAGCAGTCTTGA (Fw), ACACGTGTGGCCAT TGTAGTTG (Rv); β-actin: AGAGGGAAATCGTGCGTGAC (Fw), CAATAGTGATGACCTGGCCGT (Rv), CACTGCCGCATCCTCTTCCTCCC (Tp)

2.8. Subcellular fractionation

SVEC and SVEC-vGPCR cells were cultured in p100 dishes, treated with $1\alpha,25(OH)_2D_3$ and scrapped in ice-cold TES buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 250 mM sucrose) containing proteases inhibitors (1 mM DTT, 0.5 mM PMSF, 20 μg/ml aprotinin and 20 μg/ml leupeptin). Homogenization was carried out using a Teflon-glass hand homogenizer. The homogenate was centrifuged at 100*g* for 5 min to eliminate debris; supernatant was further centrifuged at 1500*g* for 20 min to sediment the nuclear fraction. The supernatant was further centrifuged at 14000*g* for 20 min to pellet mitochondria. The remaining supernatant was collected as cytosol fraction [24]. Protein concentration from each fraction was estimated by the method of Bradford [23]. Anti-lamin B antibody was employed for the immunodetection of the nuclear protein marker lamin B in the different fractions.

2.9. Co-immunoprecipitation

Co-immunoprecipitation assays were performed under native conditions in order to preserve protein–protein associations, and were conducted essentially as previously described [25]. Cells were lysed (15 min at 4 °C) in co-immunoprecipitation buffer (co-IP buffer) containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 3 mM KCl, 0.5 mM EDTA, 1% Tween-20, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml of each leupeptin and aprotinin. Lysates were clarified by centrifugation (14000*g*, 10 min at 4 °C). 500 μg of protein were incubated with 1 μg of rabbit anti – VDR antibody and buffer only for 5 h, followed by incubation with protein A/G plus agarose (PAG) overnight. Whole cell lysate plus PAG (L + PAG) was added as negative control. The precipitated immunocomplex was then washed 3 times with co-IP buffer and subject to Western blot with anti-NFκB (mouse) and anti-VDR (rat).

2.10. Confocal microscopy

SVEC and SVEC-vGPCR were cultured on cover slips and treated with $1\alpha,25(OH)_2D_3$. Then the cells were fixed in 3% paraformaldehyde for 15 min and permeabilized in 0.2% Triton 2% BSA in PBS for 20 min. Afterwards, the cells were incubated with mouse anti-NFκB/p65 (1:25) and rabbit anti-VDR (1: 100) for 2 h at room temperature. After 3 washes with PBS (5 min) the cells were incubated with secondary antibodies anti-mouse Alexa 467 and anti-rabbit Alexa 488. Images were taken with a Leica DM IRB2 microscope with a confocal spectral module SP2 equipped with Ar laser (458, 476, 488 and 514 nm) and HeNe laser (633 nm). Viewing was carried out with a 63 × 1.2 NA water-immersion objective.

2.11. Statistical analysis

Data are shown as means ± SD. Data from qRT-PCR and Western blot quantifications were analyzed by the two-tailed *t*-test to evaluate differences between vehicle and $1\alpha,25(OH)_2D_3$ A *P*-value <0.01 (**) and <0.05 (*) was considered highly statistically significant and statistically significant, respectively.

3. Results

3.1. $1\alpha,25(OH)_2D_3$ regulates proliferation and NFκB activity

We have previously demonstrated that 1α,25dihydroxy-vitamin D_3 ($1\alpha,25(OH)_2D_3$) has antiproliferative effects on the growth of endothelial cells transformed by the viral G protein-coupled receptor associated to Kaposi sarcoma (vGPCR) [21]. Since the Nuclear Factor κ B pathway is highly activated by vGPCR we investigated whether it is involved in $1\alpha,25(OH)_2D_3$ growth inhibitory effects. First, proliferation assays were carried out in SVEC-vGPCR, cells, which stably express the viral receptor of the herpes virus 8, treated with $1\alpha,25(OH)_2D_3$ (10 nM) or vehicle (0.01% ethanol) in the presence or absence of Bortezomib (0.5 nM) in DMEM 2% fetal bovine serum (FBS) for 48 h. Bortezomib has been shown to be an inhibitor of the proteosome 20S β 5 subunit that at low nanomolar concentration is sufficient for inhibition of the activation of NFκB in response to TNF-α and the proliferation of cancer cells [26]. Proliferation was measured using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS). The results shown in Fig. 1A demonstrate, that similarly to $1\alpha,25(OH)_2D_3$, incubation with Bortezomib (0.5 nM), a compound with NFkB inhibitory activity, significantly decreased the proliferation of SVEC-vGPCR cells. No additive effect was observed when both agents were used. To find out whether $1\alpha,25(OH)_2D_3$ treatment can alter NFκB activity and further account for the hormone inhibitory effects on the proliferation of SVEC-vGPCR cells, the transcriptional activity of the active form NFκB p65 was assayed upon $1\alpha,25(OH)_2D_3$ treatment (10 nM, 48 h). The active transcription factor bound to DNA consensus sequence was incubated with specific primary antibody (NFκB p65) followed by a secondary HRP-conjugated antibody and chemiluminesce was finally detected on a luminometer. The results in Fig. 1B show that $1\alpha,25(OH)_2D_3$ significantly decreased the activity (70%) of NFκB after 48 h of treatment.

3.2. $1\alpha,25(OH)_2D_3$ decreases NFκB and increases IκBα mRNA and protein levels

To further characterize the inhibition of NFκB activity by $1\alpha,25(OH)_2D_3$, time dependence of hormone effects on NFκB and IκBα mRNA and protein expression levels and the role of the VDR therein were studied. SVEC and SVEC-vGPCR, were plated for 1 day, then starved for 24 h followed by incubation with $1\alpha,25(OH)_2D_3$ (10 nM) or vehicle (0.01% ethanol) in presence of 2% FBS for 3–48 h. Total RNA from control (vehicle) and $1\alpha,25(OH)_2D_3$-treated SVEC and SVEC-vGPCR cells was isolated. The RNA was reverse transcribed followed by qRT-PCR using specific primers to detect NFκB and IκBα mRNA levels and β-actin mRNA to normalize gene expression. The results shown in Fig. 2A and B demonstrated that $1\alpha,25(OH)_2D_3$ significantly decreased NFκB mRNA levels in SVEC-vGPCR (20% and 30% at 12 and 24 h, respectively) and increased IκBα mRNA levels in both SVEC and SVEC-vGPCR in a time-dependent fashion (50–70%). Then, to test whether the effect was dependent on the Vitamin D receptor (VDR), a stable VDR knockdown cell line SVEC-vGPCR shVDR was used. First, VDR

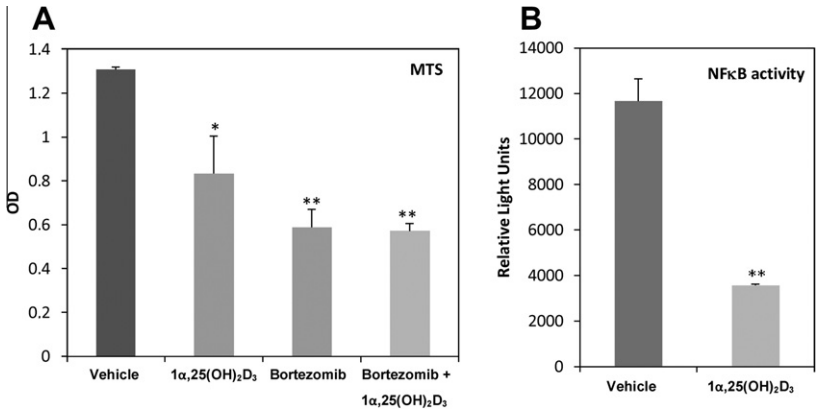

Fig. 1. $1\alpha,25(OH)_2D_3$ inhibits SVEC-vGPCR cell proliferation and NFκB activity. SVEC-vGPCR cells were treated with $1\alpha,25(OH)_2D_3$ (10 nM) or vehicle (0.01% ethanol), bortezomib (0.5 nM) and combination of both $1\alpha,25(OH)_2D_3$ and bortezomib, in DMEM 2% for 48 h. (A) Proliferation assays were carried out using MTS reagent and absorbance (OD) was measured at 490 nm (B) NFkB activity was measured by chemiluminescence reaction captured with a microplate reader as described in Experimental. The data from each experiment are means ± SE performed in triplicate. Significant differences between control (vehicle) and treated conditions are indicated.$^{*}P < 0.05$, $^{**}P < 0.01$.

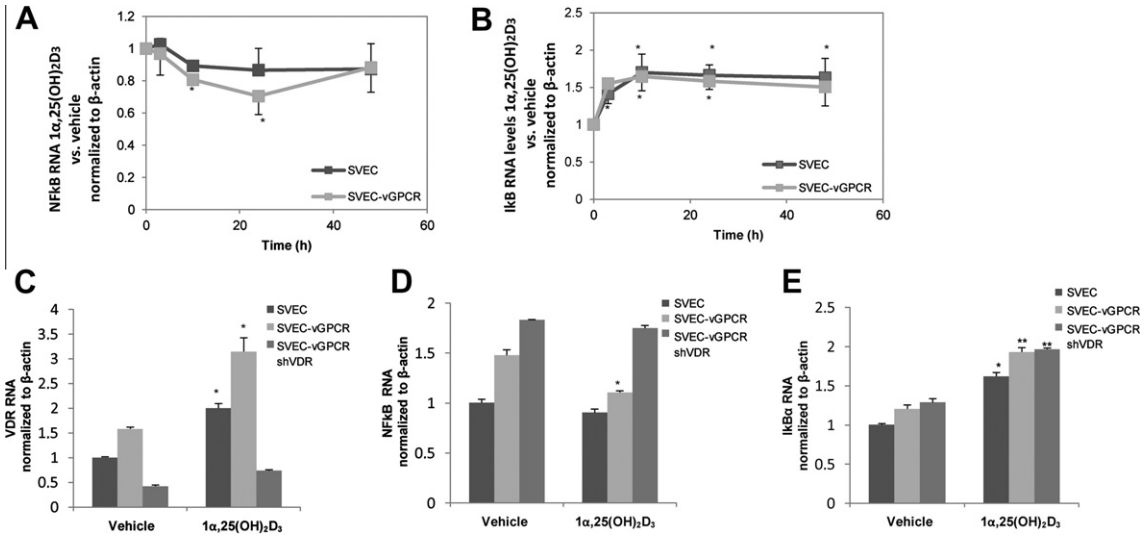

Fig. 2. VDR-dependent modulation of NFκB and IκBα mRNA in SVEC and SVEC-vGPCR cells by $1\alpha,25(OH)_2D_3$. SVEC and SVEC-vGPCR cells were treated with $1\alpha,25(OH)_2D_3$ (10 nM) or vehicle (0.01% ethanol) for 3–48 h (A and B) and SVEC, SVEC-vGPCR and SVEC-vGPCR shVDR cells for 16 h (C, D and E). Total RNA was extracted and reverse transcribed (0.5–1 μg). At each of these time points, gene expression of RelA (NFκB), IκBα and VDR was assessed by qRT-PCR analysis, normalized to β-actin RNA levels. In time course studies, data are expressed as a ratio between $1\alpha,25(OH)_2D_3$-treated and corresponding vehicle treated samples. The statistical significance of the data was evaluated using, Student's *t* test, $^{**}P < 0.01$ and $^{*}P < 0.05$. The data shown are representative of three independent experiments done in triplicate.

expression was evaluated (Fig. 2C). Upon $1\alpha,25(OH)_2D_3$ treatment (10 nM, 16 h), VDR mRNA significantly increased in both SVEC and SVEC-vGPCR and a decrease of NFκB and an increase of IκBα mRNA levels was observed in SVEC-vGPCR. The inhibition of NFκB expression by $1\alpha,25(OH)_2D_3$ was blunted in VDR knockdown SVEC-vGPCR shVDR, whereas the effect of $1\alpha,25(OH)_2D_3$ on the expression of IκBα was not statistically significant in both cells lines (Fig. 2D and E). To further investigate whether the changes in mRNA levels were reflected in changes in protein expression, Western blots from time (4–48 h) and dose (0.1–100 nM) response studies were performed (Fig. 3). Cell lysates were prepared and subject to Western blot analysis with anti NFκB and IκBα antibodies. The results in Fig. 3A and B showed that $1\alpha,25(OH)_2D_3$ significantly reduced the protein levels of NFκB in SVEC-VGPCR (20–50%) and increased IκBα in both cell types in a time-dependent manner, being more effective in SVEC-vGPCR between 10 and 100 nM of $1\alpha,25(OH)_2D_3$ (Fig. 3B).

3.3. $1\alpha,25(OH)_2D_3$ inhibits the translocation of NFκB to the nucleus

To elucidate in more depth the inhibitory effect of $1\alpha,25(OH)_2D_3$ on the NFκB pathway, the localization of the proteins NFκB and VDR was investigated. SVEC and SVEC-vGPCR were cultured and treated with 10 nM $1\alpha,25(OH)_2D_3$ or vehicle for 4 and 24 h. The cells were collected in TES buffer and subject to differential centrifugation to obtain enriched nuclear and cytosolic fractions, as described in Experimental. Western blot analysis with anti-NFκB, VDR, and lamin B antibodies were performed. The results shown in Fig. 4A and B demonstrate that $1\alpha,25(OH)_2D_3$ induced the translocation of VDR to the nucleus at 4 and 24 h in

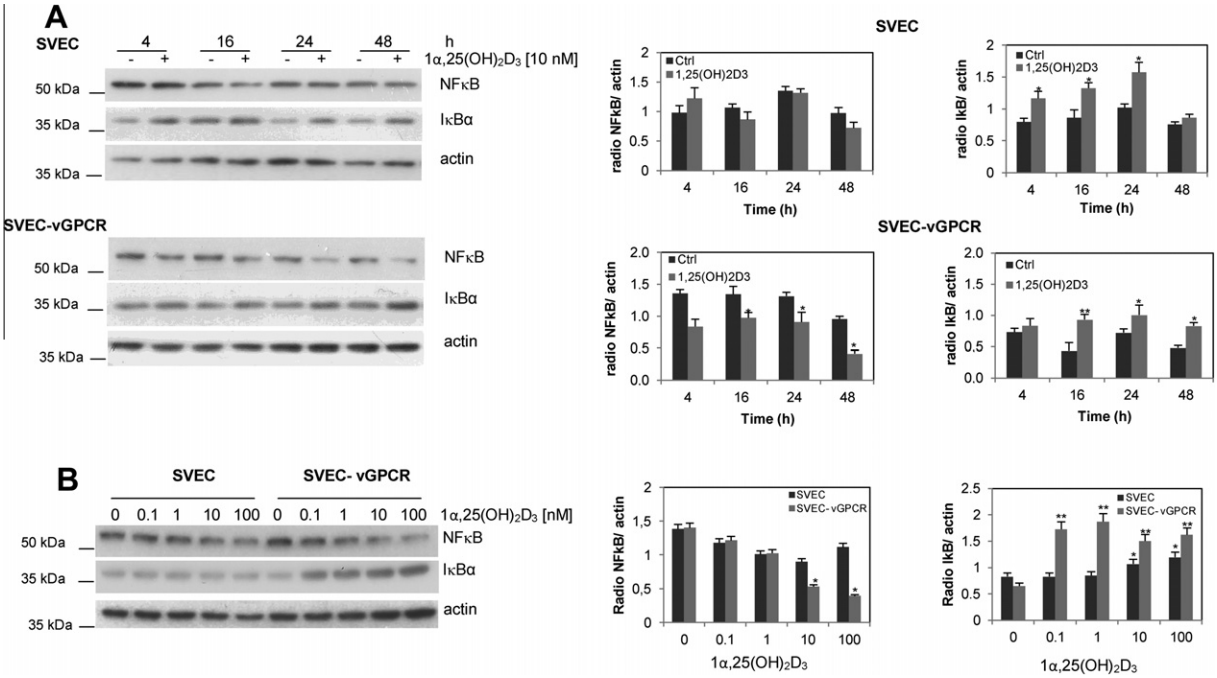

Fig. 3. $1\alpha,25(OH)_2D_3$ decreases NFκB and increases IκBα protein levels in a time- and dose-dependent manner in SVEC and SVEC-vGPCR cells. SVEC and SVEC-vGPCR were cultured and treated with 10 nM $1\alpha,25(OH)_2D_3$ or vehicle for 4–48 h as described in Fig 1 (A) and 0.1–100 nM $1\alpha,25(OH)_2D_3$ for 16 h (B). Cell lysates were prepared and subject to Western blot analysis with anti-NFκB, IκBα and actin antibodies. The bands were quantified by densitometry and the ratios between VDR and actin levels were taken into account for statistical analysis. $^{**}P < 0.01$ and $^{*}P < 0.05$. The data shown are representative of three independent experiments.

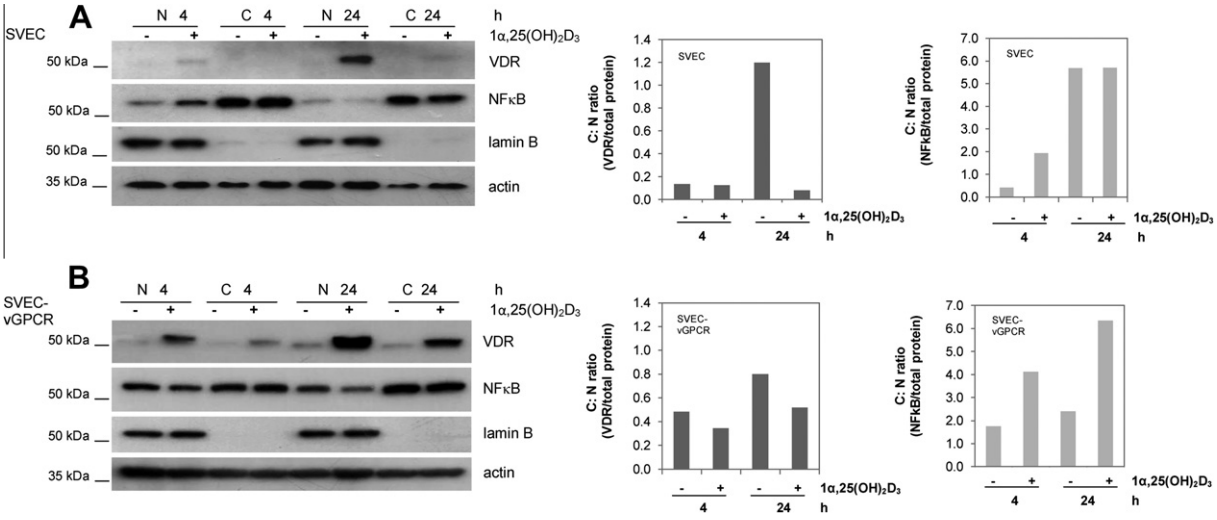

Fig. 4. Translocation of NFκB to the nucleus is inhibited by $1\alpha,25(OH)_2D_3$. SVEC (A) and SVEC-vGPCR (B) cells were cultured and treated with 10 nM $1\alpha,25(OH)_2D_3$ for 4 and 24 h as described in Fig. 1 legend. Cells were collected in TES buffer and subject to differential centrifugation to obtain enriched nuclear (N) and cytosolic fractions (C). Western blot analysis with anti-NFκB, VDR, lamin B and actin antibodies were performed. The western blots and their quantification by Image J (barh graphs) shown are representative of three independent experiments.

both cell lines and inhibited the translocation of NFκB after 24 h of treatment in SVEC-vGPCR.

3.4. Down-regulation of the NFκB pathway by $1\alpha,25(OH)_2D_3$ is independent of NFκB-VDR association

Recently, it has been demonstrated that VDR interacts physically with p65 in human osteoblasts [13], but the functional relevance of this interaction in NFκB signaling remains unclear. We investigated whether the association of VDR with NFκB was part of the inhibitory mechanism of action of $1\alpha,25(OH)_2D_3$ on the NFκB pathway. To that end, SVEC and SVEC-vGPCR were treated with 10 nM $1\alpha,25(OH)_2D_3$ or vehicle (0.01% ethanol) for 16 h. The cells were collected in co-immunoprecipitation buffer (co-IP) as described in Experimental. The immunocomplexes were washed with co IP buffer and then subject to Western blot analysis using anti-NFκB (mouse) and VDR (rat) antibodies. As shown in Fig. 5A, no association between VDR and NFκB was detected. To confirm this observation, we analyzed the interaction between these two proteins by confocal microscopy. To that end, both cell lines were cultured on cover slips, treated with 10 nM $1\alpha,25(OH)_2D_3$ or vehicle for 16 h, then fixed and permeabilized as described in

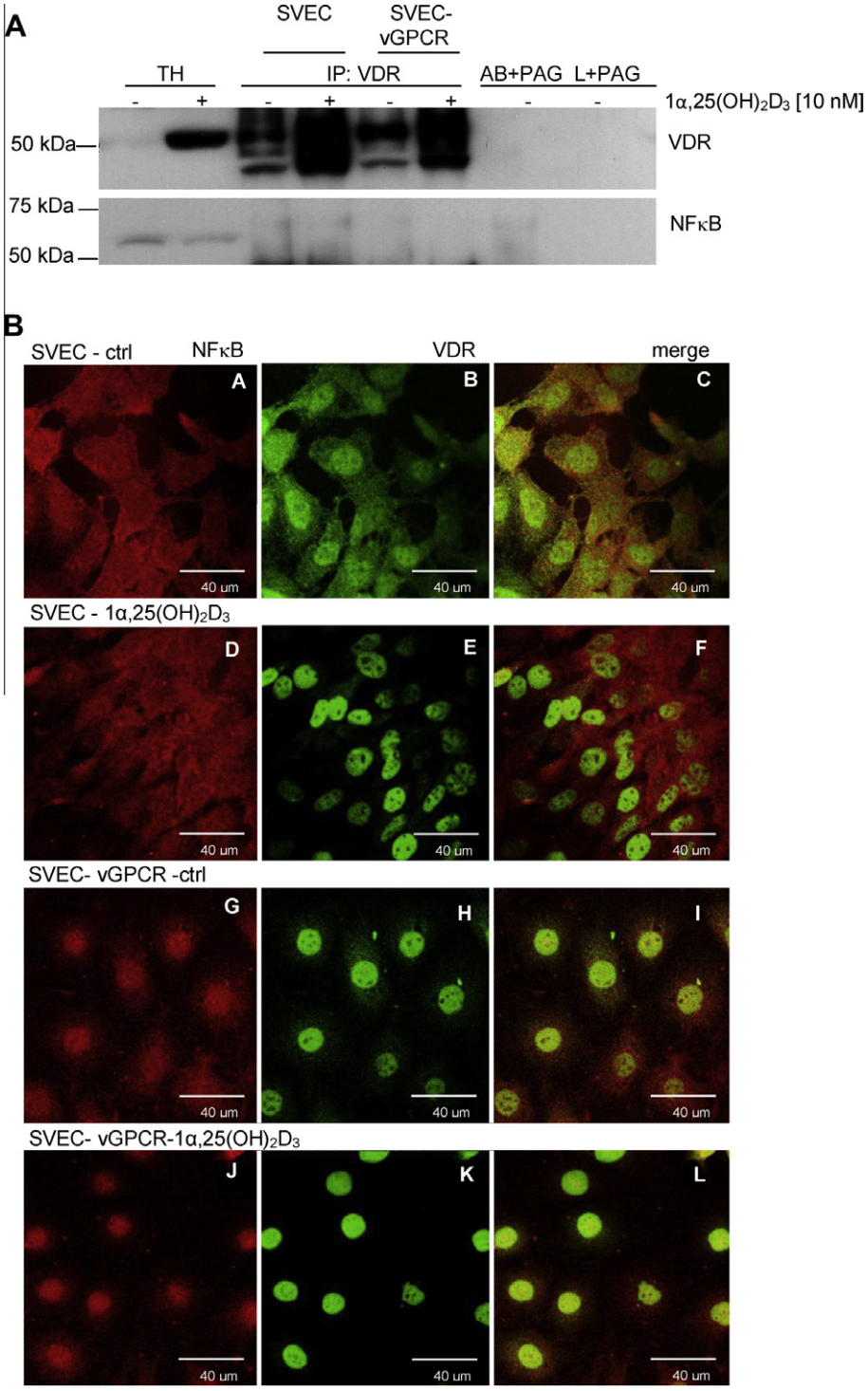

Fig. 5. Down-regulation of the NFκB pathway by $1\alpha,25(OH)_2D_3$ is independent of NFκB-VDR association. SVEC and SVEC-vGPCR cells were cultured and treated with $1\alpha,25(OH)_2D_3$ (10 nM) for 16 h as described in Fig. 1 legend. (A) Cells were scrapped and collected in co-IP buffer. Samples from control (−) and treated (+) cells and buffer alone were incubated with anti-VDR (rabbit) antibody for 5 h, followed by incubation with protein A/G plus agarose (PAG) overnight. Whole cell lysate plus PAG (L + PAG) was added as negative control. TH: total homogenate, and AB: antibody. Western blot analyses with anti-NFκB (mouse) and VDR (rat) analysis were performed. (B) Confocal microscopy images were obtained from control (A, B, G, H) and $1\alpha,25(OH)_2D_3$ (D,E, J, K) treated cells as described in Experimental. Magnification 630×. Representative Western blot and images of three independent experiments are shown.

Experimental. Then, the cells were first incubated with NFκB and VDR antibodies and then with secondary antibodies conjugated with anti-mouse Alexa 467 and anti-rabbit Alexa 488, respectively. Fig. 5B shows that under control conditions the localization of NFκB was mainly nuclear and cytoplasmic in SVEC and mostly nuclear in SVEC-vGPCR as expected due to its oncogenic role on the pathway, whereas VDR was cytoplasmic and nuclear. Upon $1\alpha,25(OH)_2D_3$ treatment (10 nM, 16 h), the translocation of VDR to the nucleus was observed, while NFκB intensity decreased and remained in the nucleus in SVEC-vGPCR. No merge was obtained when both images were mixed supporting the idea that both transcription factors do not physically interact.

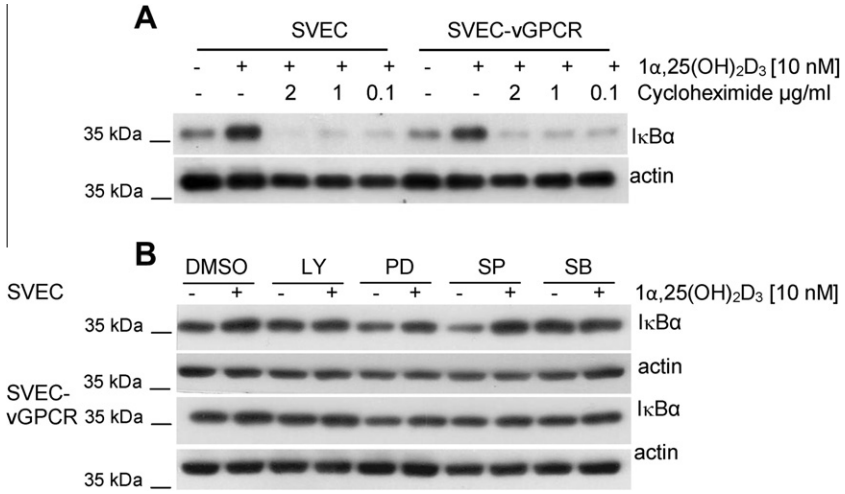

Fig. 6. $1\alpha,25(OH)_2D_3$ induces de novo IkBα synthesis independently of PI3K/Akt and MAPK pathways. SVEC and SVEC-vGPCR cells were plated for 1 day and starved for 24 h thereafter. Then, the cells were first incubated with cycloheximide (0.1–2 μg/ml) for 15 min followed by treatment with $1\alpha,25(OH)_2D_3$ and 2% FBS for 16 h (A) in the presence or absence of 10 μM LY294002 (LY), PD98059 (PD), SP600125 (SP) and SB203580 (SB), inhibitors of PI3 K/Akt, ERK1/2, JNK and p38 MAPK, respectively (B). Cell lysates were prepared and subject to Western blot analysis with anti-IκBα and actin antibodies. The data shown are representative of three independent experiments.

3.5. $1\alpha,25(OH)_2D_3$ induces de novo IκBα protein synthesis independently of MAPKs and PI3K/Akt pathways

$1\alpha,25(OH)_2D_3$ was reported to induce IκBα expression and therefore inhibits NF-κB activity [15,20]. Herein, RT-qPCR and Western blot analysis showed that $1\alpha,25(OH)_2D_3$ induced an early expression of IκBα at the mRNA (Fig. 2B) and protein level (Fig. 3) starting at 3–4 h. Moreover, in the presence of the protein synthesis inhibitor cycloheximide (CHX), $1\alpha,25(OH)_2D_3$-induced IκBα protein levels were reduced (Fig. 6A). It has been reported that vGPCR expression on endothelial cells not only activates the NFκB pathway but also Akt/mTOR and MAP kinases [27]. Moreover, PI3K/Akt inhibitors blocked $1\alpha,25(OH)_2D_3$ induced IκBα phosphorylation and NF-κB controlled gene expression in Leukemia cells [16]. In order to find out whether the MAPKs (ERK1/2, p38 and JNK) and PI3K/Akt pathways were involved upstream in $1\alpha,25(OH)_2D_3$ modulation of IκBα protein synthesis, SVEC and SVEC-vGPCR cells were incubated in the presence or absence of 10 μM PD98059 (MEK1/2 inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), and LY294002 (PI3K inhibitor). At this concentration, each inhibitor blocks the phosphorylation of its respective kinase in endothelial cells (data not shown). The results shown in Fig. 6B demonstrated that pretreatment with MAPKs and PI3K/Akt inhibitors had no effect on IκBα induction by the hormone.

4. Discussion

$1\alpha,25(OH)_2D_3$ has long been known to possess immunomodulatory activity, affecting both innate and adaptive immune responses. $1\alpha,25(OH)_2D_3$ can suppress the production of a spectrum of inflammatory cytokines in the immune cells and other cells (such as keratinocytes), including IL-1, IL-2, IL-6, IL-8, INF-γ and TNF-α [8,28]. In addition to its principal function in physiological immune reactions, NFκB plays a pivotal role in the generation and maintenance of malignancies [29]. NFκB is responsible for promoting breast carcinogenesis involving tumor initiation, hyperinsulinemia, proliferation, antiapoptosis, angiogenesis, chemoresistance and metastasis [30].

Since NFκB plays a key role in inflammation [4] and tumorogenesis [2,30] and is highly activated by viral receptor of Kaposi Sarcoma (vGPCR), the question arises whether the antiproliferative effects of $1\alpha,25(OH)_2D_3$ on SVEC and SVEC-vGPCR [21] could be due to the inhibition of the NFκB pathway. To address this issue the activity of NFκB and its participation in SVEC-vGPCR proliferation was first examined. The results demonstrated that the transcriptional activity of NFκB and proliferation was inhibited by $1\alpha,25(OH)_2D_3$ and also in the presence of bortezomib which has been shown to inhibit NFκB activity. Since no additive effect was observed when both agents were added together, it could be suggested that the hormone and bortezomib may act by the same mechanism (Fig. 1). We found that $1\alpha,25(OH)_2D_3$ decreased NFκB and increased IκBα mRNA and protein levels in SVEC-vGPCR. Although the expression of the inhibitory protein IκBα was increased in SVEC cells, the hormone did not change NFκB gene expression (Fig. 2). This could be explained by the lack of expression of the viral receptor vGPCR, which would result in reduced activation of the NFκB pathway. Moreover, the percentage of inhibition of NFκB was more noticeable at the protein level in SVEC-vGPCR (Fig. 3) suggesting that in addition to regulate RNA expression, the hormone could also affect protein degradation. Inhibition of NFκB mRNA and protein levels has been shown to occur in other cell types such as myelomonocytic, dendritic and lymphocyte cells [31–33]. On the other hand, some reports demonstrated that the inhibition of the pathway may occur by inhibiting the proteasome regulating A20 (Tumor Necrosis alpha induced protein 3 or *TNFAIP3*) activity and thus, IκB maintains its cytoplasmic levels sequestering NFκB and retarding its traslocation to the nucleus [34]. More studies are need to ascertain whether proteosome inhibition is part of the mechanism of action of $1\alpha,25(OH)_2D_3$ in NFκB regulation. In this work, we also showed that $1\alpha,25(OH)_2D_3$ inhibited the translocation of NFκB to the nucleus. Although the association of NFκB to VDR has been shown to be part of the mechanism of action of $1\alpha,25(OH)_2D_3$ [13], our immunoprecipitation and confocal microscopy studies strongly suggest that the VDR does not interact with NFκB in SVEC and SVEC-vGPCR cells (Figs. 4 and 5). In addition, it was shown that the early suppression of NFκB activity by $1\alpha,25(OH)_2D_3$ is associated with increased IκBα expression. This increase is independent of MAPKs and PI3K/Akt pathways but depends on *de novo* protein synthesis (Fig. 5). One previous report has suggested that $1\alpha,25(OH)_2D_3$ elevates IκBα level by prolonging IκBα mRNA half-life and suppressing IκBα phosphorylation/degradation [20]. Others have suggested direct gene activation since a DR3-type vitamin D responsive element (VDRE) was found in IκBα (NFκB1a) gene promoter. Other nuclear receptors like the gluco-

corticoid receptor inhibit NFκB activity through induction of IκBα synthesis [35,36].

In conclusion, these results demonstrate that $1\alpha,25(OH)_2D_3$ down regulates the NFκB pathway, not only by inhibiting NFκB mRNA and protein levels, but also increasing IκBα expression and interfering with the translocation of NFκB to the nucleus in endothelial cells transformed by vGPCR.

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