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Vitamin D receptor deletion leads to reduced level of I κ B α protein through protein translation, protein-protein interaction, and post-translational modification

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Abstract

Vitamin D receptor plays an essential role in regulation of inflammation. Previous studies demonstrate that vitamin D receptor negatively modulates the pro-inflammatory NF- κ B pathway. However, it is unknown how vitamin D receptor regulates I κ B α , the endogenous inhibitor of NF- κ B. Here we investigated the molecular mechanism of vitamin D receptor deletion and I κ B α expression. We found that cells lacking vitamin D receptor had significantly increased levels of I κ B α mRNA and simultaneously decreased levels of I κ B α protein. Lacking vitamin D receptor abolished its binding to the I κ B α promoter. Moreover, the levels of protein translation regulators and the rate of protein synthesis were both decreased in cells lacking vitamin D receptor. At the post-translational level, I κ B α ubiquitination was enhanced, indicating increased degradation of I κ B α in the absence of vitamin D receptor. We further transfected cells with a plasmid carrying either wild-type or mutant I κ B α . The expression of wild-type I κ B α was much higher in the cells with vitamin D receptor than in the cells without vitamin D receptor, whereas the expression of exogenous I κ B α was equally high in both cell lines. In summary, vitamin D receptor deletion affects I κ B α through mRNA transcription, protein translation, protein-protein interaction, post-translational modification, and protein degradation, thus reducing the level of I κ B α protein. Cells lacking vitamin D receptor are known in a proinflammatory state with activation of NF- κ B. Our study provides new insight into vitamin D receptor regulation of an inhibitor of NF- κ B in inflammation. Deletion of vitamin D receptor contributes to the activation of NF- κ B on multiple levels.

Keywords

Vitamin D; Vitamin D receptor; I κ B α ; Inflammation; NF- κ B

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Introduction

The active form of vitamin D, 1, 25-Dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}_3$), is known to have anti-inflammatory activity. For example, vitamin D is an environmental factor that influences the course and severity of Inflammatory Bowel Disease (IBD) (Lim et al., 2005). Low levels of vitamin D have been reported in patients with IBD (Sentongo et al., 2002). In animal models, $1, 25(\text{OH})_2\text{D}_3$ suppressed the development of IBD (Cantorna et al., 2004). The vitamin D receptor (VDR) is required for all known biological effects of vitamin D. Accumulated evidences suggest that VDR signaling plays an essential role in the regulation of inflammation. Therefore, extensive studies are investigating the mechanism and potential application of $1, 25(\text{OH})_2\text{D}_3$, analogues and VDR agonists in the autoimmune diseases, such as type 1 diabetes, IBD, and multiple sclerosis (Giarratana et al., 2004, Gregori et al., 2002, Adorini et al., 2008, Nagpal et al., 2001, Hewison, 2008).

The nuclear factor- κB (NF- κB) family is a group of transcription factors that plays an essential role in inflammation. NF- κB is active in the nucleus, and its activity is inhibited by the inhibitor of $\kappa\text{B}\alpha$ (I $\kappa\text{B}\alpha$). I $\kappa\text{B}\alpha$ binds to NF- κB and blocks the nuclear localization signal so that the NF- κB dimer (p50 and p65) is retained in the cytoplasm. Phosphorylation of I $\kappa\text{B}\alpha$ by I κB kinase (IKK) initiates the ubiquitination and degradation of I $\kappa\text{B}\alpha$, leading to nuclear translocation and activation of NF- κB (Bonizzi et al., 2004). VDR has been shown to interact physically with NF- κB p65 in human osteoblasts (Lu et al., 2004) and mouse embryonic fibroblast cells (Sun et al., 2006), and VDR expression negatively regulates NF- κB activity (Sun et al., 2006). Of interest, the expression of I $\kappa\text{B}\alpha$ is also affected by the status of VDR. In mouse embryonic fibroblast cells (MEF) lacking VDR, the total level of I $\kappa\text{B}\alpha$ protein is only 40% of that in VDR^{+/-} cells (Sun et al., 2006). However, the functional relevance of VDR and I $\kappa\text{B}\alpha$ in regulating the activity of NF- κB remains unclear.

It is reported that $1, 25(\text{OH})_2\text{D}_3$ increases I $\kappa\text{B}\alpha$ levels by stabilizing I $\kappa\text{B}\alpha$ mRNA and decreasing the level of I $\kappa\text{B}\alpha$ phosphorylation, thus decreasing NF- κB activity in macrophages and keratinocytes (Cohen-Lahav et al., 2007, Cohen-Lahav et al., 2006, Riis et al., 2004). The vitamin D analog significantly down-regulates proinflammatory chemokine production by islet cells. Giarratana et al. found that the inhibition of islet chemokine is associated with up-regulation of I $\kappa\text{B}\alpha$ transcription and with arrest of NF- κB p65 nuclear translocation (Giarratana et al., 2004). Our data demonstrate that VDR ablation leads to a marked reduction in I $\kappa\text{B}\alpha$ protein in fibroblasts (Sun et al., 2006) and intestinal epithelial cells (Sun's unpublished data). By inference, $1, 25(\text{OH})_2\text{D}_3$ -bound VDR may help stabilize I $\kappa\text{B}\alpha$ in fibroblasts and epithelial cells. This may partially explain why VDR ablation leads to a decrease in I $\kappa\text{B}\alpha$ levels (Sun et al., 2006). However, the molecular mechanism of VDR regulation of I $\kappa\text{B}\alpha$ expression levels requires further investigation.

In the current study, we investigated the molecular mechanism by which VDR ablation leads to the reduction of the total I $\kappa\text{B}\alpha$ protein level. We used VDR^{+/-} and VDR^{-/-} cells as our experimental models to explore VDR regulation of I $\kappa\text{B}\alpha$ in terms of mRNA and protein expression levels, post-translational modification, and degradation. We chose VDR^{+/-} and VDR^{-/-} MEF cells because we wanted to compare the difference between VDR-null cells and those carrying one allele of the *Vdr* gene. We found that the VDR regulates I $\kappa\text{B}\alpha$ at multiple levels including mRNA transcription, protein translation, post-translational modification, and degradation by the proteasome. Results in this study may provide new insight into vitamin D receptor regulation of an inhibitor of NF- κB in inflammation.

Materials and Methods

Mouse Embryonic fibroblast (MEF) isolation and culture

MEFs were isolated from E13.5 embryos generated from $VDR^{+/-}$ x $VDR^{+/-}$ mouse breeding (Sun et al., 2006). Briefly, the embryos were harvested and placed in PBS during the removal of the internal organs, head, and four limbs. The remaining embryo bodies were minced individually and digested with 0.5% trypsin and 10 mM EDTA for 0.5 h at 37°C. The digested materials were gently pipetted to single-cell suspension. The cells were cultured in DMEM (high glucose, 4.5 g/l) containing 10% fetal bovine serum, 50 µg/ml streptomycin, and 50 U/ml penicillin. Cells from each embryo were genotyped by PCR using genomic DNA isolated from the cells. $VDR^{+/-}$ and $VDR^{-/-}$ MEFs were used in experiments after more than 15 passages, at which point they had been immortalized. In the present study, we used these MEFs to investigate the effect of VDR ablation on the IκBα expression. We used $VDR^{+/-}$ and $VDR^{-/-}$ MEFs in order to compare VDR-null cells with those carrying one allele of the *Vdr* gene, as we did in the previous studies (Sun et al., 2006, Sun et al., 2008).

Reverse transcription polymerase chain reaction (RT PCR) of mRNA IκBα

Total RNA was extracted from MEF cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. First-strand cDNA synthesis was performed with 1 µg of total RNA using the Superscript III First-strand synthesis system kit (Invitrogen). The primer sequences used were as follows: IκBα; 5'-ACGAGGAGTACGAGCAAATGGT-3' and 5' GTGAGTAGAGGCTAGGTGCAGACA-3' (464bp), GAPDH; 5'-GGCATGGACTGTGGTCATGAG-3' and 5'-CTTACCACCATGGAGAAGGC-3' (237 bp). The reaction mixture was subjected to 26 cycles of PCR amplification. PCR products were separated on 2% agarose gels.

Immunoblotting

MEF cells were rinsed twice in ice-cold HBSS, lysed in protein loading buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), and sonicated. Equal amount of protein was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with primary antibodies: anti-VDR, anti-p65, anti-IκBα, anti-eIF2α, anti-PKR, anti-phosphor-eIF2α, anti-phosphor-PKR, anti-IKKα, anti-IKKβ (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-phospho-IκBα (Cell Signal, Beverly, MA, USA), anti-ubiquitin antibody (Affiniti, Mamhead, Exeter, UK), or anti-β-actin (Sigma-Aldrich, Milwaukee, WI, USA) antibodies and visualized by ECL.

Treatment with actinomycin D

To determine whether transcription is required for VDR regulation of IκBα, MEF cells were treated for two hours with the RNA polymerase II inhibitor Actinomycin D (1 µg/ml) which was added directly to the culture medium. Actinomycin D was removed after two hours and cells were cultured for an additional 22 hours at 37°C with 5% CO₂. Cells were then treated with TNF for 0.5 hours and harvested. Levels of the indicated proteins were determined by Western blotting as described above.

Coimmunoprecipitation assay

Mouse colonic epithelial cells were collected by scraping from mouse colon including proximal and distal regions as previously described (Duan et al., 2007). Cultured MEF cells were rinsed twice in ice-cold HBSS. Cells or colonic tissues were lysed in cold immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM sodium orthovanadate) containing protease inhibitor cocktail (Boehringer Mannheim, Roche, Indianapolis, IN, USA). Samples were pre-cleared with protein A-agarose

(Invitrogen). Pre-cleared lysates were then incubated with 2 μ g of anti-p65 antibody (Santa Cruz Biotechnology) for 1 h at 4°C. A 50% slurry of Protein A-agarose was added to the lysate and incubated for 30 min with agitation at 4°C and then washed with cold immunoprecipitation buffer. The pellet was resuspended in 0.1 M glycine, pH 2.5, incubated with agitation for 10 min at 4°C and then centrifuged at 9,000 *g* for 2 min. The supernatant was removed and neutralized with 1 M Tris-HCl, pH 8.0. The samples were diluted with concentrated (5x) electrophoresis sample buffer (125 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β -mercaptoethanol), boiled for 5 min, separated by SDS-polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane. Membrane blots were probed with anti-I κ B α antibody.

Chromatin immunoprecipitation (CHIP) assay

Binding of VDR to the I κ B α promoter was investigated using the ChIP assay according to the protocol of Upstate Biotechnology, Inc. Briefly, MEF cells were treated with 1% formaldehyde for 10 min at 37°C. Cells were washed twice in ice-cold phosphate-buffered saline containing protease inhibitor cocktail tablets (Roche). Cells were scraped into conical tubes, pelleted and lysed in SDS Lysis Buffer. The lysate was sonicated to shear DNA into fragments of 200 to 1000 bp (4 cycles of 10 second sonication, 10 second pausing, Branson Sonifier 250, USA). The chromatin samples were pre-cleared with salmon sperm DNA-bovine serum albumin-sepharose beads, then incubated overnight at 4°C with VDR antibody (Santa Cruz Biotechnology). Immune complexes were precipitated with salmon sperm DNA-bovine serum albumin-sepharose beads. DNA was prepared by treatment with proteinase K, extraction with phenol and chloroform, and ethanol precipitation. PCR was performed using the following promoter-specific primers: I κ B α forward, 5'-TGGCGAGGTCTGACTGTTGTGG-3'; I κ B α reverse, 5'-GCTCATCAAAAAGTTCCCTGTGC-3' (230 bp); β -actin forward, 5'-TGCAGTGTGCGCGAAGC-3'; β -actin reverse 5'-TCGAGCCATAAAAGGCAA-3' (Anest et al., 2003).

Plasmids

Plasmid expression vectors encoding wild-type I κ B α or a hemagglutinin-tagged mutant I κ B α (pI κ B α S32/36A) were obtained from Sanjay B. Maggirwar (Sui et al., 2007).

Transient transfection

Cells were grown in 24-well plates in triplicate. At 70–80% confluence, the cells were transfected with DNA plasmid using LipofectAMINE (Invitrogen). After 24 h, the cells were lysed for Western blot analysis.

Statistical Analysis

Data are expressed as the mean $\pm$ SD. Differences between two samples were analyzed using Student's *t* test. P-values of 0.05 or less were considered significant.

Results

Cells lacking VDR have decreased total I κ B α protein and increased I κ B α mRNA

As measured by Western blotting, the total level of I κ B α protein in the VDR^{-/-} MEF cells was reduced significantly compared to the VDR^{+/-} MEFs (Fig. 1A). The total amount of I κ B α in VDR^{-/-} cells is about 40% that of the cells containing VDR. After determining that deletion of VDR reduces the amount of I κ B α protein in intestinal epithelial cells, the effect of VDR on I κ B α RNA levels was examined using RT PCR. We observed significantly higher levels of I κ B α mRNA in VDR^{-/-} MEFs compared to the VDR^{+/-} cells (Fig. 1B).

To examine the possibility that the difference in I κ B α protein levels between VDR^{+/-} and VDR^{-/-} cells was caused by the level of mRNA transcription, cells were treated with the RNA polymerase II inhibitor actinomycin D. RT-PCR experiments showed that actinomycin D treatment reduced the level of I κ B α mRNA below the detection threshold in VDR^{+/-} and VDR^{-/-} cells (Fig. 2A). As shown in Fig. 2B, actinomycin D was able to reduce the total level of I κ B α protein in VDR^{+/-} and VDR^{-/-} cells. TNF treatment induced I κ B α protein degradation. However, the effect of actinomycin D seemed to be similar in VDR^{+/-} and VDR^{-/-} cells, suggesting that exposure to actinomycin D resulted in transcriptional down regulation of I κ B α mRNA expression and resulting in reduction of protein levels in a VDR-independent manner.

A previous study in cells treated with TNF demonstrated that TNF induced transcription of the I κ B α mRNA while inducing the degradation of the I κ B α protein (Sun et al., 1993). The increased level of mRNA may complement the degradation of the I κ B α protein. Our data also showed an increase in I κ B α mRNA after only 30 minutes of TNF treatment (Fig. 2A), whereas TNF induced I κ B α protein degradation in VDR^{+/-} and VDR^{-/-} cells (Fig. 2B).

Deletion of VDR abolishes the binding of VDR and the I κ B α promoter

VDR acts as a transcription factor when it translocates into the nucleus. Our CHIP assay determined that VDR was able to bind with the I κ B α promoter in the VDR^{+/-} cells, whereas the binding was undetectable in the VDR^{-/-} cells (Fig. 2C). This result suggests that VDR deletion may affect I κ B α mRNA transcription.

Reduced total acetylation and transcription in VDR^{-/-} cells

Acetylation of the lysine residues at the N-terminus of histone proteins removes positive charges, thereby reducing the affinity between histones and DNA. This provides RNA polymerase and other transcription factors easy access to the promoter region (Turner et al., 1990). The level of total acetylated lysine (ace-lysine) was also determined here. The total level of acetylated lysine level was very low in the VDR^{-/-} cells, whereas acetylated lysine was easily detected in the VDR^{+/-} cells (Fig. 2D). These data suggest that the general acetylation of lysine was significantly decreased in the VDR knockout cells and may affect the transcription of many genes other than I κ B α .

Reduced translation regulated by eIF-2 α and PKR in VDR^{-/-} cells

We further tested the level of protein translation, which is regulated by eukaryotic initiation factor 2 (eIF-2 α) and PKR. In mammals, eIF-2 promotes Met-tRNAⁱ delivery to the 40S ribosome to initiate polypeptide chain synthesis. EIF-2 is composed of three subunits (α , β and γ) and binds Met-tRNAⁱ in a GTP-dependent manner to form the ternary complex, which then binds to the 40S subunit (Garcia et al., 2006). Once Met-tRNAⁱ is delivered, eIF-2 is released from the 48S initiation complex after eIF-5-mediated GTP hydrolysis (Garcia et al., 2006). The activity of eIF-2 is regulated by phosphorylation at S51 of its α subunit. Small increases in eIF-2 α phosphorylation can lead to an exacerbated effect on protein synthesis (Garcia et al., 2006). Our Western blot data showed a reduced level of phosphorylated-eIF2 α (p-eIF2 α) in the VDR^{-/-} MEF cells (Fig. 3).

PKR is the most-studied member of the eIF-2 α -specific kinase subfamily (de Haro et al., 1996). It is a serine/threonine kinase, characterized by two distinct kinase activities: autophosphorylation, which causes activation, and the phosphorylation of eIF-2 α (Galabru et al., 1987), which impairs eIF-2 activity, resulting in inhibition of protein synthesis (Rhoads, 1993). Our data showed a significant reduction of PKR and phosphorylated PKR in the VDR^{-/-} MEF cells (Fig. 3A and 3B).

Deletion of VDR affects I κ B α protein synthesis

We further hypothesized that protein synthesis is affected by the status of VDR in cells. Cycloheximide (CHX) was used to inhibit the protein synthesis. Cells were treated with CHX (20 μ g/ml) for 4 hours. We found that the I κ B α protein synthesis was reduced after only 1 hour of treatment. The level of I κ B α is reduced by about 40% after CHX for 4 hours. This indicates that the level of protein synthesis was higher in the VDR^{+/-} MEFs than in the VDR^{-/-} MEFs, thus, the rate of I κ B α reduction is slower in the VDR^{+/-} MEFs (Fig. 3C). A line chart further shows that the level of I κ B α protein synthesis is higher in VDR^{+/-} cells than in VDR^{-/-} cells (Fig. 3D). Taken together, these data indicate that the protein synthesis is reduced in VDR^{-/-} cells due to the VDR knockout.

Reduction of binding proteins leads to less I κ B α in VDR^{-/-} cells

I κ B α is known to bind to NF- κ B and block the nuclear localization signal, causing retention of the NF- κ B dimer (p50 and p65) in the cytoplasm. Interestingly, there is less total P65 protein in the VDR^{-/-} MEFs than in the VDR^{+/-} MEFs (Fig. 4A). In mouse colonic epithelial cells, increased VDR expression is correlated with increased levels of both I κ B α and p65. We reasoned that VDR deletion reduces the total amount of p65, and thus less I κ B α is needed to bind with p65. Our immunoprecipitation data showed that less I κ B α was pulled down by the anti-p65 antibody in VDR^{-/-} cells compared to VDR^{+/-} cells. Reduction of the level of binding proteins may contribute to the low level of I κ B α protein in VDR^{-/-} cells (Fig. 4B).

There is no difference in the phosphorylation of I κ B α protein in untreated VDR^{+/-} and VDR^{-/-} cells

I κ B α is modified at the post-translational level through phosphorylation and ubiquitination. Phosphorylation of I κ B α by I κ B kinase (IKK) initiates the ubiquitination and degradation of I κ B α , leading to nuclear translocation and activation of NF- κ B (Hayden et al., 2004, Neumann et al., 2007). We examined the expression of IKK proteins including IKK α and IKK β in MEF cells and intestinal epithelial cells. We found that the VDR^{-/-} MEF cells had lower levels of IKK α and IKK β (Fig. 5A). A similar trend was also observed in the VDR knock out mouse intestinal cells. The total level of IKK was only 57% of that in the wild-type (WT) cells *in vivo* (Fig. 5B). However, at the post translational level, the level of I κ B α phosphorylation was not higher in the MEF VDR^{-/-} cells than in the VDR^{+/-} cells in the absence of any treatment (data not shown).

VDR expression affects I κ B α Ubiquitination

I κ B α is phosphorylated and ubiquitinated, which targets the protein for degradation by the proteasome. We investigated whether VDR knockout reduced the level of I κ B α protein through increased ubiquitination. A major pathway for protein degradation is initiated by the addition of multiple 76-amino acid ubiquitin monomers via a three-step process of ubiquitin activation and substrate recognition. Polyubiquitination targets proteins for recognition and processing by the 26S proteasome, a cylindrical protein assembly that recognizes ubiquitinated proteins, degrades the proteins, and recycles ubiquitin. In the Western blot assay, ubiquitinated protein appears as a smear of bands above the regular band of the target protein. Cells were treated with the proteasome inhibitor MG262 to prevent the degradation of ubiquitinated proteins by the proteasome. We know that the total I κ B α in the VDR^{-/-} MEFs is only 40% of that in the VDR^{+/-} cells. To compare the degree of I κ B α ubiquitination based on equal amounts of total I κ B α , we adjusted the loading volume of the proteins (showed in Fig. 5C). The level of ubiquitinated I κ B α was higher in the VDR^{-/-} cells than in the VDR^{+/-} MEFs. In the presence of MG262, the level of ubiquitinated I κ B α was more enhanced in the VDR^{-/-} cells compared to the VDR^{+/-} cells (Fig. 5C). These data showed that I κ B α degradation was enhanced in the VDR^{-/-} MEFs.

Enhanced I κ B α protein degradation in VDR^{-/-} cells

Increased ubiquitination of I κ B α may lead to the enhanced protein degradation. To test our hypothesis that VDR deletion results in enhanced I κ B α protein degradation, we transfected a plasmid carrying full-length wild-type I κ B α into the MEF cells. Interestingly, we found that the transfected exogenous I κ B α protein was also expressed at low levels in the VDR^{-/-} cells. This could be explained by low transfection efficiency or increased protein degradation in VDR^{-/-} cells. We transfected cells with green-fluorescence protein tagged vector. There was no difference in transfection efficiency between these two cell lines (Supplemental data Fig. S1). We also used a plasmid expression vector encoding a mutant I κ B α (p I κ B α S32/36A) for transfection of the MEFs. It is known that this mutant I κ B α cannot be degraded by the proteasome (Sui et al., 2007). We found that the level of mutant I κ B α protein is much higher than that of WT I κ B α in the MEF VDR^{-/-} cells. In addition, we used the proteasome inhibitor MG262 to treat the cells after transfection and found that MG262 treatment stabilized I κ B α and enhanced the level of total protein in the MEF^{-/-} cells (Fig. 6). Taken together, these data indicate that degradation of I κ B α is enhanced in the VDR^{-/-} cells.

Discussion

The aim of the present study is to examine the cause of the reduction in the level of the I κ B α protein induced by the deletion of VDR. We examined the regulation of I κ B α at multiple levels including mRNA expression, protein expression, modification, and degradation. We found that VDR knockout cells have significantly increased levels of I κ B α mRNA and decreased levels of I κ B α protein. In the VDR knockout cells, the binding of VDR and the I κ B α promoter is abolished. The level of translation of I κ B α is lower in VDR^{-/-} MEFs than in VDR^{+/+} cells. At the post-translational level, I κ B α modification is also affected by the presence of VDR in the cells. Ubiquitination of I κ B α is enhanced and the rate of protein degradation is increased in VDR^{-/-} cells. Moreover, exogenous wild-type I κ B α introduced by transfection was degraded in VDR^{-/-} cells, whereas the expression of a phosphorylation-deficient mutant of I κ B α is much higher in the VDR^{-/-} cells. Overall, VDR deletion causes enhanced I κ B α degradation in VDR^{-/-} cells.

1,25(OH)₂D₃ is known to promote I κ B α mRNA stability and to decrease I κ B α phosphorylation, thus decreasing NF- κ B activity in macrophages, keratinocytes, and islet cells (Cohen-Lahav et al., 2007, Cohen-Lahav et al., 2006, Riis et al., 2004, Giarratana et al., 2004). Our previous results demonstrated by inference that vitamin D-liganded VDR may help stabilize I κ B α protein in fibroblasts and epithelial cells (Sun et al., 2006, Sun et al., 2008). In VDR^{-/-} cells, vitamin D cannot exert its effects through VDR activation. This partially explains why VDR deletion leads to a decrease in I κ B α levels. Our current studies further investigate the molecular mechanism of VDR regulation of I κ B α by examining mRNA expression, protein expression, post-translational modification, and degradation. We also tried to restore the VDR expression in VDR^{-/-} cells to investigate the level of I κ B α . We found that during VDR expression from the plasmid cDNA-hVDR in VDR^{-/-} MEFs, the level of I κ B α expression is only partially restored compared to VDR^{+/+} cells (data not shown). We suspect that the exogenous VDR has some functional alterations in comparison to endogenous VDR. However, 1,25(OH)₂D₃ treatment can increase the level of I κ B α protein when combined with VDR expression in the VDR^{-/-} cells. Moreover, when VDR was overexpressed in the cells, it inhibited TNF-induced I κ B α degradation (data not shown).

Our data show a reduction in translation regulated by eIF-2 α and PKR in VDR^{-/-} cells. In addition to its function in translational regulation, eIF-2 α phosphorylation has emerged not only as the main regulation point in protein synthesis but also as a critical trigger of the stress response (Garcia et al., 2006). PKR has a role in signal transduction and transcriptional control

through the I κ B α /NF- κ B pathway (Kumar et al., 1994). These aspects of cellular regulation require further investigation in the VDR^{-/-} cells under inflammatory stimulation.

Although we did not find any difference in the phosphorylation of I κ B α in unstimulated VDR^{-/-} and VDR^{+/-} cells, we found that the phosphorylation of I κ B α was enhanced in VDR^{-/-} immediately to the similar level as that in the VDR^{-/-} cells upon TNF treatment (data not shown). In addition, I κ B α was degraded rapidly in VDR^{-/-} cells upon TNF stimulation (data not shown). This indicates that cells lacking VDR are susceptible to inflammatory stimulation, as we previously reported (Sun et al., 2006). The protein enzyme A20 regulates NF- κ B activity through ubiquitination (Hitotsumatsu et al., 2008, Boone et al., 2004, Wertz et al., 2004). We measured the level of A20 expression in these two cell lines. Our data showed that the level of A20 is lower in the VDR^{-/-} MEFs compared with VDR^{+/-} MEFs. Lower levels of A20 would result in less inhibition of NF- κ B activity (Supplementary data Fig. S2). Therefore, not surprisingly, NF- κ B activity is enhanced in VDR^{-/-} cells compared to that in VDR^{+/-} cells (Sun et al., 2006).

I κ B α binds to NF- κ B and blocks the p56 nuclear localization signal. Our study showed reduced I κ B α /NF- κ B p65 physical interaction in VDR^{-/-} cells. Interestingly, NF- κ B p65 can bind to VDR (Sun et al., 2006). Because of the lack of VDR, the physical interaction between VDR and p65 was also absent in VDR^{-/-} cells. Consequently, the combined alterations including less protein-protein binding and less I κ B α inhibition may lead to free p65 and increase p65 translocation to the nuclei. Previous study showed a marked increase in nuclear p65 in VDR^{-/-} MEF without any stimulation (Sun et al., 2006).

In summary (Fig. 7), our current study answers fundamental questions about how VDR expression regulates the level of I κ B α . VDR deletion reduces the levels of the NF- κ B p65 protein and the translation regulators eIF2 α and PKR, and increases the level of I κ B α mRNA. Additionally, the absence of VDR prevents the binding of VDR with the I κ B α promoter. Post-translational modification of I κ B α is also affected by VDR status in these cells. In VDR^{-/-} cells, the ubiquitination of I κ B α and its resulting degradation are both enhanced. These factors all contribute to the reduction of total I κ B α in VDR^{-/-} cells. With reduced quantities of the NF- κ B inhibitor and no VDR/p65 binding in the cells, the activity of NF- κ B can reach high levels in the VDR^{-/-} cells in the absence of stimulation (Sun et al., 2006). The expression of inflammatory cytokines, such as TNF, is high in VDR^{-/-} cells (Sun et al., 2006). As a consequence of the proinflammatory state and activation of the NF- κ B pathway, VDR^{-/-} cells have significantly elevated levels of I κ B α mRNA as a response to the degradation of the I κ B α protein. Overall, deletion of VDR reduces the level of I κ B α protein, a potent inhibitor of NF- κ B, and activates NF- κ B and inflammation at multiple levels. Our studies provide new insight into the regulation of the I κ B α -NF- κ B pathway and inflammation by VDR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Levels of IκBα protein in VDR^{-/-} and VDR^{+/-} MEF cells

(A) IκBα expression in VDR^{-/-} and VDR^{+/-} MEF cells. MEF cells were cultured to 80% confluence. Cell lysates were immunoblotted with antibodies against IκBα and β-actin. The relative intensity of the IκBα band from MEF cell lysates was analyzed with NIH image. (B) IκBα RT-PCR in VDR^{-/-} and VDR^{+/-} MEFs. GAPDH was used as the internal control. Data are reported as the mean ± SD of three independent experiments. * $P < 0.05$ was considered significant.

Figure 2. The effects of VDR deletion on IκBα transcription in MEF cells

(A) Actinomycin treatment reduces the level of IκBα mRNA in MEF cells. MEF cells were treated for two hours with the RNA polymerase II inhibitor Actinomycin D (1 μg/ml) added to the culture medium. Actinomycin D was removed after two hours and cells were kept for an additional 22 hours. Cells were then treated with TNF for 0.5 hours and harvested. Levels of the indicated proteins were determined by Western blotting as described above. (B) Actinomycin treatment reduces the level of IκBα protein in MEF cells. (C) VDR deletion abolished the binding of VDR to the IκBα promoter. Binding of VDR to the IκBα promoter was investigated using the ChIP assay. PCR was performed using primers specific for the promoter of IκBα. Beta-actin was used as an internal control. (D) The acetylation of lysine in MEF cells. The total cell lysate was processed for immunoblotting to detect lysine acetylation and β-actin.

Figure 3. The effects of VDR deletion on the translation regulators eIF2 α and PKR in MEF cells
 (A) Western blot assay of the expression levels of eIF2 α , PKR, and their phosphorylated forms in VDR^{-/-} and VDR^{+/-} MEF cells. (B) Relative protein band intensities in MEF cells were analyzed with NIH image. Data are reported as the mean $\pm$ SD of three independent experiments. (C) Protein synthesis levels were affected by VDR status in MEF cells. Cells were treated with Cycloheximide (CHX) (20 μ g/ml) over 4 hours. Cell lysates were immunoblotted with antibodies against I κ B α and β -actin. (D) A line-chart shows the I κ B α protein density in the VDR^{+/-} and VDR^{-/-} cells treated with CHX.

Figure 4. The expression and regulation of p65 in MEF cells

(A) NF- κ B p65 expression is reduced in the VDR $^{-/-}$ cells. MEF cells were collected, and cell lysates were immunoblotted with antibody against NF- κ B p65. Relative NF- κ B p65 band intensity in VDR $^{-/-}$ and VDR $^{+/-}$ MEFs. (B) I κ B α physically interacts with NF- κ B p65 in intestinal epithelial cells *in vivo*. VDR mutation abolishes this interaction. Cell lysates were immunoprecipitated with anti-I κ B α antibody, and the precipitated complex was probed with anti-p65 antibody by Western blot (W.B. *upper*). An aliquot of the cell lysates was probed with antibodies against p65, I κ B α , and β -actin to confirm equal input (*lower*).

Figure 5. IkB α phosphorylation, ubiquitination, and degradation

(A) IKK α and IKK β expression in the VDR^{-/-} and VDR^{+/-} MEF cells. MEF cells were cultured to 80% confluence. Cell lysates were immunoblotted with antibodies against IKK α , IKK β , and β -actin. (B) IKK α and IKK β expression in VDR^{-/-} and VDR^{+/-} mouse intestinal epithelial cells. Mouse intestinal epithelial cells were scraped and collected as previously described (Duan et al., 2007). Cell lysates were immunoblotted with antibodies against IKK α , IKK β , and β -actin. Relative IkB α band intensities were analyzed with NIH image. (C) Ubiquitination of IkB α in VDR^{+/-} and VDR^{-/-} MEF cells treated with MG262 for 4 h. Please note the bands above the regular IkB α protein. Data are representative of at least three independent experiments.

Figure 6. Enhanced IκBα protein degradation in VDR^{-/-} cells

(A) Less exogenous IκBα was present after transfection of VDR^{-/-} cells compared to VDR^{+/-} cells. MEF cells were transfected with a plasmid carrying T7-wild-type IκBα for 30 hours. Lipo: lipofectamine 2000. The expression of IκBα was detected by Western blotting. Please note that there are two bands for endogenous and exogenous IκBα respectively. (B) Mutant IκBα was stabilized in VDR^{-/-} cells. Cells were transfected with a wild-type IκBα or a hemagglutinin-tagged mutant IκBα with modified phosphorylation sites (IκBα S32/36A). The level of IκBα was determined by Western blotting. (C) MG262 treatment stabilized the IκBα protein. Cells were transfected with wild-type IκBα or a hemagglutinin-tagged mutant IκBα (S32/36A). The expression of IκBα was detected by Western blotting. Cells treated with MG262 (20 μM) for 2 hours had increased levels of IκBα protein. Data are representative of at least three independent experiments.

Figure 7. Schematic illustration of VDR deletion and NF-κB activation

The VDR deletion contributes to the reduction of the IκBα, activation of the NF-κB pathway, and inflammation at multiple levels.