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Inhibitory effect of NF-κB on 1,25-dihydroxyvitamin D_3 and retinoid X receptor function

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Farmer, Paul K., Xiaofei He, M. Lienhard Schmitz, Janet Rubin, and Mark S. Nanes. Inhibitory effect of NF-κB on 1,25-dihydroxyvitamin D_3 and retinoid X receptor function. *Am J Physiol Endocrinol Metab* 279: E213–E220, 2000.—Responsiveness to 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] may be diminished in osteoporosis and inflammatory arthritis. The inflammatory cytokine tumor necrosis factor-α (TNF-α) is produced in excess in these disorders and has been shown to decrease osteoblast transcriptional responsiveness to vitamin D and to inhibit the binding of the vitamin D receptor (VDR) and its nuclear partner the retinoid X receptor (RXR) to DNA. Previous studies have shown that a vitamin D (VDRE) or retinoid X DNA response element (RXRE) is sufficient to confer TNF-α inhibition of vitamin D or retinoid-stimulated transcription in the absence of known TNF-α-responsive DNA sequences. We tested the hypothesis that the TNF-α-stimulated transcription factor nuclear factor (NF)-κB could, in part, mediate TNF-α action by inhibiting the transcriptional potency of the VDR and RXR at their cognate *cis* regulatory sites. Osteoblastic ROS 17/2.8 cells transfected with a dose of NF-κB comparable to that stimulated by TNF-α decreased 1,25$(OH)_2D_3$-stimulated transcription. This inhibitory effect of NF-κB was not observed on basal transcription of a heterologous reporter in the absence of the VDRE. The effects of NF-κB and TNF-α were comparable but not additive. COS-7 cells were cotransfected with reporters under the regulation of VDRE or RXRE along with vectors expressing VDR, RXR, and NF-κB nuclear proteins. Reconstituted NF-κB and the NF-κB subunit p65 alone, but not p50, dose dependently suppressed basal and ligand-stimulated transcription. p65 overexpression completely abrogated enhanced VDRE-mediated transcriptional activity in response to 1,25$(OH)_2D_3$. Electrophoretic mobility shift experiments did not reveal a direct effect of recombinant NF-κB or its individual subunits on the binding of heterodimeric VDR-RXR to DNA. These results suggest that TNF-α inhibition of hormone-stimulated transcriptional activation may be mediated by activation of NF-κB. In contrast, the inhibitory effect of TNF-α on binding of receptors to DNA is unlikely to be mediated by NF-κB and is not necessary for inhibition of transcription.

nuclear factor-κB; tumor necrosis factor-α; vitamin D receptor; vitamin D

CROSS TALK BETWEEN DIFFERENT intracellular signaling pathways is important for the coordination of cell regulatory signals. The proinflammatory cytokine tumor necrosis factor-α (TNF-α) is a pleiotropic regulator that has an important role in the pathophysiology of numerous disorders, including osteoporosis and periarticular bone loss in inflammatory arthritis (5). In these disorders, TNF-α is expressed in excess, and blocking TNF-α action prevents bone loss (1, 17, 19). At the cellular level, TNF-α inhibits the production of important skeletal matrix proteins by osteoblasts and activates the recruitment of bone-resorbing osteoclasts from their progenitor cells (22). TNF-α may also cause resistance to 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], a secosteroid required for optimum fractional absorption of calcium by the intestine, a process that is decreased in osteoporosis, contributing to bone loss over time (18, 32, 33). Resistance to vitamin D also impairs stimulation of osteocalcin production by osteoblasts and suppresses the expression of β_3-integrin, proteins that are needed for cell matrix recognition in bone (1–4, 6). TNF-α could contribute toward bone loss in osteoporosis and inflammatory arthritis, in part, by stimulating cell resistance to 1,25$(OH)_2D_3$.

TNF-α controls the expression of a number of inflammatory and immune regulatory genes through activation of the nuclear transcription factor kappa B (NF-κB). NF-κB has been shown to function predominantly as a heterodimer of p65 (RelA) and p50 (RelB1). A classic pathway has been delineated in which receptor-bound TNF-α stimulates a kinase cascade that phosphorylates a large cytoplasmic multiprotein complex containing the inhibitor of NF-κB (IκB)-α protein (10, 25, 28). The TNF-α-induced NH_2-terminal phosphorylation of IκB liberates NF-κB from this complex for nuclear translocation. Prototypical NF-κB is a heterodimeric transcription factor consisting of the p50 (NF-κB1) and p65 (RelA) subunits. Nuclear binding of NF-κB to its cognate DNA response element and resulting transcriptional stimulation have been described for a variety of genes, including intracellular

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adhesion molecule (3, 8), interleukins (IL-2, IL-6, IL-8; see Refs. 21, 36, 42), and the human immunodeficiency-1 virus long terminal repeat (6, 11, 23, 37). In these examples of NF-κB-mediated gene regulation, the effects of TNF-α are generally stimulatory. Less is known about the inhibitory effects of TNF-α on transcription, particularly with regard to skeletal matrix proteins.

Our laboratory has described an inhibitory effect of TNF-α on the transcription of osteocalcin, a vitamin D-stimulated skeletal protein that is a unique product of mature osteoblasts (31). Vitamin D stimulation of the osteocalcin gene is mediated by binding of the vitamin D receptor (VDR), a member of the thyroid/steroid hormone nuclear receptor superfamily. The VDR binds to DNA as a heterodimer with the retinoid X receptor (RXR), another member of the nuclear receptor superfamily. TNF-α treatment of osteoblasts inhibits binding of the RXR/VDR heterodimer to the vitamin D response element (VDRE) and inhibits VDR-mediated transcriptional activation of the osteocalcin gene. Deletion analysis of the osteocalcin promoter revealed that the VDRE alone was sufficient to confer both transcriptional activation by the VDR and inhibition by TNF-α. This conclusion was also confirmed by using a heterologous minimal promoter containing a single copy of the osteocalcin VDRE (20, 30, 31). TNF-α-induced de novo synthesis of other protein mediators is unlikely to cause the inhibitory effect of TNF-α on VDR function, because the effect is rapid and persists in the presence of cycloheximide (12). We recently reported that TNF-α also inhibits transcriptional activation of a heterologous promoter-reporter construct containing an upstream RXR response element (RXRE) that binds an RXR homodimer (12). These results suggest that extensive cross talk may exist between one or more signaling molecules in TNF-α-stimulated pathways and members of the vitamin D/nuclear receptor superfamily.

Because NF-κB mediates many effects of TNF-α, we hypothesized that NF-κB might mediate TNF-α-induced inhibition of VDR and RXR function. NF-κB exists preformed in the cytoplasm. Therefore, new protein synthesis would not be required for its mediation of TNF-α inhibitory action. Here we report that NF-κB does indeed inhibit VDRE- or RXRE-dependent transcription through a p65-dependent mechanism. Surprisingly, p65 inhibition of vitamin D- or retinoid-stimulated transcription is not associated with inhibition of nuclear receptor binding to DNA, suggesting that NF-κB does not account for all of the TNF-α action on nuclear hormone receptors.

METHODS

Reagents. Recombinant human TNF-α was purchased from R&D Systems (Minneapolis, MN) or Peprotech (Rocky Hill, NJ). $1,25(OH)_2D_3$ and 9-*cis*-retinoic acid (9cisRA) were obtained from BioMol (Plymouth Meeting, PA) and Sigma (St. Louis, MO), respectively. A double-stranded consensus NF-κB oligonucleotide probe and purified recombinant NF-κB p50 protein were obtained from Promega (Madison, WI). The VDRE oligonucleotide probe used in gel shift assays was synthesized in the Emory University Microchemical Facility (20). Recombinant human VDR was obtained from Affinity BioReagents (Golden, CO). All polyclonal antibodies directed against NF-κB proteins were obtained from Santa Cruz (Santa Cruz, CA). 4RX-3A2.1.1 RXR and the IVG8C11 VDR antibodies were obtained from Drs. P. Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, France) and H. DeLuca (Univ. of Wisconsin). [γ-^{32}P]ATP and [^{14}C]chloramphenicol were obtained from Amersham (Arlington Heights, IL) and New England Nuclear (Boston, MA), respectively. Other reagents for cell culture, buffers, and enzymes were obtained from commercial sources.

Plasmids and protein expression. The $VDRE_2$TKCAT plasmid provided by Dr. M. Demay (Massachusetts General Hospital, Boston, MA) contains two copies of a VDRE from the rat osteocalcin gene located upstream from a minimal thymidine kinase (TK) promoter and a bacterial chloramphenicol acetyltransferase (CAT) reporter (original plasmid designation: $1D_3$#4). This construct was selected because the two backward-facing VDRE copies from the rat osteocalcin promoter provide greater responsiveness to $1,25(OH)_2D_3$ treatment than does a single response element (9, 30). The RXRE-CAT containing a single RXRE direct repeat from the RXRE multimer found in the cellular retinol binding-protein II gene promoter was described previously (12). The pGEX-2TK prokaryotic expression vector and the pSPUTK in vitro translation vector were obtained from Pharmacia Biotech (Piscataway, NJ) and Stratagene (La Jolla, CA), respectively. The pSV40-β-galactosidase and the pBLCAT2 plasmids were obtained from Promega. The pRcCMV2 control vector was purchased from Invitrogen (San Diego, CA). Cytomegalovirus (CMV)-driven eukaryotic expression vectors for human VDR and human RXRα were courtesy of Dr. L. Freedman (Sloan Kettering, NY). The CMV-driven expression vectors for human p50 and c-Rel and the $(\kappa B)_6$-luciferase reporter construct were described previously (14, 16, 39). Dr. T. Maniatis (Harvard University, Boston, MA) provided the pRcCMV-p65 vector. A CMV-driven control plasmid encoding Renilla luciferase was obtained from Promega.

*Eco*R I-containing primers were used to amplify the human RXRα cDNA contained in pRcCMV2-hRXRα (sense primer: ccggaattcttatggacaccaaacatttcctgccgc; antisense primer: ccggaattctctaagtcatttggtgcggcg). This PCR product was cloned directly into a pGEM-T vector, excised with *Eco*R I, and ligated to the pGEX-2TK vector. Induced expression of the GST-hRXRα fusion protein in *Escherichia coli*, chromatographic purification, and enzymatic cleavage to release full-length hRXRα protein were accomplished according to instructions from the manufacturer (Pharmacia). Similarly, *Bam*H I-containing primers were used to amplify the human NF-κB p65 cDNA contained in the pRcCMV2-p65 plasmid (sense primer: cgcgcggatccaccatggacgaactgttccccctc; antisense primer: cgcgcggatccttaggagctgatctgactcag). The *Bam*H I-cut PCR fragment was ligated to a *Bgl* II-linearized pSPUTK plasmid (compatible ends). This in vitro translation plasmid was used to program a quick-coupled transcription/translation wheat germ system (Promega). The identity of the translated product was verified using a Western blot and the enhanced chemiluminescence detection kit (Amersham). Some translations included [^{35}S]methionine to electrophoretically confirm expression of full-length p65.

Cell culture, transient transfection, and transcription assays. COS-7 cells originally obtained from the American Type Culture Collection (Rockville, MD) were selected because they are VDR deficient and provide a low background of RXRα and NF-κB compared with many other cells (38). The COS cells were seeded at 1×10^5/60-mm dish and were

incubated for 48 h in MEM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Hyclone, Logan, UT). Cells were then transiently transfected for 4 h in serum-free DMEM using a 1:5 mass ratio of DNA to Lipofectin according to the manufacturer's specifications (Life Technologies). The final serum concentration was adjusted to 2% by addition of an equivalent volume of MEM containing 4% FBS. Depending on the protocol, this medium was supplemented by inclusion of 100 ng/ml TNF-α, 1 μM 9cisRA, or 10 nM $1,25(OH)_2D_3$. Fresh medium containing TNF-α or ligand was applied the next morning. Cell extracts for reporter assays or nuclear extracts for electrophoretic mobility shift assay (EMSA) were harvested 48 h posttransfection. Expression vectors encoding nuclear receptors or NF-κB subunits were cotransfected in the amounts indicated for each experiment along with 4 μg of $VDRE_2TKCAT$ or RXRE-CAT reporter plasmids. Differences in cell number or transfection efficiency were controlled for by inclusion of either pSV-β-galactosidase or the CMV-Renilla plasmids. Total transfected DNA was held constant by inclusion of variable amounts of nonspecific plasmid. CAT activity was measured as previously described (40). Background CAT activity in assays was <5%. Transcriptional activity was calculated and expressed as (CAT − background)/β-galactosidase.

Nuclear extract isolation and EMSA. A rapid isolation of nuclear proteins was achieved using a procedure developed specifically for COS-7 cells (2). This method entails hypotonic lysis of cells coupled with high salt extraction of proteins from pelleted nuclei. Nuclear extracts were divided into aliquots, snap-frozen in liquid nitrogen, and stored at −70°C. Protein concentrations were determined using a Bio-Rad reagent and BSA standards from Pierce (Rockford, IL).

Blunt-ended oligonucleotide probes for the EMSA were radiolabeled using T4 polynucleotide kinase and [γ-^{32}P]ATP in an imidazole buffer. Reactions were quenched by the addition of 25 mM EDTA. Probes were then extracted with chloroform and purified on Quick Spin G50 columns (Boehringer Mannheim, Indianapolis, IN) to remove unincorporated ATP. Each 20-μl binding reaction was initiated on ice and contained a defined amount of nuclear protein in a buffer containing 10 mM HEPES-KOH (pH 7.9), 84 mM NaCl, 50 mM KCl, 0.2 mM EDTA, 2.5 mM dithiothreitol, 10% glycerol, 0.05% Nonidet P-40, and 0.05 μg/ml poly(dI-dC). When antiserum was added to induce supershifts, reactions were incubated an additional 20 min on ice. After addition of probe, samples were incubated another 30 min at room temperature. A native 5% polyacrylamide gel prepared with 1× TGE buffer, pH 8.5 (10× TGE stock contains 30 g Tris base, 147.2 g glycine, and 3.99 g EDTA-Na_2/l) was prerun at 4°C and 100 volts during this incubation. Samples were electrophoresed for 15 min at 100 volts, followed by an additional 60 min at 200 volts without recirculating the 1× TGE running buffer.

Statistics. Differences between multiple treatments were detected using one-way ANOVA. The Student-Newman-Keuls method was used for multiple comparisons among groups. Dunnett's test was employed for multiple comparisons between a control and several treatment groups. A confidence level of 0.05 was selected for all statistical analyses. Statistical analyses were performed using SigmaStat version 1.0 (SPSS, Chicago, IL).

RESULTS

Verification of NF-κB expression. The successful transfection and expression of NF-κB subunits was verified in nuclear extracts from COS-7 cells using an EMSA that employed a labeled consensus NF-κB probe and specific antiserum to supershift DNA-protein complexes (Fig. 1*A*). *Lane 1* shows unhindered migration of the free probe in the absence of nuclear extract. *Lane 2* shows only nonspecific binding of probe to nuclear extract from untransfected control cells. Transfection with the p50 expression vector produced a retarded band (*lane 3*) that was completely supershifted upon addition of p50-specific antiserum (*lane 4*). The intensity of this complex reflected both abundant nuclear p50 protein and a strong binding affinity between the p50 homodimer and the DNA response element. At least two faint retarded bands, both exhibiting reduced

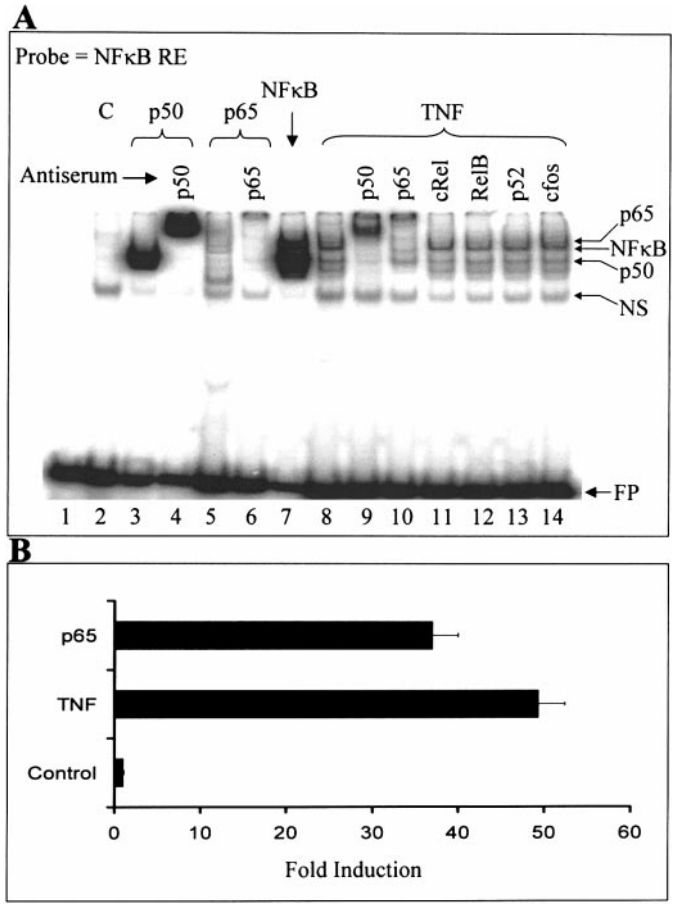

Fig. 1. Functional nuclear factor (NF)-κB can be expressed and appear in the nucleus. COS-7 cells were transfected with vectors expressing p65, p50, or both, and nuclear extract was isolated to assess NF-κB. *A*: electrophoretic mobility shift assay (EMSA) showing binding of NF-κB to a consensus ^{32}P-labeled NF-κB probe. *Lane 1*, probe only; *lanes 2–7*, nuclear extracts from cells transfected with 1 μg of empty pRcCMV2 control vector (*lane 2*) or similar cytomegalovirus (CMV)-driven expression vectors encoding p50 (*lanes 3–4*), p65 (*lanes 5–6*), or both NF-κB proteins (*lane 7*); *lanes 8–14*, nuclear extracts from cells treated with 100 ng/ml tumor necrosis factor (TNF)-α. Labels immediately above lanes indicate the addition of antibodies directed against specific NF-κB subunits or a control antibody to c-Fos. The positions of shifted bands containing homodimeric p65, NF-κB, and homodimeric p50 are shown on *right*. NS, unidentified nonspecific complex. FP, free probe. *B*: normalized luciferase reporter activities in COS-7 cells transfected with 0.1 μg of the $(κB)_6$-luciferase plasmid, ±1 μg of p65 expression vector, ±100 ng/ml of TNF-α treatment. Results are the means ± SE for triplicate samples.

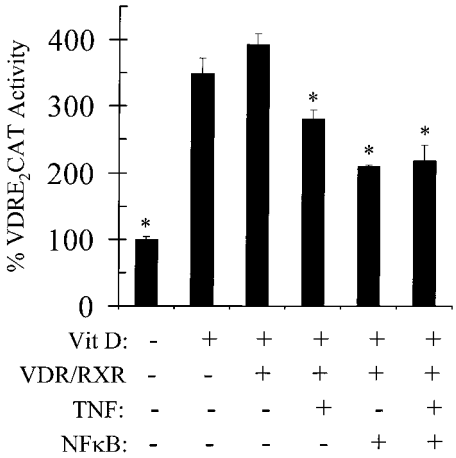

Fig. 2. Combined effects of NF-κB expression and TNF-α treatment on vitamin D receptor response element (VDRE) reporter activity in ROS 17/2.8 cells. Cells were transfected with 2.5 μg of the VDRE2TKCAT plasmid, 1 μg each of the NF-κB p50 and p65 expression vectors, and 0.1 μg of the retinoid X receptor (RXR) and vitamin D receptor (VDR) expression vectors as shown. All cells except those in the control group on the far left were then treated for 48 h with 10 ng/ml of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] either in the presence or absence of 100 ng/ml TNF-α as shown. CAT, chloramphenicol acetyltransferase; Vit D, vitamin D. Results show means ± SE; $n = 3$. *$P < 0.05$ vs. $1,25(OH)_2D_3$-treated positive control group (lacking exogenous receptor expression).

gel mobilities compared with p50, were observed in nuclear extracts from p65-transfected cells (*lane 5*). One of these bands comigrated with a DNA-protein complex produced by TNF-α treatment, indicating that this complex contained a heterodimer consisting of plasmid-encoded p65 and a small amount of endogenous p50. Because a p65-specific antiserum supershifted both bands, the second larger complex probably contained a p65 homodimer (*lane 6*). Simultaneous transfection of both subunits (i.e., reconstituted NF-κB) produced a pattern similar to that caused by TNF-α in terms of the positions of the retarded bands (*lane 7*). However, transfection yielded far greater levels of nuclear NF-κB and the putative p50 homodimer than did treatment with TNF-α (*lane 8*). Out of a panel of five Rel family-specific antisera and one control antiserum directed against c-Fos, only the p50 and p65 antisera produced supershifts, implying that TNF-α-stimulated NF-κB in COS-7 cells is primarily a p50-p65 heterodimer (*lanes 9–14*).

The weak shifts produced by p65 transfection likely reflected its low DNA binding affinity, an observation that led earlier investigators to incorrectly propose that p65, which itself mediates transcriptional activation at NF-κB-responsive elements, is devoid of DNA-binding potential (34). Nevertheless, to guarantee that expression of p65 caused nuclear translocation of an active p65 moiety, a $(κB)_6$-luciferase reporter was transfected alone or in combination with the p65 expression vector. The p65 vector caused a >35-fold increase in reporter activity compared with a 50-fold induction achieved by TNF-α (Fig. 2*B*). These observations indicate that both the amount and the activity of nuclear p65, the transcriptionally active subunit of NF-κB, were increased by transfection with the CMV-driven expression vector.

TNF-α and NF-κB block VDRE-dependent reporter activity. To evaluate whether expression of reconstituted NF-κB mimics TNF-α inhibition of VDRE-dependent activity, osteoblastic ROS 17/2.8 cells were transfected with both subunits plus a VDRE reporter. CMV-driven expression vectors encoding VDR and RXR were cotransfected to avoid decreased reporter activity that might arise from TNF-α inhibition of receptor abundance (26). ROS cells were selected for this comparison because, unlike in COS-7 cells, where nuclear NF-κB from transfection may greatly exceed that obtained from TNF-α stimulation, both transfection and TNF-α produce similar levels of nuclear NF-κB in these rat osteosarcoma cells (data not shown).

Cells treated with $1,25(OH)_2D_3$ had comparable 3.5-fold increases in $VDRE_2$TKCAT activity. Addition of excess VDR and RXR did not significantly enhance the response to $1,25(OH)_2D_3$ in these osteoblastic cells (Fig. 2). TNF-α blocked the $1,25(OH)_2D_3$-stimulated increase by ~30%. Expression of the two NF-κB subunits similarly blocked $1,25(OH)_2D_3$-stimulated transcription. Combined treatment with TNF-α and NF-κB expression was not additive, suggesting that TNF-α-induced suppression may have occurred, in part, through NF-κB activation.

NF-κB inhibition requires a hormone response element. To rule out nonspecific or generalized effects of NF-κB on transcription, a pBLCAT2 plasmid containing a minimal TK promoter and CAT reporter, similar to that in $VDRE_2$TKCAT but lacking hormone response elements, was transfected in parallel with the $VDRE_2$TKCAT reporter. As expected, treatment with $1,25(OH)_2D_3$ caused a 2.5-fold increase in $VDRE_2$TKCAT activity but did not stimulate activity from pBLCAT2 (Fig. 3). Both basal and $1,25(OH)_2D_3$-stimulated activities from $VDRE_2$TKCAT were inhibited ~50% by NF-κB. However, NF-κB had no effect on basal activity from pBLCAT2. Thus NF-κB inhibition

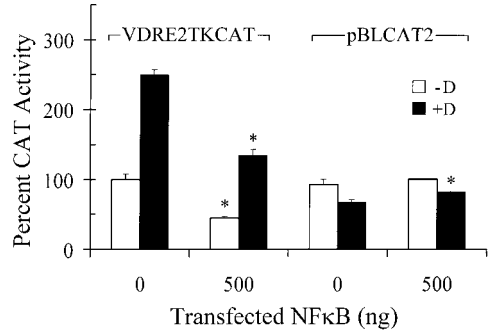

Fig. 3. NF-κB does not inhibit transcription in the absence of the hormone response element. COS-7 cells were transfected with either the VDRE2TKCAT or the pBLCAT2 transcriptional reporters (the latter lacking VDRE) and vectors expressing RXR, VDR, p50, or p65 as indicated. The effect of the NF-κB proteins on $1,25(OH)_2D_3$-stimulated transcription was then determined. Open bars, untreated (−D); filled bars, 10 nM $1,25(OH)_2D_3$ (+D). Results show means ± SE; $n = 3$. *$P < 0.05$ vs. groups lacking plasmids encoding NF-κB expression.

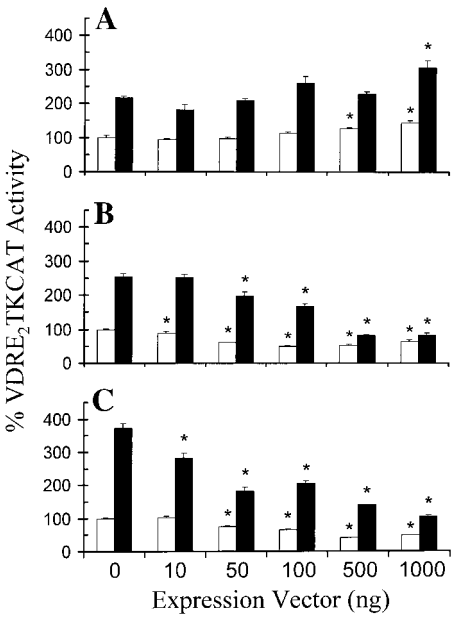

Fig. 4. NF-κB inhibition of vitamin D-stimulated transcription is dose dependent and mediated by p65. Dose-response relationship showing effect of increasing amounts of transfected NF-κB expression vectors p50 (*A*), p65 (*B*), or p50 + p65 (NF-κB; *C*) on basal and $1,25(OH)_2D_3$-stimulated transcription in COS-7 cells. Cells were transfected with the VDRE-CAT reporter as in Fig. 3. Open bars, untreated; filled bars, 10 nM $1,25(OH)_2D_3$. TK, thymidine kinase. Results show means ± SE; $n = 3$. $^*P < 0.05$ vs. groups lacking NF-κB (0 ng expression vector).

of basal and hormone-stimulated transactivation required the presence of a VDRE sequence.

NF-κB confers dose-dependent inhibition of VDRE- and RXRE-dependent activity. Because COS-7 cells represented a more responsive system for evaluating NF-κB effects on hormone-dependent reporter activity, these cells were transfected with varying amounts of the expression vectors encoding the NF-κB subunits together with vectors encoding RXR and VDR and a $VDRE_2TKCAT$ reporter. As seen in Fig. 4, VDRE-directed CAT activity increased 2.5- to 3.5-fold in response to $1,25(OH)_2D_3$. Both basal and $1,25(OH)_2D_3$-stimulated CAT activities were dose dependently suppressed in response to either p65 alone or p65 in conjunction with p50 (i.e., NF-κB). $1,25(OH)_2D_3$ stimulated VDRE-dependent activity above unstimulated levels at each of the five NF-κB doses. However, hormone-dependent stimulation was gradually lost in cells transfected with p65 alone. A small increase in both unstimulated and $1,25(OH)_2D_3$-stimulated activity occurred with increasing p50. However, significantly elevated levels were only observed at the highest doses of this expression vector. The p50 doses used to achieve this modest stimulation were at least one order of magnitude higher than the levels of p65 or NF-κB required to inhibit basal and $1,25(OH)_2D_3$-stimulated activity.

Similar experiments were performed to evaluate the potential effects of NF-κB subunits on RXR-dependent transcription. In this case, however, an RXRE-TKCAT reporter was transfected instead of $VDRE_2TKCAT$, and the VDR expression vector was excluded to prevent competitive formation of VDR-RXR heterodimers in these VDR-deficient cells. In addition, cells were exposed to the RXR-cognate ligand 9cisRA instead of $1,25(OH)_2D_3$. An approximate doubling of RXRE-dependent reporter activity occurred in response to 9cisRA (Fig. 5). Expression of p50 failed to alter basal or 9cisRA-stimulated activity. In contrast, both p65 alone and reconstituted NF-κB caused similar dose-dependent suppression of basal and ligand-stimulated activity, again raising suspicion that p65 might be the active inhibitory subunit within NF-κB. Interestingly, 9cisRA continued to stimulate RXRE-TKCAT activity at the highest p65 dose, whereas $1,25(OH)_2D_3$ stimulation of $VDRE_2TKCAT$ activity was abolished at this same DNA concentration.

A third member of the Rel family was also evaluated to determine if inhibition of receptor function might be specific to the p65 subunit or a general phenomenon among members of the Rel family. In experiments similar to those described above, transfection of a CMV-driven c-Rel expression vector did not alter either basal or ligand-stimulated reporter activities from either the $VDRE_2TKCAT$ or the RXRE-TKCAT constructs (data not shown).

NF-κB does not influence binding of VDR/RXR heterodimers to DNA. Because TNF-α perturbs binding of VDR and RXR to their cognate DNA response elements, we examined whether the NF-κB subunits might directly block the nuclear receptors from binding to DNA (12). Purified forms of recombinant human RXR and VDR proteins were assessed for their ability to bind a VDRE either alone or in the presence of

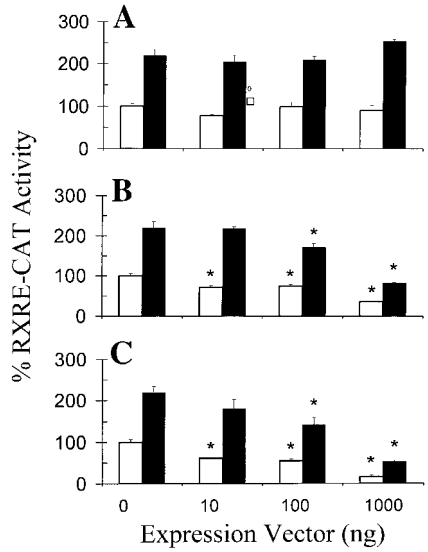

Fig. 5. NF-κB inhibition of retinoid-stimulated transcription is dose dependent and mediated by p65. Dose-response relationship showing effect of increasing amounts of transfected NF-κB expression vectors p50 (*A*), p65 (*B*), or p50 + p65 (NF-κB; *C*) on basal and 9-*cis*-retinoic acid (9cisRA)-stimulated transcription from an RXR response element (RXRE)-CAT reporter in COS-7 cells. Open bars, untreated; filled bars, 1 μM 9cisRA. Results show means ± SE; $n = 3$. $^*P < .05$ vs. groups lacking NF-κB (0 ng expression vector).

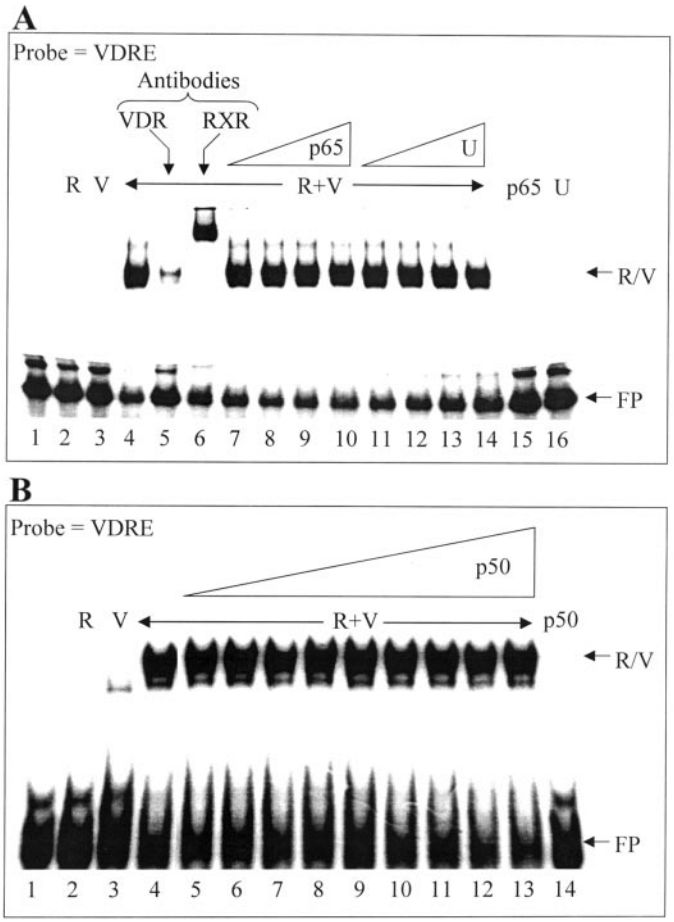

Fig. 6. NF-κB does not bind directly to the VDRE or RXRE sequence and does not inhibit binding of VDR or RXR to DNA. EMSA assessing the effects of NF-κB p65 (*A*) and p50 (*B*) on binding of recombinant VDR-RXR heterodimers to a ^{32}P-labeled rat osteocalcin VDRE probe. The positions of the free probe (FP) and the retarded probe bound by RXR/VDR heterodimers (R/V) are shown. VDR and RXR antibodies in *lanes 5* and *6* were IVG8C11 and 4RX3A2.1.1, respectively. *A*: increasing amounts of wheat germ extract from p65-programmed (p65) or unprogrammed (U) translations in the amounts of 0.01, 0.1, 1, and 5 μl were added to binding reactions in *lanes 7–10* and *11–14*, respectively. *Lanes 15* and *16* contained 5 μl of either p65-programmed or unprogrammed translations in the absence of recombinant RXR (R) and VDR (V). *B*: highly purified recombinant p50 protein in volumes ranging from 0.0001 to 1.0 μl (*lanes 5–13*) was combined with probe and both nuclear receptors. *Lane 14* contained 1.0 μl of p50 without nuclear receptors.

recombinant p50 or p65. EMSA revealed that the combination of RXR and VDR produced strong binding to a VDRE probe, but neither receptor interacted strongly with the DNA by itself (Fig. 6). The identity of the RXR-VDR heterodimer was established using VDR and RXR antibodies that either blocked protein-DNA interactions or supershifted the protein-DNA complex, respectively. Neither recombinant p65 protein that had been translated using an efficient wheat germ extract nor highly purified p50 produced in baculovirus-infected Sf9 insect cells showed any evidence of direct binding to this VDRE probe. Similarly, neither NF-κB subunit, when incubated with the two nuclear receptors, caused any change in DNA binding of the nuclear receptors.

DISCUSSION

These experiments were performed to evaluate whether TNF-α inhibition of receptor-dependent transactivation might be mediated in part by NF-κB or its protein subunits. The strong expression of p50 and p65 by our CMV promoter-driven vectors was sufficient to overwhelm cytosolic IκB binding capacity, thereby allowing functional p65/p50 translocation to the nucleus. We report here that expression of p65 or p65 in conjunction with p50 (NF-κB) caused dose-dependent declines in basal and hormone-activated VDRE- and RXRE-dependent reporter activity. Our data suggested that the p65 subunit of NF-κB conferred the inhibitory effect on nuclear receptor-stimulated transcription. This conclusion was supported by the observation that inhibition occurred when p65 was transfected alone or when p50 and p65 were cotransfected. In addition, the highest dose of the p65 expression vector completely blocked an expected increase in VDRE activity in response to treatment with $1,25(OH)_2D_3$, paralleling TNF-α effects seen previously. Third, transfection with p50 alone did not alter basal or hormone-stimulated RXRE reporter activity, and p50 modestly stimulated VDRE-dependent activity at higher DNA doses. This latter effect of p50 was unexpected in light of the fact that this subunit has been described as a DNA-binding protein lacking significant transactivational capacity and also of having the capacity to bind RXR and possibly the estrogen receptor (4, 7, 29). Although the mechanism behind this p50 stimulation remains unclear, it is conceivable that excessive p50 might bind and sequester endogenous p65, thereby decreasing a tonic p65 inhibition.

Two potential mechanisms whereby NF-κB might block nuclear receptor-dependent transactivation include direct physical interaction between the NF-κB subunits and the receptors or competitive binding of these NF-κB subunits to a hormone responsive element with subsequent displacement of nuclear receptors. These hypotheses were tested by incubating individual NF-κB subunits with a VDRE probe in either the presence or absence of VDR and RXR. Neither subunit bound this probe in the absence of receptors, nor was VDR-RXR binding to the DNA influenced by the presence of NF-κB subunits. Consequently, direct competition of NF-κB with VDR/RXR for binding to DNA does not explain our previous observation that TNF-α treatment blocks nuclear receptor binding to DNA. The current results instead support a model in which p65 interferes with receptor-dependent transactivation primarily through an indirect mechanism. The lack of detectable protein-protein interactions observed herein shows that the affinity of RXR binding to DNA must be much greater than any NF-κB-RXR interaction, if this occurs at all.

The quenching of hormone-stimulated transcription by p65 is consistent with a mechanism in which p65 competes for and sequesters an essential cofactor or activator required for VDR- or RXR-mediated transactivation. Support for this hypothesis comes from a

steadily increasing body of recent experimental evidence. In several studies, affinity "pull-down" experiments were employed to show that transcription factors such as SREBP-1a, Sp1, VP16, and the p65 NF-κB subunit interact with one or more proteins within a large activator-recruited cofactor (ARC) complex consisting of multiple proteins. Similarly, a truncated peptide containing the ligand-binding domain and α-helical region corresponding to the activation function-2 of VDR has been shown to account for ligand-dependent association of VDR with proteins within a large VDR-interacting protein (DRIP) complex (35). Some DRIPs have been found to be identical to the independently cloned steroid/thyroid receptor coactivators p100 (DRIP100/TRAP100) and p205 (PBP/RB18A/TRIP2/TRAP220/DRIP230). Furthermore, equivalent electrophoretic mobilities for the majority of ARC and DRIP proteins suggest that these two complexes are essentially the same. This large complex may bridge DNA-bound transcription factors to the RNA polymerase II preinitiation complex. VDR and p65 both form associations with proteins within these complexes and may compete for common factors that confer optimum transcriptional potency. This model of competitive sequestration of cofactors is consistent with other reports showing p65 squelches glucocorticoid-dependent transcription by competing for limited amounts of the coactivators CBP/p300 and SRC-1 (41). A reciprocal inhibition of p65-mediated transcription by glucocorticoid receptor has also been demonstrated. Similarly, NF-κB-dependent reporter activity has been shown to be inhibited by addition of RXR ligands, and both prototypical NF-κB subunits were observed to dose dependently block thyroid hormone-stimulated transcription, perhaps reflecting reciprocal inhibition between NF-κB and RXR (29). The inhibition caused by p65 in our experiments was not shared by c-Rel, another member of the Rel family of transcription factors, nor was inhibition apparent in the absence of hormone response elements. These observations confirm the specificity of p65 as a mediator of TNF-α inhibition of VDR or RXR function and ruled out a nonspecific effect of p65 on transcription.

Upon binding its receptor, TNF-α is thought to activate several protein kinase cascades, including those involving extracellular signal-regulated kinase, c-Jun NH_2-terminal kinase, and p38 mitogen-activated protein kinase (27). Potential synergy between these additional TNF-α-dependent signaling pathways and NF-κB in suppressing VDR and RXR function was examined by transfecting cells with NF-κB alone or NF-κB followed immediately by treatment with TNF-α. NF-κB significantly inhibited $1,25(OH)_2D_3$-stimulated VDRE-regulated activity, and this inhibition was not amplified further by TNF-α exposure. This finding suggests that TNF-α inhibition of transcription is mediated by NF-κB.

Previous studies from this laboratory showed that TNF-α reduces binding of heterodimeric RXR-VDR to a VDRE and RXR homodimer binding to an RXRE (12, 30). In this study, recombinant NF-κB subunits failed to block binding of VDR-RXR to a VDRE. Thus TNF-α-induced nuclear translocation of NF-κB in the absence of additional signaling events would not appear to account for decreased binding to hormone response elements. It has been suggested that phosphorylation of VDR by protein kinase C or casein kinase II may be important in regulating the activity of this receptor (13, 15). TNF-α is known to cause phosphorylation of the p50 and p65 subunits (24, 43), but it remains unclear whether changes in VDR and RXR phosphorylation occur in response to TNF-α stimulation. Such TNF-α-dependent phosphorylation events could result in a different spectrum of action compared with that observed after the expression of potentially underphosphorylated NF-κB subunits in our experimental model.

In conclusion, the p65 subunit of NF-κB appears to be one factor that mediates TNF-α inhibition of VDR- and RXR-dependent transactivation. This cross talk between previously considered independent pathways may represent a complex level of signal interaction that is important in regulating normal and pathophysiological cell processes.

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